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Characterizing the Expression of Cytochrome P450s in Breast Cancer Cells

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Characterizing the Expression of Cytochrome P450s in Breast Cancer Cells

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Résumé

Une résistance aux agents anticancéreux utilisés dans le traitement du cancer du sein est souvent associée à un échec de traitement. Des variations dans le devenir des agents anticancéreux dans l'organisme, sont des facteurs pouvant expliquer des phénomènes de résistance. Notre but était d'évaluer l'impact des isoenzymes du CYP450s, dans le métabolisme local des agents anticancéreux.

Notre premier objectif était de valider un gène rapporteur pour nos analyses de PCR en temps réel. Pour ce faire, nous avons criblé l'expression de 6 gènes rapporteurs dans 23 lignées cellulaires. *NUP-214* a été démontré comme étant le gène rapporteur le plus stable avec un écart-type de seulement 0.55 Ct.

Notre deuxième objectif était de déterminer le niveau d'expression des ARNm de 19 isoformes du CYP450 dans plusieurs lignées cellulaires du cancer du sein. Les ARNm des CYP450s ont démontré une très grande variabilité entre les lignées cellulaires. Les isoformes CYP1B1 et CYP2J2 démontrent l'expression la plus importante pour la majorité des lignées.

Notre troisième objectif était d'évaluer la corrélation entre l'expression des isoformes des CYP450s et leur activité métabolique en utilisant les substrats spécifiques du CYP1B1 et 2J2, 7-éthoxyrésorufine et ébastine, respectivement. Une forte corrélation (r^2=0.99) fut observée entre l'activité métabolique vis-à-vis l'ébastine et l'expression du CYP2J2. De même, le métabolisme du 7-éthoxyrésorufine était fortement corrélé (r^2=0.98) avec l'expression du CYP1B1.

En résumé, ces résultats suggèrent que le métabolisme local des agents anticancéreux pourrait significativement moduler le devenir des agents anticancéreux dans l'organisme, et pourrait être ainsi, une source de résistance.

Mots-clés : cytochromes P450, cancer du sein, résistance aux agents anticancéreux, métabolisme, variabilité interindividuelle

Abstract

Several types of cancer cells have shown an innate or accute resistance to anti-cancer agents which in turn causes a failure in treatment. This resistance has been suggested to be caused by the expression of membrane transporters in cancer cells, as well as inter-individual variability in metabolism. Our interest was to evaluate the implication of CYP450 enzymes in the local metabolism of cancer cells.

Our first objective was to screen the expression level of six housekeeping genes (HKG) using 23 different cell lines to determine which gene was the most stable. We found that *NUP-214* was the most stable HKG across the panel of cell lines tested, with a standard deviation of only 0.55 Ct.

Our second objective was to determine the expression level of 19 CYP450 mRNA isoforms in various breast cancer cell lines by RT-PCR. The CYP450 mRNAs showed a large variability between the different cell lines analyzed, where CYP1B1 and 2J2 were strongly expressed in most cell lines.

Our third objective was to determine if measurable metabolic activity was present and correlates with mRNA expression in these same breast cancer cell lines using the specific substrates 7-ethoxyresorufin and ebastine for CYP1B1 and 2J2 activities, respectively. The metabolism of 7-ethoxyresorufin showed an excellent correlation of 0.98 with CYP1B1 expression while ebastine demonstrates a strong correlation (r^2=0.99) with 2J2 expression.

Overall, these results suggest that local metabolism of anti-cancer agents could significantly affect drug disposition and be a source of chemoresistance.

Keywords : Cytochrome P450, breast cancer, chemotherapy resistance, drug metabolism, intersubject variability

TABLE OF CONTENTS

List of tables

Chapter 1: Introduction

Chapter 3: Housekeeping Gene Selection

Chapter 4: CYP450 expression in breast cancer cell lines

List of figures

Chapter 1: Introduction

Chapter 3: Housekeeping Gene Selection

Chapter 4: CYP450 expression in breast cancer cell lines

List of abbreviations

ADE:	Adverse Drug Event
ALDH:	Aldehyde Dehydrogenase
CD24:	Cluster of Differentiation 24
CD44:	Cluster of Differentiation 44
cDNA:	Complementary Deoxyribonucleic Acid
CSC:	Cancer Stem Cell
Ct:	Threshold Cycle
CYP450:	Cytochrome P450
DDI:	Drug-Drug Interaction
DNA:	Deoxyribonucleic Acid
dNTP:	Deoxynucleotide Triphosphate
DTT:	Dithiothreitol
H_2O:	Water
HER2:	Human Epidermal Growth Factor Receptor
EGFR:	Epidermal Growth Factor Receptor
EM:	Extensive Metabolizer
ER:	Estrogen Receptor
ERD:	Estrogen Receptor Downregulators
FACS:	Fluorescent Activated Cell Sorting
Fe:	Iron
FBS:	Fetal Bovin Serum

FFPE:	Formalin-Fixed Paraffin-Embedded
GAPDH:	Glyceraldehyde 3 Phosphate Dehydrogenase
HKG:	Housekeeping Gene
IM:	Intermediate Metabolizer
kDa:	kiloDalton
mRNA:	Messenger Ribonucleic Acid
NADPH:	Nicotinamide Adenine Dinucleotide Phopshate
NUP214:	Nucleoporin 214kDa
O_2:	Oxygen
PBS:	Phosphate Buffered Saline
PCR:	Protocol Chain Reaction
PM:	Poor Metabolizer
PPIG:	Peptidyl-prolyl cis-trans xsomerise G
PgR:	Progesterone Receptor
PR:	Progesterone Receptor
qRT-PCR:	Quantitative Real Time Protocol Chain Reaction
RNA:	Ribonucleic Acid
SNP:	Single Nucleotide Polymorphism
SERM:	Selective Estrogen Receptor Modulators
TN:	Triple Negative
VEGF:	Vascular Endothelial Growth Factor
VEGFR:	Vascular Endothelial Growth Factor Receptor

To my amazing husband, Ian

Thank you

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CHAPTER 1

INTRODUCTION

INTRODUCTION

Breast cancer is an important form of cancer that affects both men and women. In women, breast cancer accounts for 28% of all diagnosed cancers, and for 15% of the deaths due to cancer. In 2011, an estimated 6,200 women in Quebec (23,400 women and 190 men, in Canada) will be diagnosed with breast cancer, and 1,300 women in Quebec (5,100 women and 55 men, in Canada) will die due to breast cancer. Currently, it is estimated that the probability of developing breast cancer is 1 in 9, and the chance of death due to breast cancer is 1 in 29 (according to the Canadian Cancer Statistics 2011). Therefore, breast cancer is a disease which affects so many people, and merits the time and funding required to find a cure.

Great advancements have been made in the diagnosis and treatment of breast cancer. Large improvements have been made to mammography which can now detect smaller tumours while using less X-rays. Additionally, regular mammography screenings have been implemented leading to earlier detection, ultimately leading to better prognoses. Targeted treatments for breast cancers have also been developed leading to greater survival. While great advancements have been made in therapeutics, inter-subject variability and anti-cancer resistance remains to be a key issue in breast cancer patients.

Therefore, this work has been focused on finding a cause of anti-cancer resistance and inter-subject variability in the response to anticancer agents. Our focus was on the local expression of Cytochrome P450s, key enzymes in the metabolism of xenobiotics, in breast cancer.

1. BREAST CANCER PATHOLOGY

The use of molecular classifications is not only important for breast cancer patients, but also for researchers. Studies have shown that gene expression varies between subtypes, and therefore gene expression differences observed *in vitro* need to be related back to the molecular subtype, especially when large inter-subject variability is observed.

1.1. CANCER CELL LINES CHARACTERISTICS/MOLECULAR CLASSIFICATIONS

Treatment options for breast cancer tumors are based on many factors including morphology, histology, grade as well as gene expression profiles. The characteristics and molecular classifications of these tumors are necessary to properly determine tumor aggression and to choose the best treatment option. Based on gene-cluster analysis, five subtypes have been identified and described for breast cancers tumors: Luminal A, Luminal B, Basal-like, Human Epidermal Growth Factor Receptors (HER2) and Normal breast-like. [1, 2] However, the classification of subtypes used for breast cancer cell lines only consists of three groups: Luminal, Basal A and Basal B. [3, 4] The classification differences between cell lines and tumor classifications may be due to the absence of stromal and immune gene signatures in cell lines. While the cell line classification does not consist of a HER2 subtype, HER2 overexpression is observed in Luminal and Basal A subtypes. [3, 4]

Table 1 lists the cell lines used, along with various characteristics used in tumor diagnostics, which include gene cluster type, along with the presence or absence of the following receptors: estrogen, progesterone and HER2. As can be seen in Table 1, an equal amount of Luminal and Basal cell lines were selected in order to have each subtype present during our analyses, as well as the fact that each breast cancer cell line has a unique expression profile.

1.2. RECEPTORS PRESENT IN BREAST CANCER CELLS

The presence of cell surface and intracellular receptors in breast cancer is of great importance during the diagnostics of breast cancer. Hormone receptor positive tumors have available targeted treatments, which have been proven to be very effective. Hormone receptors of interest in breast cancer are Estrogen Receptor (ER), Progesterone Receptor (PR), HER1 and HER2.

Table 1: Breast cancer cell lines characteristics (Adapted from Kao, 2009, and Neve, 2006 [3, 4])

Cell Line	Cell Line Subtype	Tumor Type	Source	Estrogen Receptor	Progesterone Receptor	HER2 Receptor
Hs578T	Basal B	Invasive Ductal Carcinoma	Primary Breast	No	No	No
MCF7	Luminal	Invasive Ductal Carcinoma	Pleural Effusion	Yes	Yes	No
MCF10A	Basal B	Non-tumorigenic Epithelial Cell Line	Primary Breast	No	No	No
MDA-MB-231	Basal B	Adenicarcinoma	Pleural Effusion	No	No	No
MDA-MB-468	Basal A	Adenicarcinoma	Pleural Effusion	No	No	No
SKBR3	Luminal	Adenicarcinoma	Pleural Effusion	No	No	Yes
T47D	Luminal	Invasive Ductal Carcinoma	Pleural Effusion	Yes	Yes	No
ZR-75-1	Luminal	Invasive Ductal Carcinoma	Ascites Fluid	Yes	No	No

1.2.1. Estrogen Receptor

Many physiological processes in humans are influenced by the presence of estrogens. Estrogens mediate their effects through the binding and interactions with the estrogen receptor (ER).[5] The ER is known to be present under two forms, ERα and ERβ, which are encoded by two separate genes, ESR1 and ESR2, respectively. These two forms are tissue specific, have similar affinities for steroid ligands, and can be both localized to the nucleus, or to the plasma membrane.[6]

The development of breast cancer has been shown to be linked to the presence of estrogen.[5] Two hypotheses have been suggested to explain this phenomenon. First, the binding of estrogen to intracellular or membrane bound estrogen receptors cause an increase in cell division. This increase in cell proliferation leads to elevated DNA synthesis and therefore an elevated risk of DNA replication errors, ultimately leading to cancer development.[5] The second hypothesis is that estrogen metabolism produces genotoxic metabolites that can cause DNA damage.[5]

While estrogen receptors are linked to the activation of cancer, there are specific treatments that target the ER, called Selective Estrogen Receptor Modulators (SERMs) which include tamoxifen, a very effective anti-cancer agent for ER+ cancers.[5] Another class of treatments that target the ER are Selective Estrogen Receptor Modulators (SERDs). Currently only one SERDs is available for the treatment of breast cancer, Faslodex (Fulvestrant).[7, 8]

1.2.2. Progesterone Receptor

Progesterone is a steroid that is critical to normal breast development, and mediates its effects by binding to progesterone receptors (PR). Two isoforms have been identified for the progesterone receptor, PR-A and PR-B. Both isoforms are synthesized from the same gene, but have different translation start sites, creating two receptors of different masses, namely PR-A lacks the 164 N-terminal amino acids found in PR-B.[9, 10] The ratio of isoforms A to B is unknown, but appears to be a crucial element in cell homeostasis.[10]

One single nucleotide polymorphism (+331G/A) has been identified for this receptor, which affects the transcriptional activity of the gene, leading to a greater production of the PR-B receptor. There are some conflicting studies in whether or not there is a link between this SNP and the presence of breast cancer, but overall, there has been no association between this mutation and the risk of developing breast cancer.[9]

1.2.3. Human Epidermal Growth Factor Receptor (HER)

The Human Epidermal Growth Factor Receptor is comprised of 4 isoforms, HER1 (also known as epidermal growth factor receptor, EGFR), HER2, HER3 and HER4. These four receptors are encoded by the gene *ERBB1/2/3/4,* respectively.[11] All four of these receptors have been shown to influence tumor development by affecting cell proliferation, migration, angiogenesis and apoptosis protection.[12] While no defined ligands are known for the four HER receptors, it is known to play a key role in the amplification of cell signaling through a dimerization reaction. This dimerization has been shown to increase receptor-ligand affinity, enhancing its own activation. The activated receptor promotes the phosphorylation of tyrosines in its intracellular domain, which is subsequently recognized by specific cytoplasmic signal transducers, which inevitably leads to cell growth and proliferation.[13] Since these receptors cause increase cell proliferation and are capable of preventing apoptosis, their overexpression in breast cancer leads to very aggressive tumors.[11] HER2 has been studied the most due to its frequent overexpression in breast cancer, estimated to between 18-25% of human breast cancers, and is therefore a targeted receptor.[11, 14]

The attempt to explain the aggression observed in tumors overexpressing HER2 has been of interest. Ginestier at al. in 2007 demonstrated a strong correlation between ALDH1 overexpression (a marker of cancer stem cells, see section 4.3 for more details) and HER2 overexpression. It is believed that the aggression observed in HER2 breast cancers is due to a large population of cancer stem cells, where cancer stem cells have been linked to anti-cancer agent resistance. [15]

While HER2 overexpression is generally undesired due to its aggressive behavior, specific treatments targetting this receptor have been developed and have proven to be very effective. Treatments include Trastuzumab and Lapatinib, which are specific inhibitors of the HER2 receptor (see section 2.1.2.2.2 HER2 Inhibitor for more details).[1, 13, 16-18]

1.3. MOLECULAR SUBTYPES

Molecular subtypes used in the description of breast cancer cell lines and breast cancer tumors are slightly different. In order to better understand how these two classification systems overlap, Kao et al looked at the various gene expression patterns of all subtypes to determine how cell lines would be classified using the breast cancer tumor subtypes. It was determined that the Luminal cell lines most resembled Luminal A and Luminal B tumors, while Basal A cell lines were most similar to basal-like tumors, and Basal B cell lines were similar to basal-like or HER2 overexpressing tumors.[2, 3] While the Basal B cell lines displayed expression patterns that are similar to HER2 overexpressing tumors, Basal B cell lines do not overexpress HER2 and are referred to as as triple negative cell lines.[3]

For breast cancer cell lines, the molecular subtypes are important to take into consideration because previous studies have shown that gene expression profiles are dependent on the molecular subtype. Meaning that some genes may demonstrate an overexpression in one molecular subtype, whereas a downregulation is observed in others. Therefore gene expression variations observed may simply be due to its cell line characteristics.[3]

Since each molecular subtype is very different, and requires a different treatment plan, molecular subtypes are always determined for breast cancer patients. Much detail is available for breast cancer tumors subtypes; therefore a more comprehensive assessment of these subtypes follows.

1.3.1. Luminal A

Luminal A cancers originate from the inner cells, called luminal cells, which line the mammary duct.[1] Luminal cells are responsible for the secretion of milk, are highly differentiated and are glandular.[19] Luminal A tumors are mainly characterized by the presence of Estrogen Receptor (ER) and, or the Progesterone Receptor (PR, or PgR), but the absence of the Human Epidermal Growth Factor Receptor 2 (HER2). Luminal A cancers have been associated with a good prognosis, where the survival

rate is high, and the rate of reccurrence is low because they can be treated with hormone therapies, including Tamoxifen.[1, 2, 20]

1.3.2. Luminal B

Luminal B cancers also originate from the inner cells, called the luminal cells.[1] While this subtype is similar to the Luminal A subtype, where there is the presence of Estrogen Receptor (ER) and, or the Progesterone Receptor (PR, or PgR), Luminal B tumors often express the HER2 receptor. [1, 2, 21, 22] Hormone treatments are available; however because of the presence of the HER2 receptor, Luminal B tumors tend to have a higher tumor grade, and exhibit high proliferation rates and DNA amplifications, and therefore are associated with a poor prognosis.[1-3]

Treatments of Luminal B tumors include hormone therapy, Trastuzumab-based, chemotherapy and/or surgery.[1]

1.3.3. Basal-Like

Basal-like cancers originate from the outer cells, called the basal cells (myoepithelial), which line the mammary duct [1, 3]. Basal cells do not have a role in secretion, but rather in muscle contraction, and are undifferentiated.[19] These tumors are often associated with the hereditary BRCA1 breast cancer tumors and have been known to be the most aggressive subtype. [1] Most Basal-like tumors are negative for all three receptors, and are commonly referred to as the Triple Negative tumor (TN). However, this is not always the case. It is estimated that 5-45% of basal tumors are ER+ and that 14% are HER2+.[1, 17] For patients diagnosed with a tumor that is both basal-like and TN, the prognosis tends to be very poor because of the lack of targeted treatment (such that are available for hormone responsive tumors) and because the cancer is very aggressive.[1]

Stem cells, or cells with stem cell like properties are believed to be the source of basal-like cancers. There have been reports showing that the Basal phenotype are largely composed of the

$CD44^+/CD24^-$ polulation, otherwise known as a Cancer Stem Cell population (see section 4.3 for more details).[4] The expression of markers such as cytokeratins (specifically CK5/6, 14 and 17) and the transcription factor p63 are found in basal-like breast cancers.[23]

Treatment options for Basal-Like tumors depend on the presence of receptor overexpression. If ER, PR or HER2 is overexpressed, hormone, or trastuzumab treatments are used. For TN tumors, treatment options include chemotherapy and surgery.[1]

1.3.4. HER2 overexpressing

HER2 overexpression has been linked to mammary tumorigenesis, tumor aggression and metastasis and therefore tends to have a poor prognosis.[14] HER2+ tumors are negative for both ER and PR, while they overexpress the HER2 receptor. While the tumor is known to be aggressive, targeted treatments for HER2 positive breast cancers are available. Trastuzumab (or Herceptin®), and Lapatinib are two targeted treatments for HER2 positive tumors that have proven to be very effective.[1, 13, 16-18, 24]

1.3.5. Normal breast-like

The normal breast-like cancers are cancers which do not fit within the other predefined subtypes.[1] This subtype has been shown to demonstrate similar expression patterns as a normal breast tissue. [3] Overall, these tumours are small, and usually have a good prognosis.[1]

2. ANTI-CANCER AGENTS

Anti-cancer agents, such as chemotherapy and targeted treatments, are used in the treatment of breast cancer. However, some patients experience resistance to these treatments, which inevitably ends in treatment failure. Research has shown that the presence of breast cancer stem cell, as well as proteins involved in drug bioavailability, such as membrane transporters and drug metabolizing enzymes, are the sources of resistance (see Section 4 for more details). Our focus is on the effects of the Phase I drug metabolizing enzymes, Cytochrome P450s on the bioavailability of anti-cancer agents. First the agents used in breast cancer will be described.

2.1. TYPES OF TREATMENTS

Two main groups of treatments are used in the treatment of breast cancer, chemotherapy agents, and targeted treatments. Different classes of each group are available; however, chemotherapy agents destroy all quickly dividing cells, whereas targeted treatments attack cells which overexpress specific proteins or processes observed in breast cancer cells.

2.1.1. Chemotherapy Classes

Chemotherapy is the process where anticancer medications are used to treat cancer. The goal of chemotherapy agents is to prevent the growth and spreading of cancer cells by interfering with normal cell processes at different points. Some chemotherapy agents mechanism of action occurs at the genetic level (DNA and RNA damage), while other interfere with normal protein function.[25] Chemotherapy agents have been categorized in different chemotherapy classes based on their mechanism of action. These classes include alkylating agents, anti-metabolites, plant alkaloids (vinca alkaloids and Taxanes), Topoisomerase inhibitors and cytotoxic antibiotics (anthracylines). See Table 2 for more details on chemotherapy agents used in breast cancer treatments.

Table 2: List of chemotherapy agent classes used for breast cancer and their mechanism of action

Chemotherapy Classes	Action Mechanism	Examples used in breast cancer
Alkylating Agents	DNA damage through alkylating nucleophilic sites of DNA bases. [26]	Cyclophosphamide and carboplatin
Anti-metabolites	DNA and RNA damage through masquerading as pyrimidine and purine bases. [27]	Methotrexate, 5-fluorouracil, Gemcitabine and Capecitabine
Plant Alkaloids	Microtubule binding [27, 28]	Vinca Alkaloids and Taxanes
Vinca Alkaloids	Natural product that destabilizes microtubule by binding to the β-subunit of tubulin destroying mitotic spindles and blocks mitosis. [27, 28]	Vinblastine and Vinorelbine
Taxanes	Natural product that stabilizes microtubule by binding to the β-subunit of tubulin inhibiting depolymerisation and slowing down mitosis. [27, 28]	Paclitaxel and Docetaxel
Topoisomerase inhibitors	Relaxes DNA supercoiling by Topoisomerases, causing DNA vulnerability to intercalating agents.[29]	Irinotecan (TOP1) and Anthracyclines (TOP2); currently in clinical trials.
Cytotoxic antibiotics -Anthracyclines	Intercalating agents, that inhibit topoisomerases and generated reactive oxygen species.[30]	Doxorubicin and Epirubicin[31]

2.1.2. Targeted Treatments

Targeted treatments are often used in the treatment of breast cancer, specifically cancers that are receptor positive. Treatment groups include hormone therapies and specific targeted enzymes.

2.1.2.1. Hormone Therapy

The majority of breast cancer tumors are hormone receptor positive and therefore can benefit from hormone therapies. Hormone receptors, namely estrogen and progesterone receptors, respond to intra- and extracellular hormone levels, which act as a signal to turn on cell growth. Therefore, hormone therapies work by blocking the hormone action potential at their specific receptors, as well as to lower the amount of hormone in the body.[7] Hormone therapies include aromatase inhibitors, Selective Estrogen Receptor Modulators and Estrogen Receptor Downregulators.

2.1.2.1.1. Aromatase inhibitors

Aromatase, otherwise known as CYP19A1, is an enzyme which catalyzes the conversion of the hormone androgen to estrogen. [32, 33] Aromatase Inhibitors are molecules that inhibit aromatase activity by blocking the production of estrogen in the body (through CYP19A1 activity). These inhibitors only work in post-menopausal women, who are diagnosed with ER+ cancers, because pre-menopausal women produce significant amounts of estrogen in the ovaries without aromatase activity.[34] Aromatase inhibitors include Anastrozole, Exemestane and Letrozole.[7, 34]

2.1.2.1.2. Selective Estrogen Recetor Modulators (SERMs)

Selective Estrogen Receptor Modulators (SERMs) work by competitively binding to the active site of Estrogen Receptors. Since estrogen is unable to bind to the estrogen receptor in the presence of SERMs, the breast cancer cell does not receive a signal to grow and divide, and therefore the proliferation of breast cancer is blocked.[35] SERMs include Tamoxifen, Raloxifene and Toremifen.[7]

2.1.2.1.3. Seletive Estrogen Receptor Downregulators (SERDs)

Selective Estrogen Receptor Downregulators (ERDs) have a similar action mechanism as SERMs, however, they also work to reduce the amount of Estrogen receptors in the breast cell, and change the shape of the Estrogen Receptor active site, so that the receptor cannot recognize the hormone as efficiently.[8] Currently there is only one SERDs commercially available, Faslodex (Fulvestrant).[7, 8]

2.1.2.2. Specific Targeted Enzymes

Certain enzymes or receptors are overexpressed in breast cancer. By blocking the activity of these specific enzymes, the proliferation rate of cancer cells is blocked. These enzymes include the Epidermal Growth Factor Receptor, HER2 and VEGFR (enzyme responsible for angiogenesis).

2.1.2.2.1. Human Epidermal Growth Factor Receptor (HER) Inhibitor

The Human Epidermal Growth Factor Receptor (HER) is a family of receptors which play a key role in the development and proliferation of cancer as well as in the prevention of apoptosis.[12-14] The HER family is known to require tyrosine kinase activitation. In breast cancer, HER1, also known as the epidermal growth factor receptor (EGFR) and HER2 are most often studied members of the HER family, where HER2 is considered to be the most important because of its frequent overexpressed breast cancer. Several molecules have been used to inhibit this family of receptors. Gefitinib is an EGFR inhibitor that blocks the kinase activity of the EGFR receptor, however it is not approved for breast cancer.[36] Lapatinib (Tykerb®) is a small molecule which inhibits the tyrosine kinase of both the HER1 and HER2 receptors.[36] Finally, Trastuzumab (Herceptin®) is a monoclonal antibody which specifically recognizes the HER2 receptor.

2.1.2.2.2. Angiogenesis Inhibitors

The vascular endothelial growth factor receptor (VEGFR) is responsible for the development and maintenance of tumor vasculature, or angiogenesis through a signal transduction process. [37] When the vascular endothelial growth factor (VEGF) binds to its receptor, VEGFR, signals are sent to initiate the growth of new blood vessels and to promote the maintenance of old ones. This is a vital process to all cells, and has been seen to be overexpressed in some tumors. This process has also been suggested as a possible source of cancer metastasis. Therefore, by targeting angiogenesis, cells which overexpress VEGF, such as cancer cells, would be most affected by angiogenesis inhibitors. Several treatments are available and include: monoclonal antibodies that binds specifically to VEGF, Bevacizumab (no longer approved for the treatment of breast cancer), small-molecule inhibitors of tyrosine kinase, Axitinib (not yet approved in Canada), Sunitinib, Sorafenib, as well as endogenous inhibitors of angiogenesis, Endostatin and Angiostatin. [36, 38-40] Currently no Angiogenesis inhibitors are approved for the treatment of breast cancer.

Table 3: List of hormonal and targeted treatment classes used for breast cancer and their mechanisms of action

Chemotherapy Classes	Action Mechanism	Examples used in breast cancer
Aromatase Inhibitors	Reduction of estrogen production through the inhibition of aromatase activity (through CYP19A1) [32, 33]	Anastrozole, Exemestane and letrozole [7, 34]
Selective Estrogen Receptor Modulators (SERMs)	SERMs bind to the ER preventing the growth signal of ER+ breast cancer to grow and divide. [35]	Tamoxifen, Raloxifen and Toremifen [7]
Selective Estrogen Receptor Downregulators (SERDs)	Reduction of the amount of ER present, and change in the active site shape to reduce hormone binding efficiency. [8]	Faslodex [7, 8]
Human Epidermal Growth Factor Receptor (HER) Inhibitors	Inhibition of HER to prevent receptor activation which allow for the apoptosis of cancer cells.[12-14]	Gefitinib (not approved for breast cancer) Trastuzuab and Lapatinib [13, 36]
Angiogenesis Inhibitors	Blocking of VEGFR to reduce blood flow to tumors by reducing the formation of new blood vessel formation and maintenance of old vessels. [37]	Axitinib (not yet approved in Canada), Bevacizumab (no longer approved for breast cancer), Sunitinib, Sorafenib, Endostatin and Angiostatin (not yet approved) [36, 38, 39]

2.2. METABOLISM

The clearance of chemotherapeutics is an essential aspect to consider for drug administration. Many of the chemotherapy agents used in the treatment of breast cancer are known substrates and or inhibitors of the superfamily of metabolizing enzymes called Cytochrome P450 (CYP450s). Table 3 lists the various anti-cancer agents used in the treatment of breast cancer, along with the CYP450s isoforms that are responsible for their clearance. As can be seen, some agents are not metabolized by CYP450s and therefore may require other pathways for drug clearance, such as by drug transporters.

Table 4: Anticancer agents used in the treatment of breast cancer, and their metabolism by CYP450s

Anti-Cancer Agent	**CYP450**
Cyclophosphamide	CYP2B6, 2A6, 3A4/5, 2C8,2C9, 2C19 [41-44]
Carboplatin	None[42-44]
Methotrexate	None [43]
5-fluorouracil	Inhibitor of CYP2C9 [45]
Gemcitabine	None
Capecitabine	None
Vinblastine	CYP3A4 [44]
Vinorelbine	CYP3A4 [44, 46, 47]
Paclitaxel	CYP2C8, 3A4, 3A5 [41, 43, 44]
Docetaxel	CYP3A4/5 [43, 44, 48]
Doxorubicin	Inhibitor of CYP2D6 [45]
Epirubicin	None [43]
Anastrozole	CYP3A4/5 [44, 49]
Exemestane	CYP3A4 [44, 50]
Letrozole	CYP2A6 and 3A [44, 51]
Tamoxifen	CYP1A1/2, 1B1, 2B6, 2C9/19, 2D6, 2J2 3A4/5 [44, 45, 52-55]
Raloxifene	Inhibitor of and metabolism by CYP3A4[56-58]
Toremifene	CYP3A4 [44, 59]
Fulvestrant	None
Gefitinib	CYP3A4, 2D6, minor 3A5 and 1A1 [60]
Trastuzumab	None
Lapatinib	CYP3A4/5 and minor by 2C8/19 [12, 60]
Bevacizumab	None

3. CYTOCHROME P450S

Cytochrome P450s (CYP450) are enzymes which have been shown to be involved in the bioavailability of anti-cancer agents through phase I metabolism. Each CYP450 has a specific role in drug metabolism and tissue localization pattern. CYP450 isoforms which are locally expressed in breast cancer tissue and breast cancer cell lines are of interest because they may play an important role in the local drug metabolism of various anti-cancer agents.

3.1. DESCRIPTION

Drug metabolism, an important role in drug disposition, is executed by many enzymes in the human body, but more specifically in the liver. The most important family of enzymes responsible for the metabolism of medications is the cytochrome P450 (CYP450) superfamily. These enzymes work to detoxify the body of many xenobiotic molecules through a biotransformation reaction, rendering the molecule more hydrosoluble. This in turn facilitates the secretion of xenobiotics into urine and bile. [61] In human, this superfamily is composed of 57 known CYP450 genes, which are classified into families and subfamilies, depending on sequence homology. [62, 63] Isoforms which display greater than 40% homology are grouped within the same family, while enzymes with greater than 55% are classified within the same subfamily.[64, 65] Each CYP450 isoform has various roles in metabolism and in the synthesis of molecules, where similar functions are common within the same family.

While CYP450s localized in the liver play a major role in drug metabolism, either in drug clearance or in the activation off pro-drugs, extrahepatic CYP450s are implicated in both local drug metabolism, as well as in the synthesis and degradation of endogenous molecules, such as steroids, and fatty acids. [32, 33, 45, 63, 66-70]

3.1.1. Structure

All isoforms of the CYP450 superfamily are relatively small proteins with molecular weights ranging between 45 and 66 kDa.[71] CYP450s are found in every tissue at varying concentrations, and are all membrane bound protein located in the endoplasmic reticulum.[72] All CYP450s contain a ferroprotoporphyrin IX heme group, which constitutes the active site (the heme moiety). However, the specificity of each isoform is dependent upon the apoprotein and not the active site. [65]

In order for CYP450s to oxidize a molecule, these isoenzymes require two things; the presence of an energy source, which in the case of CYP450s is the reducing agent, nicotinamide adenine dinucleotide phosphate (NADPH), as well as atmospheric oxygen.[65]

3.1.1.1. Oxydation Cycle

The oxidation cycle of CYP450s has been well described, and is summarized and simplified in Figure 1. [73] Overall, the cycle is described as a dynamic process, which means that these steps do not necessarily occur in a sequential fashion. [73] In brief, a substrate binds to the active site in a reversible fashion. Using NADPH, the heme centre is reduced from the ferric state (Fe^{3+}) to the ferrous state (Fe^{2+}). One molecule of oxygen then binds generating a ferrous CYP-Substrate complex. The oxygen molecule is then cleaved by using a second electron (either from another molecule of NADPH or by Cytochrome b_5. The molecule is then oxidized and released from the active site.[65] In the end, the iron is returned to the ferric state, and is ready for another cycle of oxidation.

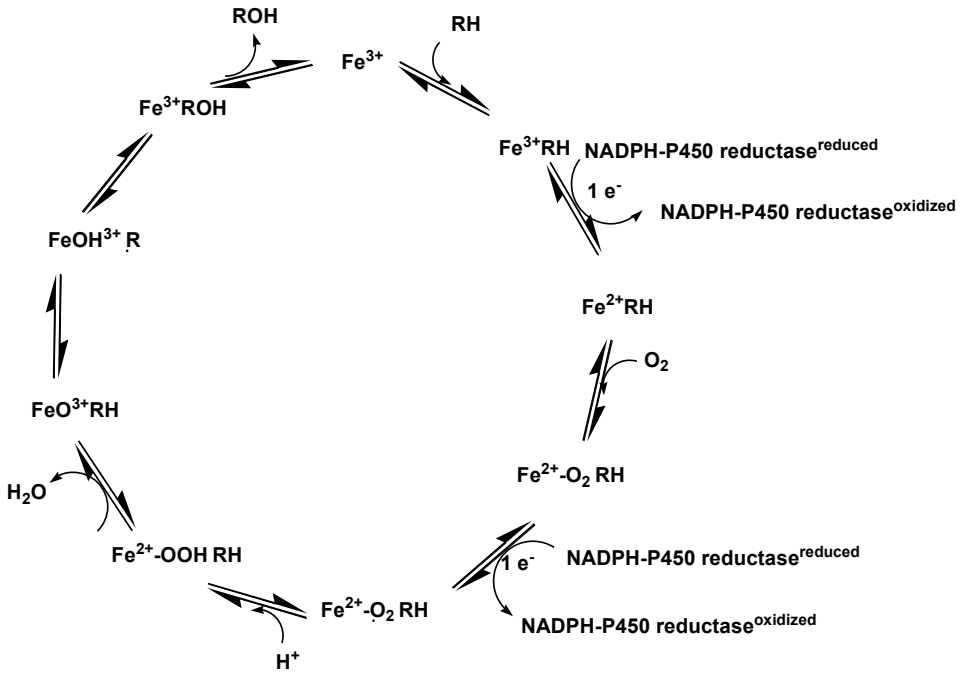

Figure 1: Oxydation cycle of CYP450s. (Adapted from Guengerich, 2007 [73])

3.2. FAMILIES

Cytochrome P450 isoforms are grouped according to their families and subfamilies. In human, the CYP450s are grouped into 18 families, and 43 subfamilies.[74, 75] Not all families have been shown to be influenced in drug metabolism or steroid biosynthesis; therefore specific CYP450s were selected based on their role in drug metabolism, steroid metabolism, or link to breast cancer. Below is a table of all CYP450s analyzed, categorized by their families, and describing their roles and localization in human.

3.3. ROLES OF CYP450S

3.3.1. Systemic Metabolism (Exogenous molecules)

Hepatic CYP450 isoforms have been well characterized. These enzymes account for ~75% of the drug metabolizing enzymes found in the liver. The five most abundant hepatic CYP450s, accounting for 95% of hepatic metabolism are CYP3A4, 2D6, 1A2, 2C9 and 2C19, where 3A4 is the most important.[73]

Table 5: Table of CYP450s of interest describing their roles and localization

Family	Subfamily	Enzymes	Roles	Localization
CYP1	CYP1A	CYP1A1	Hydroxylation of Steroids and Xenobiotic metabolism [63, 66-69]	Hepatic[73]
		CYP1A2	Hydroxylation of Steroids and Xenobiotic metabolism [63, 66-69]	Hepatic[73]
	CYP1B	CYP1B1	Detoxification of steroidal hormones, therapeutic drugs and environmental toxins [76]	Adrenal glands, ovary, testis, lung, prostate[72]
CYP2	CYP2A	CYP2A6	Xenobiotics Metabolism[63]	Hepatic [77]
	CYP2B	CYP2B6	Xenobiotics Metabolism[63]	Hepatic[73]
	CYP2C	CYP2C8	Xenobiotics Metabolism[63]	Liver, kidney, adrenal gland, brain, uterus, breast, ovary and duodenum[78]
		CYP2C9	Xenobiotics Metabolism[63]	Hepatic[73]
		CYP2C19	Xenobiotics Metabolism[63]	Hepatic[73]
	CYP2D	CYP2D6	Xenobiotics Metabolism[63]	Hepatic[73]
	CYP2E	CYP2E1	Xenobiotics Metabolism (Ethanol)[45, 63]	Hepatic[73]
	CYP2J	CYP2J2	Fatty Acid and xenobiotic Metabolism [53, 63]	Heart, kidney, lung [72, 79, 80]
	CYP2W	CYP2W1	Procarcinogens [81]	Prostate, pancreas, placenta, lung, colon, intestine[63, 82]
CYP3	CYP3A	CYP3A4	Xenobiotics Metabolism[63]	Hepatic[73]
		CYP3A5	Xenobiotics Metabolism[63]	Hepatic, lung, small intestine, prostate[83]
		CYP3A7	Xenobiotics Metabolism[63]	Prenatal Tissue (liver) [65, 84]
CYP4	CYP4A	CYP4A11	Fatty Acid Metabolism[63]	Kidney and liver[72]
		CYP4Z1	Lauric Acid Metabolism[70]	Breast, breast carcinoma, kidney and liver[63, 82, 85]
CYP17	CYP17A	CYP17A1	Androstenedione synthesis[33, 67]	Adrenal cortex[72]
CYP19	CYP19A	CYP19A1	Aromatase Activity[32, 33]	Breast Brain, placenta gonads[33, 72]

3.3.2. Local Metabolism (Exogenous molecules)

The local metabolism of exogenous molecules by various Cytochrome P450 isoforms has become of greater importance. The presence of many CYP450 enzymes has been identified in extra-hepatic tissues, such as the intestines, kidneys, brain, lungs and heart. [86-92] While concentrations of these enzymes may be inferior to those found in the liver, their implications in drug disposition cannot be ignored. Above all, the expression of the CYP450 isoforms appear to be tissue specific, where many isoforms identified in the extra-hepatic tissues are not found in the liver. For example, the mRNA expression of CYP2J2, an isoform not present in the liver, has been identified in abundance in cardiac

tissue.[79] Since many extra-hepatic CYP450s are not evaluated during the drug discovery process, the metabolism potential of these tissue specific CYP450s are unknown, and could locally metabolize exogenous molecules. As is seen in the liver, inter-subject variability is observed in the expression of CYP450s, and therefore, this same variability could be present in other tissues, such as the heart, or breast. This inter-subject variability may be another source of variation in drug effect.

3.3.3. Synthesis of endogenous molecules

The presence of steroids in breast cancer has been shown to be important, especially in hormone receptor positive breast cancers. Therefore, the expression of various CYP450s responsible for the synthesis and degradation of steroids expressed in breast cancer could be of great importance. The members of the Family 1 isoforms, namely CYP1A1, 1A2 and 1B1, have been shown to play a role in the hydroxylation of progesterone, testosterone and estrogen.[66-69] Therefore the local expression of these three isoforms may lead to the growth and proliferation of breast cancers which are stimulated by the presence of these hormones, (namely ER+ cancers being stimulated by estrogens). The expression of two other CYP450 isoforms which may be of interest in breast cancer are CYP17A1, mainly expressed in prostate tissue and 19A1, mainly expressed in breast tissue. CYP17A1 plays a role in the conversion of pregnenolone to androstenedione, wherease CYP19A1 converts androstendione to estrone through the use of aromatase activity.[33, 67, 93] Therefore the presence of these five CYP450s may be greatly implicated in the local synthesis of estrogens and other steroids.

3.3.4. Adverse Drug Events/Drug-Drug Interactions

Adverse drug events (ADEs) are events where an injury occurs from the use of a medication when the drug has been administered at a normal dose. ADE can lead to toxicity or loss of treatment efficiency, and can occur due to genetic alterations (such as polymorphisms), drug-drug interactions, or food-drug interactions. Overall, ADE results in 6.7% of hospitalizations in the United States, which has been estimated to cost 1.5 million dollars. [94, 95]

Drug-drug interactions (DDIs) are highly studied ADE, where interactions with the major hepatic CYP450s, such as CYP3A4 and 2D6 are commonly evaluated (substrates, inhibitors or inducers of these enzymes).[73] One example of a DDI for ER+ breast cancer patients would be the co-administration of tamoxifen, a substrate of CYP2D6, and the anti-depressant fluoxetine, a substrate of CYP2D6.[45, 52, 96] Under this situation, tamoxifen, which requires metabolism by CYP2D6 to become endoxifen, its active form, would be affected by the presence of the anti-depressant, due to competitive inhibitions of the enzyme. Therefore, a lack of active metabolite would be present, and a treatment failure would ensue.

Overall, DDI need to be avoided in all patients to ensure that no toxicity or treatment failure occurs.

3.4. IMPORTANCE IN BREAST CANCER

Two CYP450 Isoforms have been identified, through this work, to be highly expressed in the breast cancer cell lines analyzed: CYP1B1 and CYP2J2 (See Chapter 4 for more details). Both isoforms have a different role and importance in metabolic reactions, where 1B1 is involved in hormone and toxin detoxification, while 2J2 is involved in the hydroxylation of fatty acids. [63, 76]

3.4.1. CYP1B1

The expression of CYP1B1, a 58 kDa protein, has been found in many extra-hepatic tissues such as the uterus, ovaries, testis, prostate and adrenal glands.[97, 98] CYP1B1 has been shown to catalyze the hydroxylation of 17β-estradiol and testosterone.[99] However, CYP1B1s metabolic activity has also been linked to the activation of several pre-carcinogenic molecules such as benzanthracene, benzo(a)pyrine, DMBA, 1-ethynyl-pyrene, 3-methyl-cholantrene and oestradiol.[97] Since an overexpression of this isoforms has been found in tumors, such as in breast cancer, it is believed that CYP1B1 may be a source of steroid hormone-mediated cancer.[99, 100]

In order to understand the local metabolism potential of CYP1B1 in breast cancer cells, 7-ethoxyresorufin, a substrate which is common to CYP1A1, 1B1 and 1A2, may be used.[101] The rate of metabolism of 7-ethoxyresorufin is quite different in each of these enzymes. The maximum activity observed in CYP1A1 is greater than what is observed by CYP1B1, however, the substrate demonstrates a stronger affinity towards CYP1B1 than 1A1 (lower Km for 1B1).[101] However, since CYP1B1 is preferentially expressed in breast cancer cell lines, the metabolic activity of 7-ethoxyresorufin corresponds to CYP1B1 expression.

3.4.1.1. Homology

CYP1B1 shares a strong homology with the two other members of the CYP1 family, CYP1A1 and 1A2. According to sequence alignment, CYP1B1 shares 39% and 37% homology with CYP1A1 and 1A2, respectively. The active site is where the most homology is seen where many of the amino acids are conserved; this leads to large substrate overlap.[102] However, there are small differences in the amino acids in the active site which leads to substrate affinity differences (see figure 2).

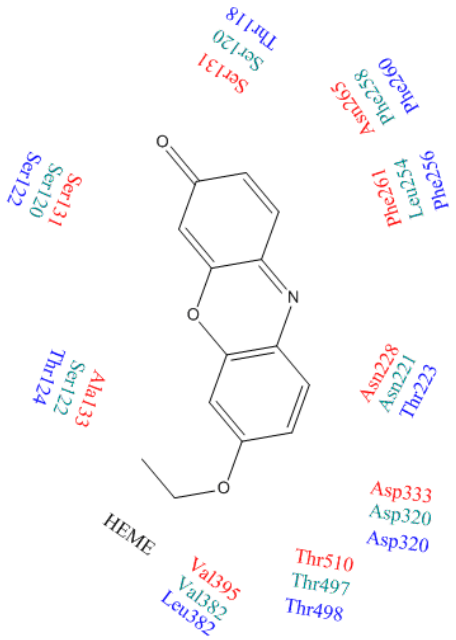

Figure 2: Active site of CYP1A1 (green), 1A2 (blue) and 1B1 (red). 7-Ethoxyresorufin is shown in the active site. (Adapted from Takemura, 2010.[102])

3.4.1.2. Polymorphisms in *CYP1B1*

There are many known Single Nucleotide Polymorphisms (SNPs) for CYP1B1, both in the coding and non-coding regions. Some of these SNPS have been further characterized and have been given specific variant names (see Table 5). As can be seen, many of the CYP1B1 variants do not lead to altered metabolic activity with the exception of variant *6 and *7 which demonstrate a significant decrease in enzyme efficiency.[103] This finding was of interest because *6 and *7 variants are a combination of 3 and 4 SNPs, respectively, and when only one or two of these mutations are present, no change in enzyme activity was observed. Therefore it is hypothesized that only in the presence of all three SNPs (found in *6 and *7 variants), a change in protein folding results in a conformational change of the enzymes active site.[103] Others have reported that the *2 and *3 variants have a 2-4 increase in metabolic activity and an increased risk of developing certain types of cancers.[76] Since CYP1B1 has been linked to activation of certain pre-carcinogenic molecules, it is believed that an increased activity of CYP1B1 may lead to a greater production of carcinogens, and hence cancer development, specifically in tissues where elevated expression of CYP1B1 is found.[76]

Table 6: Known polymorphisms of *CYP1B1* and their effects on metabolic activity (Adapted from Aklillu, 2001, [103])

Variant	Nucleotide Change	Amino Acid Substitution	Protein Activity
CYP1B1*1	None	None	Wild Type Activity
CYP1B1*2	142C>G, 355G>T	Arg48Gly, Ala119Ser	Lower expression level, similar kinetic activity as wild type
CYP1B1*3	1294C>G	Leu432Val	Lower expression level, similar kinetic activity as wild type
CYP1B1*4	1328A>G	Asn453Ser	Lower expression level, similar kinetic activity as wild type
CYP1B1*5	142C>G, 1294C>G	Arg48Gly, Leu432Val	Unknown
CYP1B1*6	142C>G, 355G>T, 1294C>G	Arg48Gly, Ala119Ser, Leu432Val	Similar expression level, kinetic activity significantly lower
CYP1B1*7	142C>G, 355G>T, 1294C>G, 1328C>G	Arg48Gly, Ala119Ser, Leu432Val, Ala443Gly	Lower expression level, kinetic activity significantly lower

3.4.2. CYP2J2

CYP2J2, a 56 kDa protein, is localized primarily in the heart, kidneys, lungs and breast. [72, 79, 80] CYP2J2 has been shown to be involved in the metabolism of endogenous molecules, such as fatty acids, arachidonic and linoleic acid, as well as xenobiotic molecules such as ebastine, terfenadine, astemizole, amiodarone, albendazole, danazol, thioridazine, tamoxifen, cyclosporin A, nabumetone and mesoridazine. [53, 63, 104-106] Substrate overlap has been observed between CYP2J2 and 3A4, because both isoforms have large active sites. However, because CYP2J2 has a slightly more cylindrical and narrow active site, substrates are more restricted and therefore can only be metabolized at a single site.[53] This restriction leads to differences in regioselectivity between CYP2J2 and 3A4.[107] Figure 3 shows the active site of CYP2J2 where ebastine has been shown in place.

In order to understand the metabolism of CYP2J2, ebastine can be used. While CYP3A4 can metabolize ebastine, a different metabolite is formed by CYP3A4 (namely N-desmethyl-ebastine) than by CYP2J2 (namely Hydroxyebastine).[106] In addition, the expression of CYP3A4 mRNAs is almost non-detectable in breast cancer cell lines, so that the metabolism of ebastine within these cells is specific to CYP2J2.

3.4.2.1. Polymorphisms in *CYP2J2*

There are many known Single Nucleotide Polymorphisms (SNPs) for CYP2J2, both in the coding and non-coding regions. Some of these SNPS have been further characterized and have been given specific variant names (see Table 6). Significant loss in activity has been observed for variant *2, *3 and *6 in the metabolism of arachidonic acid and linolein acid, while *4 only showed a loss of function for arachidonic acid metabolism, and *5 showed no loss in functional activity. [108, 109] Variants *8 and *9 were analyzed using astemizole and ebastine as metabolites, where *8 showed almost a complete loss of function, while *9 showed wild type activity. [109, 110]

Since CYP2J2 has been shown to be capable of metabolizing xenobiotics, such as tamoxifen, an anti-cancer agent, the loss of function of this isoform, which is highly expressed in breast cancer cells, may have important implications on local drug metabolism.[53]

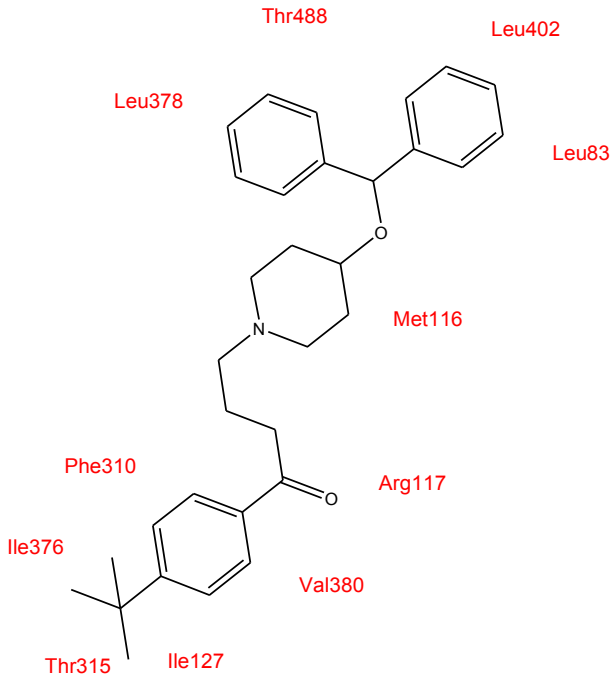

Figure 3: Active site of CYP2J2 (red). Ebastine is shown in the active site. (Adapted from Lafite, 2007.[111])

Table 7: Known polymorphisms of CYP2J2 and their effects on metabolic activity

Variant	Nucleotide Change	Amino Acid Substitution	Protein Activity
CYP2J2*1	None	None	Wild Type Activity
CYP2J2*2	427A>G	Thr143Ala	Significant decreased in metabolism of arachidonic and linoleic acid [108, 109]
CYP2J2*3	472C>T	Arg158Cys	Significant decrease in metabolism of arachidonic and linoleic acid [108, 109]
CYP2J2*4	575T>A	Ile192Asn	Significant decrease in metabolism of arachidonic acid, but no difference of linoleic acid [108, 109]
CYP2J2*5	1024G>A	Asp342Asn	Wild Type activity for arachidonnic and linoleic acid [108, 109]
CYP2J2*6	1210A>T	Asn404Tyr	Almost complete loss of metabolism for arachidonic and linoleic acid [108, 109]
CYP2J2*7	-50G>T	Promotor	Decreased promotor activity [109, 112]
CYP2J2*8	934G>A	Gly312Arg	Almost complete loss of catalytic activity for Astemizole and Ebastine [109, 110]
CYP2J2*9	1052C>T	Pro351Leu	Wild type activity for Astemizole and Ebastine [109, 110]
CYP2J2*10[109]	434C>T	Pro155Leu	No determined

4. ANTI-CANCER RESISTANCE

Several types of cancer cells have shown an innate or acute resistance to anti-cancer agents which in turn causes a failure in treatment. Even though our work has been focussed on metabolic factors that could affect anti-cancer resistance, it is also important to acknowledge other sources of resistance, such as the impact of membrane transporters on drug bioavailability, as well as the presence of Tumor-Initiating cells (T-ICs) and Cancer Stem Cells (CSCs). [113, 114]

4.1. DRUG BIOAVAILABILITY-MEMBRANE TRANSPORTERS

Many anti-cancer agents are known substrates of the membrane transporters, Multidrug Resistance Protein 2 (MRP2 encoded by the gene *ABCC2*), Breast Cancer Resistance Protein (BCRP encoded by the gene *ABCG2*) and P-glycoprotein (P-gp, encoded by the gene *ABCB1*), which are responsible for the efflux of these medications. Table 7 lists the efflux transporters responsible for the efflux of anti-cancer agents used in the treatment of breast cancer. [43, 44, 114-119] These three transporters, MRP2, BCRP and P-gp, are highly expressed in the liver membrane, specifically the cannulicular side. Therefore the bioavailability of anti-cancer agents, which are substrates of these transporters, will be greatly affected by the presence of these transporters.

However, these transporters are also present on the basolateral membrane of other tissues, acting as efflux transporters, where an elevated expression of BCRP is observed in breast cancer. [115, 120, 121] Some studies have shown that an overexpression of membrane transporters occurs when cells are treated with anti-cancer agents. One particular study treated the breast cancer cell line, MCF-7 with Adriamycin, and demonstrated that an overexpression of P-gp was present in MCF-7-Adriamycin resistant cells. [122] Other studies demonstrated an overexpression of BCRP in mitoxantrone resistant cells while VP-16 resistant cells overexpressed MRP transporters. [121, 123] Therefore, cancer cells are either upregulating their expressions of specific membrane transporters to reduce intracellular exposure to anti-cancer agents, or some cancer cells naturally have an overexpression of the specific membrane

transporters which aid in their resistance, resulting in a population selection. In either case, membrane transporters are locally expressed in breast cancer, which results in a resistance to anti-cancer agents.

Table 8: Anticancer agents used in the treatment of breast cancer, and transport by membrane efflux transporters

Anti-Cancer Agent	Efflux Transporters
Carboplatin	MRP2 [43]
Docetaxel	MRP2 and P-gp [43, 44, 114, 117]
Doxorubicin	MRP2, BCRP and P-gp [43, 44, 115-117]
Epirubicin	MRP2, BCRP and P-gp [43, 115, 116]
Gefitinib	BCRP [115]
Methotrexate	MRP2 and BCRP [43, 115, 116]
Paclitaxel	P-gp [43, 44, 117]
Raloxifene	P-gp and MRP2 [58, 118]
Tamoxifen	P-gp and BCRP [44, 115]
Toremifene	BCRP [115]
Vinblastine	MRP2, P-gp [44, 117]
Vinorelbine	P-gp [119]

4.2. STOCHASTIC CELL THEORY AND TUMOR-INITIATING CELLS

Stochastic cell theory states that every cell has the potential of becoming a tumor-initiating cell (T-IC). However, this property is not present in every cell, and the chance of developing this property is very low.[113] Therefore, only a small population of cells within a tumor are able to initiate tumor growth.[113] It is believed that the cause of the tumor-initiation process is due to random mutations and subsequent clonal selection.[124] However, this process leads to the production of a homogenous tumor.[113]

The stochastic theory states that T-ICs are resistant to chemotherapy agents and will ultimately lead to the relapse of cancer. However, because it is impossible to predict which cells are T-ICs, and they cannot be separated from non-T-ICs, T-ICs cannot be targeted to prevent relapses.[113] It is also unclear as to why T-ICs have developed a resistance to chemotherapy agents.

4.3. CANCER STEM CELL THEORY

The cancer stem cell (CSC) theory states that all tissues are derived from organ-specific stem cells. These cells have the capacity of self-renewal and differentiation, which ensures tissue integrity. The CSC hypothesis states that cancer develops from normal stem cells that have undergone oncogenic transformation.[124] Since stem cells are believed to be long living, slow dividing cells, they have a longer period of toxin exposure than regular cells, ultimately leading to the development of cancer and cancer stem cells.[125] Since stem cells have the properties of self renewal and differentiation, the expansion of the cell population can then lead to additional genetic and epigenetic changes.[124] In the breast, it is believed that the differentiation of CSC is limited to specific cell types, and therefore leads to the development of specific breast cancer molecular subtypes.[124]

4.3.1. Isolation of Cancer Stem Cells

Several methodologies for the isolation and purification of CSC have been suggested: dye exclusion (side population), cell culture selection through tumorospheres, cell surface marker ($CD44^{+}/CD24^{-/low}$), and an enzymatic assay for ALDH+ cells (ALDEFLUOR assay).[15, 124, 126, 127] The two most commonly used methods for the isolation of CSC are the cell surface markers and the ALDEFLUOR enzymatic assay.

4.3.1.1. $CD44^{+}/CD24^{-/low}$

CD44 and CD24 are cell surface markers that are often present in breast cancer cells. Cells which express CD44, but do not express CD24 have been described as being highly resistant to chemotherapy agents. Previous studies showed that placing MCF-7 cells in the presence of chemotherapy agents caused some cells to die, but others resisted. The remaining cells showed increased concentration of $CD44^{+}/CD24^{-/low}$ cells. [124]

$CD44^{+}/CD24^{-/low}$ cells have also demonstrated high tumorigenicity, where only 100-200 cells were required for tumor growth, whereas, tens of thousands of other phenotypes failed to form a tumor.[124, 126] $CD44^{+}/CD24^{-/low}$ cells have also been shown to be strongly correlated to the triple negative (TN) diagnosis of breast cancer, where TN demonstrates a high level of expression of $CD44^{+}/CD24^{-/low}$ cells. Specific antibodies tagged with fluorescent probes can be used to identify cells expressing CD44 and not expressing CD24.

4.3.1.2. ALDH

Aldehyde dehydrogenase (ALDH) is a superfamily of enzymes which consists of 19 isoforms.[128] ALDH is an intracellular detoxifying enzyme which is known to metabolize aldehydes to carboxylic acids through an oxidation reaction. [128, 129] These enzymes are also known to play a role in the oxidation of retinal to retinoic acid, [128, 130] as well as in the metabolism of some chemotherapy agents, such as cyclophosphamide.[128, 131, 132]

The isolation of $ALDH^{+}$ cells is possible using a kit called ALDEFLUOR which is manufactured by Stemcell Technologies.[124] Using the kit, $ADLH^{+}$ cells are able to be identified and sorted by Fluorescence Activated Cell Sorting (FACS).

Using the ALDEFLUOR kit, some clinical studies have shown that an elevated expression of ALDH activity had been linked to poor clinical outcomes, and that ALDH+ cells are capable of self-renewal *in vitro*. [15] Studies have also been performed to determine the tumorigenicty of ALDH+ cells. It was observed that NOD/SCID mice injected with as few as 500 ALDH+ cells developed a tumor. [133] Combination of ALDH and the cell surface markers (CD44+/CD24-) further amplified the tumorigenicity where only 20 cells were required for tumor growth. [133]

4.3.2. Resistance of Cancer Stem Cells

CSC are believed to show similar properties to normal stem cells including relative order, resistance to drugs and toxins, active DNA repair capacity and resistance to apoptosis. Therefore, CSCs are pluripotent, chemotherapy resistant cells that are capable of reinitiating tumor growth.[124]

CSCs have also been shown to be resistant to radiotherapy. The resistance is believed to be due to DNA damage checkpoints and an increase in the DNA repair cycle, through cell cycle-regulating proteins CHEK1 and CHEK2. This same resistance was observed in the breast cancer cell line, MCF-7, where following radiotherapy an increase in the CSC population (CD44+/CD24-/low) was observed.[124]

4.3.2.1. Membrane Efflux Transporters

The overexpression of membrane efflux transporters, namely the ABC transporter family (ATP-binding cassette transporters), have been identified as a potential source of CSC resistance. The three genes that have been most studied have been *ABCB1*, *ABCG2* and *ABCC1*, which encode the proteins, P-glycoprotein, Breast Cancer Resistance Protein, and MRP1, respectively.[124]

Breast cancer resistance protein (BCRP) or ABCG2 appeared to be upregulated in breast cancer stem cells isolated from breast cancer cell lines MCF-7 and MDA-MB-231 cells by 5.8 and 3.6 fold.[115, 134] The selection of cells that overexpress BCRP through the dye exclusion assay are referred to as the side population (rather than a CSC population).[124] Therefore, the elevated expression of BCRP in breast cancer stem cells could decrease the intracellular concentrations of certain anti-cancer agents, which could lead to the resistance of anti-cancer agents by CSCs.

5. CONCLUSIONS

Breast cancer cell lines and breast cancer tumors have been classified into various subtypes using two different classification systems. For breast cancer cell lines, three subtypes are used, Luminal, Basal A and Basal B, whereas breast cancer tumors are grouped into five subtypes, Luminal A, Luminal B, Basal-Like, HER2 overexpressing and normal-like. Generally, luminal subtypes contain membrane and intracellular receptors, such as estrogen receptors, progesterone receptors, and the human epidermal growth factor receptor 2 (HER2), and therefore can be treated with effective targeted treatments against these receptors. Basal subtypes are often associated with the triple negative cancer because they do not contain breast cancer receptors, and therefore cannot be treated with targeted treatments.

A resistance to anti-cancer agents used in the treatment of breast cancer is often observed, and this leads to a treatment failure for breast cancer patients. Some research shows that this resistance is due to the presence of breast cancer stem cells, as well as the presence of enzymes involved in the bioavailability of medications. Membrane transporters have been shown to be overexpressed in cells resistant to anti-cancer agent as well as in breast cancer stem cells.

However, our focus has been on the effects of the phase I metabolizing enzymes, Cytochrome P450s on the bioavailability of anti-cancer agents because many of these agents are metabolized by CYP450s. Some anti-cancer agents used in the treatment of breast cancer that are metabolized by CYP450s include tamoxifen, paclitaxel and cyclophosphamide.

CYP450s are involved in the systemic metabolism and local metabolism of xenobiotics as well as in the metabolism of endogenous molecules (such as steroids). The overexpression of some CYP450s, such as CYP1B1, has been associated with the development of breast cancer. CYP1B1 is particularly associated with breast cancer because many of its substrates, such as benzanthracene, benzo(a)pyrine, DMBA, 1-ethynyl-pyrene, 3-methyl-cholantrene and oestradiol, form metabolites which are considered

carcinogenic.[97] CYP1B1 and 2J2 are two isoforms which are of particular interest in breast cancer because they demonstrate an elevated expression in breast cancer cell lines. CYP1B1 is mainly implicated in the metabolism of endogenous molecules such as estrogens, while CYP2J2 plays a role in fatty acid metabolism. However, high active site homology is present between CYP1A1/2 and 1B1 as well as between CYP3A4 and 2J2 which suggests that many xenobiotics which are metabolized by CYP1A1/2 and 3A4 could also be substrates of CYP1B1 and 2J2, respectively.

While there is a lack of metabolism studies of anti-cancer agents by CYP1B1 and 2J2, it has been shown that these two enzymes are implicated in the metabolism of the anti-cancer agent tamoxifen. Therefore, other anti-cancer agents may also be metabolized by these two enzymes. Therefore the local metabolism potential of CYP1B1 and 2J2 are of interest as a potential source of resistance to anti-cancer agents.

Many SNPs have been documented for CYP1B1 and 2J2, some of which result in a loss of function. Therefore, the presence of these mutations may result in a decrease in enzyme efficiency and affect the intracellular concentrations of some anti-cancer agents.

In conclusion, CYP450s may play a crucial role in the local metabolism of anti-cancer agents, which may explain the resistance that is observed in patients. This may also help explain why there is such a larger inter-subject variability present between patients.

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CHAPTER 2

HYPOTHESIS AND OBJECTIVE OF STUDY

HYPOTHESIS

Knowing that many chemotherapy agents are substrates of various isoenzymes from the superfamily Cytochrome P450 (CYP450), these enzymes may play an important role in the bioavailability of many chemotherapy and anti-cancer treatments. The work executed was to determine if local metabolism, at the level of breast tissue, could have a significant impact on the local concentrations of chemotherapy agents, and hence be a source of anti-cancer treatment resistance observed in many patients.

OBJECTIVES OF STUDY

This project consisted of several objectives. However, the majority of the work consisted of Real-Time PCR experiments for the determination of mRNA expression of Cytochrome P450s. In order to complete reliable and concrete results, Housekeeping Genes are used to correct of concentration errors and product degradation. Therefore, the first objective was to screen the expression level of six potential Housekeeping Genes (*β-Actin, GAPDH, NUP-214, PPIG, RPLPO and TBP)* in the twenty-three cell lines used in house to determine which Housekeeping Gene demonstrated the most stable expression. Most of the screened housekeeping genes were chosen based on their common use in RT-PCR studies (*β-Actin, GAPDH, RPLPO and TBP)*, whereas *NUP-214* and *PPIG,* were selected because of their stability in paraffin-embedded breast samples.

Our secondary objective was to identify and characterize the role of Cytochrome P450s in breast cancer. Using *NUP-214* as the most stable Housekeeping Gene, the mRNA expression levels of nineteen specific CYP450 isoenzymes were determined by Real-Time PCR.

The third objective was to determine if the mRNA expression of the CYP450 isoforms 2J2 and 1B1 were sufficient to observe the metabolism of ebastine and 7-ethoxyresorufin, specific substrates of CYP2J2 and 1B1, respectively. Finally, correlation studies were completed to determine if mRNA expression was capable of predicting metabolic activities in these cell lines.

CHAPTER 3

SELECTION OF A STABLE HOUSEKEEPING GENE FOR RT-PCR ANALYSIS OF CULTURED CELLS; NUP-214 A PREFERRED CHOICE

Selection of a Stable Housekeeping Gene for RT-PCR Analysis of Cultured Cells: NUP-214 a Preferred Option

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ABSTRACT

Background: The accuracy of quantitative Real-Time PCR is highly dependent on a stable and reliable housekeeping gene. Certain endogenous genes, such as glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and *β-Actin* are commonly used to correct for mRNA degradation and concentration differences. However, these genes have been shown to be affected by experimental conditions and therefore cannot serve as ubiquitous controls. The need to identify a more stable control housekeeping gene is therefore required.

Results: The expression levels of six potential control genes (*β-Actin*, *GAPDH*, *NUP-214*, *PPIG*, *RPLPO*, *TBP*) were tested in a variety of cancer and non-cancer cell lines (n=23) (including breast cancer (n=7), endometrial cancer (n=5) and ovarian cancer (n=4)) in order to determine which gene could serve as a stable housekeeping gene. The results showed that *NUP-214* was the most stable control gene for all samples analyzed with a standard deviation of 0.55 Ct (CV=2.5%). *β-Actin* and *GAPDH*, showed greater variabilities, with standard deviations of 0.96Ct (CV=5.4%) and 1.01 Ct (CV=5.7%), respectively. *PPIG* which demonstrates a standard deviation of 1.243Ct (CV=4.3%) was not expressed in muscle cells.

Conclusions: Therefore, *PPIG, β-Actin* and *GAPDH* may be useful housekeeping genes under selected conditions. However, we propose that *NUP-214* can be used with less restriction as a housekeeping gene for RT-PCR analyses for cultured cells.

BACKGROUND

The correction or normalization of mRNA expression data by Real-Time Polymerase Chain Reaction (RT-PCR) is required to determine if relevant expression differences are present between samples. Commonly, normalization is performed using an endogenous gene, such as a housekeeping gene (HKG). Housekeeping genes are genes which are constitutively expressed because they are required for basic cell maintenance, and therefore should show stable expression despite treatments, stresses or experimental conditions.[1] However, many reports have shown that commonly used HKGs are not appropriate for all experimental conditions and therefore cannot be used to correct for RNA integrity.[2]

Most commonly used reference genes include: *GAPDH*, *β-Actin*, TATA-box binding protein (*TBP*) and Large Ribosomal Protein (*RPLPO*) (See Table 1 for gene details). Each of these genes plays a crucial role in the cell maintenance and growth. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) is essential for the production of cellular ATP, *β-Actin* is a cytoskeletal protein, TBP is a transcription factor while the RPLPO is required for Peptide Synthesis.

Previously Nucleoporin 214 (*NUP-214*) and Peptidyl-prolyl cis-trans isomerase G *(PPIG)* were demonstrated to be the most stable HKG in breast tumour formalin-fixed, paraffin-embedded samples.[3] *NUP-214* (also known as CAN), is a protein localized to the Nuclear Pore Complex (NPC), which is responsible for the transport of macromolecules inside and out of the nucleus.[4] *PPIG* is an enzyme which is responsible for the cis/trans isomerase reactions of amino acid side chains during the protein folding process.[5] Both these genes encode for proteins which play a crucial roles in the maintenance and growth of cells, which suggests mRNA stability of these genes.[3]

Therefore, the goal of this study is to evaluate the expression levels of six different HKGs in a variety of cell lines (coming from different pathologies and tissues), to determine which gene shows the

most stable expression pattern. If a stable HKG is present across the various cell lines, it could be utilized to correct for concentration differences and RNA integrity.

MATERIALS AND METHODS

Materials.

RPMI, DMEM, DMEM/F12 medias, Trypsin/EDTA and Fetal Bovine Serum (FBS) were purchased from Wisent Inc (St-Bruno, QC, Canada). MEGM bulletkit and SkGM bulletkit were purchased from Lonza (Walkersville, MD, USA). The following cell lines from various cancer sources were purchased from the ATCC (Manassa, VA, USA): Breast cancer (Hs578T, MCF-7, MDA-MB-231, MDA-MB-468, SKBR3, T47D, and ZR-75-1), Ovarian and endometrial cancer (AN3 CA, CaOV-3, ES-2, Hec-1B, KLE, NIH:OVCAR-3, PA-1, RL-95-2, and SKOV-3), Cervix Cancer (Hela), Hepatocellular Cancer (HepG2), and Colorectal Cancer (Caco2). Three benign cell lines served as controls, i.e. SkMC (Human skeletal muscle cells) purchased from Lonza, while Hek293T (embryonic kidney cell line), and MCF-10A (breast tissue cell line) were purchased from the ATCC. RNA extractions were performed using the QIAGEN RNA extraction Kit (Quiagen Sciences, MD, USA). SuperScript II Reverse Transcriptase, random primers and RNaseOUT Recombinant Ribonuclease Inhibitor were purchased from Invitrogen (Carlsband, CA, USA). 25mM dNTP mix was purchased from Wisent (St-Bruno, QC, Canada). Taqman Universal PCR Mix, probes and HKG were purchased from Applied Biosystems (Foster, CA, USA).

Cell Culture.

Caco2, CaOV-3, HELA, HepG2, Hs578T, MCF-7, MDA-MB-231, MDA-MB-468 and SKBR3 were cultured in DMEM media supplemented with 10% fetal bovine serum (FBS). NIH:OVCAR-3, T47D, and ZR-75-1 were cultured in RPMI supplemented with 10% FBS. AN3-CA, Hec-1-B, Hek293T and PA-1, were cultured in EMEM supplemented with 10% FBS, 5mL NEAA and 5 mL L-Glutamine. ES-2 and SKOV-3 were cultured in McCoy's 5A media supplemented with 10% FBS. MCF-10A was grown in MEGM media (Lonza) while KLE was grown in DMEM/F12 supplemented with 10% FBS and RL-95-2 in DMEM/F12 supplemented

with 10% FBS and 0.005ng/mL insulin. SkMC cells were grown in SkGM media (Lonza), while SkMC statin-induced cells were induced for 6 days with 2 µM Simvastatin. All cells were cultured at 37°C with 5% CO_2.

RNA Extraction.

Cell lines were grown to 70% confluency before RNA isolation. Briefly, cells were washed with PBS, trypsinized, and harvested at 100 x g. Under RNase free conditions, and using the QIAGEN RNA extraction kit, RNA was harvested and quantified by UV absorption at 260 and 280nm. RNA was stored at -80°C until use.

Reverse Transcriptase.

Isolated RNA was used to synthesize cDNA, where each 20 µL mixture contained 1 µg of RNA, 40 units of RNaseOUT inhibitor, 200 units of Superscript II reverse transcriptase, 3 µg of random primers, 1.25 mM dNTP, 10 mM DTT in first-strand buffer (at a final concentration of 50 mM Tris-HCl, pH 8.3; 75 mM KCl; 3 mM $MgCl_2$). Reverse transcriptase cycle was performed as per manufacturer's protocol. Briefly, RNA, random primers and water was heated at 65°C for 5 minutes. Contents were placed on ice. A second mixture containing buffer, DTT, RNaseOUT and Superscript was added to the RNA mix, which was then placed back in the thermal cycler (2720 Thermal Cycler, Applied Biosystems) and heated at 42°C for 50 minutes. Mixture was then inactivated by heating at 70°C for 15 minutes. cDNA was then aliquoted and stored at -80°C.

Real-Time-PCR Standard Curves.

All RT-PCR runs were performed using the RotorGene RG6000 (Corbett Research, Mortlake, Australia). Standard curves were prepared for each HKG using the RNA isolated from various cell lines known to highly express the RNA of interest. Samples contained 1 µL of 20X probes, 10 µL of 2X TaqMan Universal Mix and 4 µL of cDNA (final concentration ranging from 40-0.156 ng), where the final assay volume was completed to 20 µL with water. All samples were prepared on ice. Thermal cycling conditions were as follows: 95°C for 10 minutes, followed by 45 cycles of 95°C for 10 seconds and 60°C for 45 seconds. Every standard curve was validated using the technique described by Livak et al. in 2001, using Ct/log of cDNA concentration curve. [6]

Housekeeping Gene Selection.

Six commonly used HKG (*GAPDH*, *NUP-214*, *PPIG*, *β-Actin*, *RPLPO* and *TBP*) were tested in the twenty-three cell lines to determine which HKG would be the most stable. Samples were prepared in triplicate and contained 1 µL of 20X probes, 10 µL of 2X TaqMan Universal Mix and 4 µL of cDNA (final concentration of 10 ng), where the final assay volume was completed to 20 µL with water. The thermal cycling was as described for Standard Curves. Cycle thresholds (Ct) were determined and the deviation from average was determined.

Housekeeping Gene Relative Expression.

The stability of the six HKGs (*GAPDH*, *NUP-214*, *PPIG*, *β-Actin*, *RPLPO* and *TBP*) was assessed by calculating the relative expressions for each gene. Comparative relative expression levels were determined using the following equation:

Relative Expression: $2^{-(\text{Ct HKG-Ct HKG Average})}$

Housekeeping Gene Stability Analysis.

The stability of the six HKGs (*GAPDH*, *NUP-214*, *PPIG*, *β-Actin*, *RPLPO* and *TBP*) was assessed using an online database (Cotton EST Database). [7] This database gives a comprehensive assessment of the reference genes stability using four different methods; Delta CT, BestKeeper, Normfinder and Genorm.

RESULTS

Six commonly used HKG were tested in twenty-three cell lines to determine which gene would be the most stable and therefore better able to correct for RNA integrity. Threshold cycles (Ct) were determined for the six HKG, and the averages and standard deviations were calculated (Table 2). The stability or deviation of the cell lines HKG expression was analyzed to determine which HKG showed the smallest variations between samples (Figure 2). The online database Cotton EST was also used to determine the stability of these reference genes (Table 3).

The expression of each HKG, from most expressed to least expressed is as follows, where the values in Ct are shown: *RPLPO* (14.624), *GAPDH* (17.792), *β-Actin* (17.824), *NUP-214* (21.750), *TBP* (22.356) and *PPIG* (28.723). The expression level of the HKG is an important aspect to consider when choosing a candidate gene. When the expression of a gene is very strongly expressed, slight degradation of the product is going to be less significant than a gene that is more weakly expressed. However, a gene that is weakly expressed will generate more variability in RT-PCR analyses, and therefore yield less reliable results. Thus, a gene which is moderately expressed (around 20 Cts) like *NUP-214* will be sensitive enough to RNA degradation, but will be very stably expressed.

In order to evaluate the stability of each gene, the standard deviations for the inter-cell line expression and percent CVs were calculated, and is as follows: *RPLPO* (0.587Ct, 4.0%), *GAPDH* (1.014Ct, 5.7%), *β-Actin* (0.961Ct, 5.4%), *NUP-214* (0.546Ct, 2.5%), *TBP* (0.715Ct, 3.2%) and *PPIG* (1.243, 4.3%), respectively (see Table 2). Therefore, based on standard deviations, the order of stability for these genes are the following: *NUP-214*, *RPLPO*, *TBP*, *β-Actin*, *GAPDH*, and finally *PPIG*. However, based on percent CV, the stability order would be: *NUP-214*, *TBP*, *RPLPO*, *PPIG*, *β-Actin*, and *GAPDH*. In either case, it can be seen that *NUP-214* is the most stable.

Relative expressions can be calculated to evaluate the inter-cell line variability, and therefore gene stability. Figure 1 shows the relative expressions of each gene in each cell line. When a value is around 1, it signifies that the gene demonstrates around average expression. When a value is greatly above 1, the gene is more greatly expressed in that cell line compared to the other cell lines. Therefore, genes which demonstrate the least amount of deviation from 1 (or average) can be said to be more stable. As it can be seen, both *NUP-214* and *RPLPO* demonstrate relative expressions that are less that 2, but greater than 0.5 Cts. Since two cell lines did not express the gene *PPIG*, this gene shows large variations in relative expression, and should not be used as a reference gene.

Another way to evaluate the stability of these genes is to look at how much variability in gene expression is present between cell lines. Figure 2 shows the frequency distribution of variability for each gene, where the average expression (in Ct), was set at 0. Therefore, if a cell line has a much greater or weaker expression of a particular gene, there would be a greater difference from the average. The largest ΔCt that is present for each gene is as follows: *β-Actin* (1.5), *GAPDH* (2.0), *NUP-214* (1.0), *PPIG* (4.0), *RPLPO* (1.0) and *TBP* (1.5). This data demonstrates the same order of stability for each gene as when looking simply at the standard deviation.

Finally, the online database Cotton EST was used to determine the stability of each reference gene. This database uses four methods (Delta CT, BestKeeper, Normfinder and Genorm) in order to determine which reference gene is the best. Table 3 lists the rankings of each method, along with a comprehensive ranking. The data shows that *NUP-214* is the most stable reference gene using each system, whereas *PPIG* is the worst.

Therefore, *NUP-214* has the smallest overall standard deviation, of 0.55 Ct, demonstrates a ΔCt of 1.0, and was evaluated as the best HKG by the Cotton EST database. Overall, all analyses lead to the same conclusion; *NUP-214* is the most stable HKG candidate.

DISCUSSION

Results in this study demonstrate that *NUP-214* is the most stable of the HKGs analyzed in the twenty-three cell lines tested, despite their tissue source or pathology. Among the results, *PPIG*, *GAPDH* and *β-Actin* were the least stable or variably expressed genes in these same cell lines. Therefore, *NUP-214* is the superior choice as a HKG and can be used to effectively analyze mRNA differences between samples.

Housekeeping genes are genes which are constitutively expressed due to a cellular need for these specific proteins. In RT-PCR, HKGs are used to correct for RNA integrity as well as small concentrations differences which may exist from one sample to another. However, in some cases, classic HKG genes cannot be used when experimental conditions cause large variations in the gene's expression. Therefore, the selection of a stable HKG is important to ensure that the RNA is of good quality. The goal of this study was to determine if there was a HKG (among *GAPDH*, *β-Actin*, *RPLPO*, *TBP*, *NUP-214* and *PPIG*) that is stable across many different types of cells and which contain a variety of pathologies.

While *NUP-214* and *PPIG* are not commonly evaluated or used as HKGs, these two genes had been previously shown to be stably expressed in formalin-fixed paraffin-embedded (FFPE) breast cancer tissue [3]. Since *NUP-214* and *PPIG* were so stable in FFPE breast cancer tissue, the stability of these two genes in breast cancer cell lines were of interest. *NUP-214* proved to be the most stable HKG candidate for the 7 breast cancer cell lines analysed (and 1 non-cancerous breast cell line). Since *NUP-214* is almost never evaluated as a HKG candidate (except for in the paper by Iverson AA in 2009), we wanted to determine if *NUP-214* was universally stable in cell lines isolated from various tissues and pathologies, or if its expression stability was purely related to breast cancer cells. As can be seen in Figure 1 and 2, *NUP--214* is truly the most stable HKG candidate among the various cell lines evaluated.

The second most stable HKG determined was *RPLPO* with a standard deviation of 0.59 Ct. While this gene is a much better choice compared to *GAPDH* or *β-Actin*, it's stability is less than *NUP-214*. One issue that can be foreseen for *RPLPO* is the fact that it's expression level is much stronger than that of *NUP-214* (with an average Ct of 14.62 compared to 21.75 for *NUP-214*). Currently these results are based on RNA that was freshely isolated from cell lines that are grown under ideal conditions. Therefore, little to no degradation is present in the RNA. Since degradation is less apparent for genes which are very strongly expressed, small degradation of RNA samples may not be accurately corrected for when using *RPLPO* due to its strong expression.

Overall, *NUP-214* proves to be an excellent option as a HKG candidate. Not only is the gene stabilily expressed between cell lines, but is also stable within the cell line (ie, the Ct has very low variation, and so has a small standard deviation). *NUP-214* is also an excellent choice because of it's expression level (average Ct of 21.75). Therefore, its expression is weak enough that it will correct for RNA integrity, but strong enough that it shows very little variations between repeats. Therefore, based on this data, *NUP-214* could serve as a universal HKG candidate for a variety of cell lines.

CONCLUSION

In conclusion, we performed a thorough evaluation of the stability of six candidate HKGs (*β-Actin*, *GAPDH*, *NUP-214*, *PPIG*, *RPLPO*, *TBP*) in twenty-three different cell lines (coming from different pathologies and isolated from different tissues). This study serves to determine which candidate HKG is the most stablily expressed to determine if a universal HKG can be idenified. Overall, the results demonstrate that *NUP-214* is the most stable HKG identified in the different cell lines tested, and could serve as a universal HKG.

Table 1: Panel of 6 candidate housekeeping genes

General information for each of the candidate housekeeping genes evaluated.

Gene Symol	mRNA Accession Number	Gene Name	Function	Gene Aliases
NUP-214 (Hs01090093_m1*)	NM_005085	Nucleoporin 214kDa	Nuclear Pore Complex	CAIN CAN D9S46E MGC104525 N214 RP11-544A12.7
PPIG (Hs01081188_gH*)	NM_004792	Peptidylprolyl isomerase G (cyclophilin G)	Cis/Trans Isomerization enzyme	CARS-Cyp CYP MGC133241 SCAF10 SRCyp
ACTB	NM_001101	Beta-Actin (*β-Actin*)	Cytoskeletal structural protein	
GAPDH	NM_002046	Glyceraldehyde-3-phospate dehydrogenase	Glycolytic enzyme	G3PD GAPD
RPLPO	NM_001002	Large Ribosomal Protein	Peptide Synthesis	L10E MGC111226 MGC88175 P0 PRLP0 RPP0
TBP	NM_003194	TATA-box binding protein	Transcription factor	GTF2D GTF2D1 HDL4 MGC117320 MGC126054 MGC126055 RP1-191N21.3 SCA17 TFIID

Table 2: Expression of housekeeping genes

The expression of each housekeeping gene in all cell lines is listed in Threshold Ct. The data was performed in triplicate and the table lists the standard deviation of the gene for each cell line. The average Ct, overall standard deviation and percent CV was calculated for each gene using all cell lines. (ND: Not detected).

Gene	*β-Actin*	*GAPDH*	*NUP-214*	*PPGI*	*RPLPO*	*TBP*
KLE	16.15 ± 0.06	17.68 ± 0.38	22.00 ± 0.59	28.35 ±0.08	15.18 ± 0.32	22.50 ± 0.91
RL-95-S	17.21 ± 0.07	18.57 ± 0.36	21.56 ± 0.09	27.83 ± 0.23	15.09 ± 0.05	23.63 ± 1.23
SK-OV-3	17.65 ± 0.17	18.05 ± 0.04	21.58 ± 0.08	27.82 ± 0.09	15.29 ± 0.21	21.29 ± 0.60
CA-OV-3	16.28 ± 0.24	15.54 ±0.06	21.46 ± 0.21	28.78 ± 0.32	13.82 ± 0.18	21.74 ± 0.15
ES-2	19.42 ± 0.29	18.19 ± 0.05	22.04 ± 0.09	28.88 ± 0.31	14.55 ± 0.20	23.65 ± 0.30
PA-1	16.31 ± 0.04	17.9 ± 0.02	21.01 ± 0.10	32.67 ± 0.29	14.2 ± 0.07	21.71 ± 0.09
Hec-1B	17.88 ± 0.04	18.2 ± 0.01	22.26 ± 0.07	29.65 ± 0.23	15.09 ± 0.09	22.53 ± 0.66
OVCAR-3	17.38 ± 0.03	17.31 ± 0.06	20.82 ± 0.06	28.28 ± 0.12	14.84 ± 0.26	21.57 ± 0.17
AN3 CA	18.42 ± 0.03	18.52 ± 0.40	22.18 ± 0.09	30.27 ± 0.18	15.48 ± 0.10	22.28 ± 0.25
HS578T	17.26 ± 0.04	17.17 ± 0.18	21.7 ± 0.28	28.76 ± 0.17	14.53 ± 0.22	22.04 ± 0.16
SKBR3	18.31 ± 0.02	17.7 ± 0.13	21.3 ± 0.19	27.44 ± 0.12	14.00 ± 0.17	20.81 ± 0.25
MDA-MB-468	17.78 ± 0.13	16.81 ± 0.04	21.94 ± 0.22	28.72 ± 0.16	14.99 ± 0.01	22.54 ± 0.18
MDA-MB-231	17.74 ± 0.13	16.97 ± 0.12	21.78 ± 0.10	29.57 ± 0.26	14.09 ± 0.07	23.17 ± 0.60
MCF-7	19.24 ± 0.17	18.36 ± 0.03	22.83 ± 0.12	28.46 ± 0.28	13.39 ± 0.01	22.49 ± 0.17
MCF-10A	14.68 ± 0.01	16.54 ± 0.23	21.8 ± 0.14	28.18 ± 0.14	15.15 ± 0.24	22.28 ± 0.26
T47D	18.19 ± 0.07	18.83 ± 0.03	21.1 ± 0.06	27.47 ± 0.29	14.44 ± 0.11	23.11 ± 0.01
ZR-75-1	17.17 ± 0.02	15.66 ± 0.05	21.6 ± 0.05	26.48 ± 0.13	14.54 ± 0.10	22.41 ± 0.14
HeG2	18.99 ± 0.02	19.48 ± 0.05	21.73 ± 0.06	28.42 ± 0.13	14.92 ± 0.10	22.43 ± 0.03
Caco2	17.84 ± 0.18	17.41 ± 0.09	21.65 ± 0.06	28.27 ± 0.11	14.98 ± 0.12	21.69 ± 0.24
Hela	17.68 ± 0.41	17.49 ± 0.16	21.55 ± 0.22	29.01 ± 1.21	15.29 ± 0.5	22.14 ± 0.55
Hek293	17.02 ± 0.23	17.65 ± 0.15	20.93 ± 0.37	29.53 ± 1.9	15.49 ± 0.73	21.88 ± 1.2
SkMC	19.34 ± 0.14	19.37 ± 0.01	22.8 ± 0.08	ND	14.13 ± 0.41	23.06 ± 0.28
SkMC Statin Induced	19.16 ± 0.17	19.15 ± 0.06	22.6 ± 0.12	ND	14.09 ± 0.47	22.88 ± 0.25
Average	**17.824**	**17.792**	**21.75**	**28.723**	**14.624**	**22.356**
Standard Deviation	**0.961**	**1.014**	**0.546**	**1.243**	**0.587**	**0.715**
Percent CV	**5.40%**	**5.70%**	**2.50%**	**4.30%**	**4.00%**	**3.20%**

Table 3: Cotton EST database evaluation of reference gene expression

Data imported into online database, were reference gene expression was evaluated using four different methods (Delta CT, BestKeeper, Normfinder, and Genorm). For the cell line that did not express *PPIG* a value of 40 was used for calculation purposes.

Method	Ranking (best to worst)					
	1	2	3	4	5	6
Delta CT	*NUP-214*	*TBP*	*β-Actin*	*GAPDH*	*RPLPO*	*PPIG*
BestKeeper	*NUP-214*	*RPLPO*	*TBP*	*β-Actin*	*GAPDH*	*PPIG*
Normfinder	*NUP-214*	*GAPDH*	*β-Actin*	*TBP*	*RPLPO*	*PPIG*
Genorm	*NUP-214/TBP*		*β-Actin*	*GAPDH*	*RPLPO*	*PPIG*
Recommended comprehensive ranking	*NUP-214* (1.00)	*TBP* (2.21)	*β-Actin* (3.22)	*GAPDH* (3.56)	*RPLPO* (3.98)	*PPIG* (6.00)

Figure 1: Housekeeping gene stability

Relative expression calculated using the average threshold cycle as the equilibrator for each gene. All 23 cell lines are shown for each gene. *PPIG* was not expressed in muscle cells, so the relative expression was calculated using 40 as a Ct value

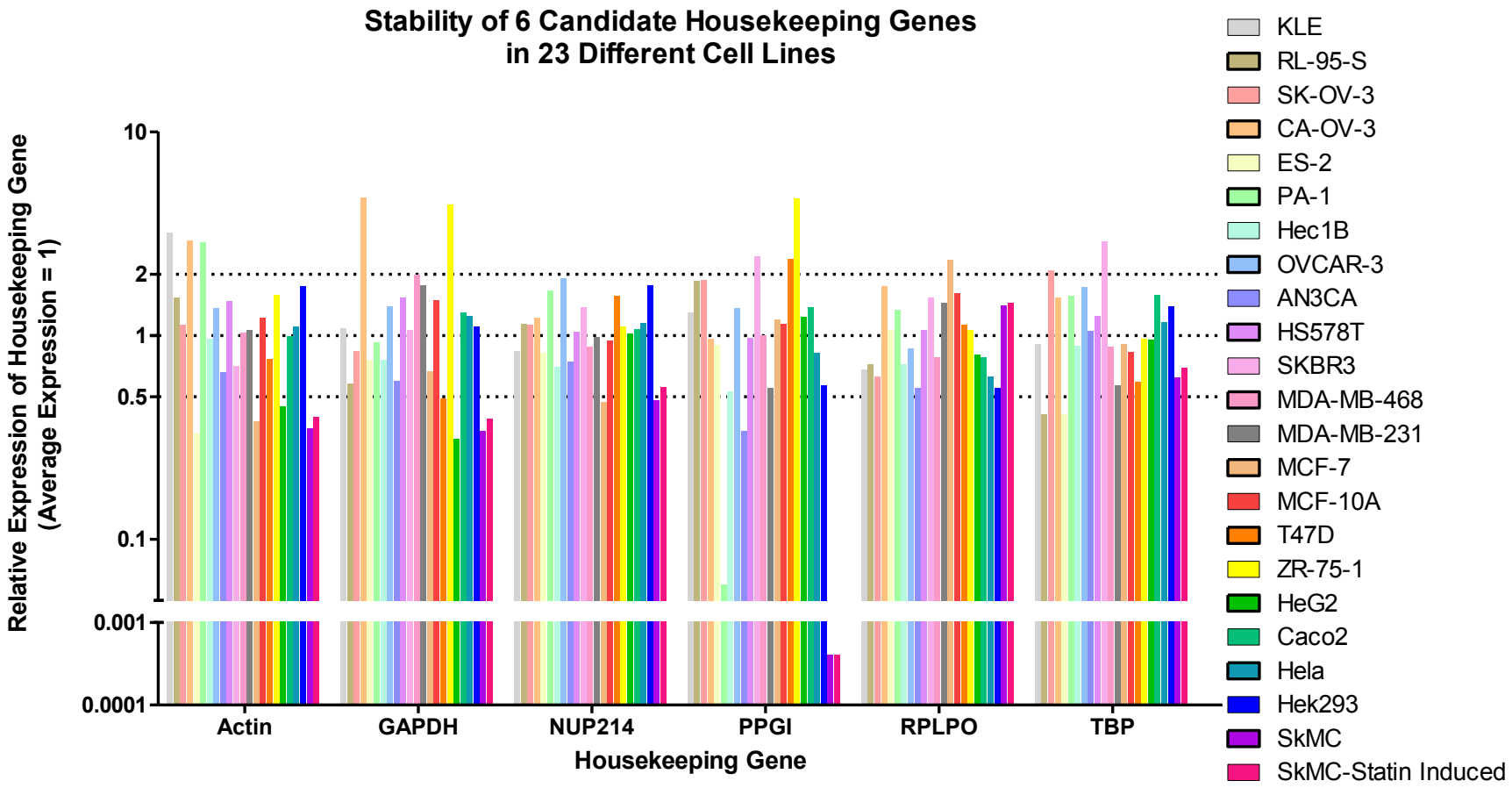
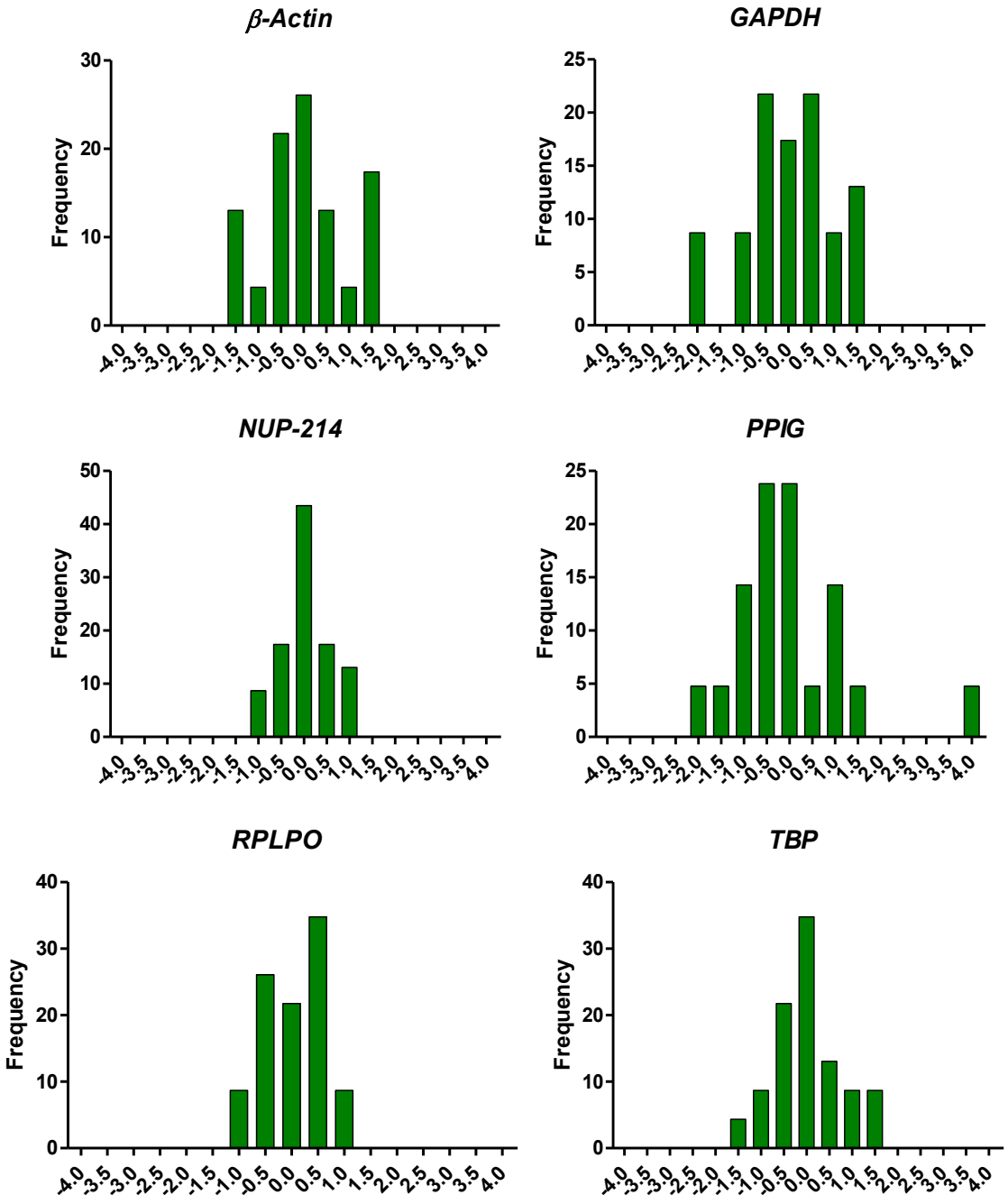


Figure 2: Frequency distributions of housekeeping gene expression

Average threshold cycle was calculated for each gene, and is placed at 0. Data shows that *NUP-214* has the least deviation between the cell lines for the housekeeping genes tested.



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CHAPTER 4

EXPRESSION OF CYP450 MRNAS IN VARIOUS BREAST CANCER CELL LINES

Characterization of CYP450 mRNAs relative expression and metabolic activities of CYP1B1 and 2J2 in various breast cancer cell lines

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Short title: CYP450 expression in breast cancer

ABSTRACT

Background : Cytochrome P450 (CYP450) enzymes are known to be involved in the metabolism of chemotherapy agents that are commonly used in the treatment of breast cancer. While CYP450s are primarily found in the intestines and liver, where they likely control the systemic exposure to drugs, they are also present at significant levels in other tissues. In such tissues, they would be responsible for the local metabolism of drugs. Hence, variable expression of CYP450 genes at the tissue level, next to their effector site, could explain inter-subject variability in the local metabolism, efficacy and toxicity of drugs. In order to determine if gene expression is variable for these enzymes, their expression level of mRNAs in breast cancer cell lines and functional CYP450 activities were evaluated. **Methods:** Seven commonly used breast cancer cell lines, (Hs578T, MDA-MB-231, MDA-MB-468, MCF-7, SKBR3, T47D, ZR-75-1) and one benign breast cell line (MCF-10A) were cultured and then extracted for RNA. The RNA was reverse transcribed to cDNA where the mRNA levels of 19 CYP450 isoenzymes were determined by Quantitative Real-Time PCR. Functional activities of CYP1B1 and 2J2 were determined by incubating whole cells with 7-ethoxyresorufin and ebastine, respectively, and measuring the metabolite produced by LC-MS. **Results:** The relative mRNA levels of the different CYP450 enzymes showed a large variability between the different cell lines. CYP1B1 mRNA was highly expressed in most of the breast cancer cell lines; CYP2J2 mRNA is the most abundant CYP450 found in SKBR3 and ZR-75-1 cell lines contributing 62.9 and 15.4% of their total CYP450 make-up respectively; CYP3A4/5/7 mRNAs expressions were 41.5, 13.9 and 43.3 times greater in the benign control compared to the mean value; whereas 2C9 mRNAs showed no expression in any cell line analyzed. The metabolism of 7-ethoxyresorufin was observed in 4 of the 8 cell lines, where the Km observed was between 0.05 to 0.1 µM and Vmax ranged from 0.04 to 3.7 pmol/mg protein/min. The metabolism of ebastine was observed in 7 of the 8 cell lines, where the Km was between 0.2 and 3.1 µM and Vmax

between 0.58 and 2.6 pmol/mg protein/min. Correlation studies between mRNA expression of CYP1B1 and CYP2J2 and the metabolism of 7-ethoxyresorufin and ebastine, respectively, revealed very strong correlations of 0.98 and 0.99, respectively. **Conclusion:** The variability in expression levels of CYP450 mRNAs appears to be characteristic of the different cell lines analyzed. We propose that the variable expression of CYP450s in breast cancer cells could explain part of the inter-subject variability in response to chemotherapy agents. The variability of CYP450 mRNAs expression may also identify new targets for the synthesis of anti-cancer agents.

Keywords: Breast Cancer, mRNA, qualitative RT-PCR, CYP1B1, CYP2J2

INTRODUCTION

The cytochrome P450 (CYP450) superfamily is a family of enzymes which work to detoxify the body of many xenobiotic molecules by biotransforming them into more hydrosoluble molecules.[1] In human, there are 57 known CYP450 isoforms which have been classified based on their sequence homology, and have various roles in the metabolism and synthesis of molecules. CYP450s isoforms play an important role in the synthesis and degradation of many endogeneous molecules, such as steroids. CYP1A1, 1A2 and 1B1 have been shown to be implicated in the hydroxylation of progesterone, testosterone and estrogen. [2-5] CYP17A1 and 19A1 are two isoforms which are also important in the formation of androstenedione through the use of aromatase activity. [3,6] Since these steroids have been shown to impact breast cancer survival and treatment, these isoforms will be important to evaluate. [7]

CYP450s are most abundantly found in the liver, where CYP3A4 is considered the most important for drug metabolism. However, the presence of CYP450 enzymes, with tissue specific expression, have been shown in other tissues such as the intestines, kidneys, brain, lungs and heart at varying concentrations. [8-15] The expression of specific enzymes in extra hepatic tissue shows that local metabolism could play an important role in drug metabolism and cause a source of variation in drug effects.

Many chemotherapy agents, both active drug and pro-drug, are known substrates of CYP450s.[16] Therefore, the presence of CYP450s at both the intestinal/hepatic level and the tissular level will influence the response of chemotherapy agents. At the hepatic level, the CYP450s present cause

decreased bioavailability of medications. The portion of the medication that reaches the targeted cells would then encounter other CYP450s which would locally metabolize the drug (either for clearance or activation purposes). For active drugs, if the molecule is a substrate of a specific CYP450 highly expressed in the cells of interest, it would be metabolized too quickly from the targeted cell and cause no local effect; whereas a pro-drug which requires CYP450 metabolism could be activated locally due to the presence of a specific enzyme at the targeted site. Since tissue specific expression of CYP450 enzymes are expected so is the metabolism potential.

Inter-subject variability of CYP450 mRNA expression would be expected in breast tissue based on similar results observed in other tissues. This can also be suggested when evaluating the efficacy of chemotherapy agents in breast cancer cell lines. A study performed in 2009 evaluated the effects of three chemotherapy agents in nineteen breast cancer cell lines to determine the efficacy of the chemotherapy agents. The study showed that not every chemotherapy agent was effective in all cell lines analyzed which shows that inter-subject response is present.[17] Since chemotherapy agents are substrates of CYP450 enzymes, the response difference of the cell lines may be partly explained by the presence of differing CYP450 expression.

Therefore, we believe that breast tumors will display variations in CYP450 expression, and this tissue specific expression could cause a variation in local metabolism which is an important phenomenon to consider in breast cancer treatment and survival. To evaluate this point, commonly used cell lines were analyzed for mRNA expression of CYP450 enzymes, which we believe will display inter-cell line variation, which would indicate a potential variation in breast

cancer patients. In addition, functional activities of CY1B1 and 2J2, two isoforms which have been shown to be highly expressed through this work, were determined using two specific substrates, 7-ethoxyresorufin and ebastine, respectively. These differences could help identify new pathways for the synthesis of new anti-cancer agents, either as pro-drug or active drugs.

MATERIALS AND METHODS

Materials. RPMI, and DMEM medias, trypsin/EDTA and fetal bovine serum (FBS) were purchased from Wisent Inc (St-Bruno, QC, Canada). MCF-7, SKBR3, Hs578T, MDA-MB-231, MDA-MB-468, T47D, ZR-75-1 and MCF-10A were purchased from the American Type Culture Collection, ATCC (Manassa, VA, USA). RNA extractions were performed using the QIAGEN RNA extraction kit (Quiagen Sciences, MD, USA). Superscript II reverse transcriptase, random primers and RNaseOUT recombinant ribonuclease inhibitor were purchased from Invitrogen (Carlsband, CA, USA). 25mM dNTP mix was purchased from Wisent (St-Bruno, QC, Canada). Taqman universal PCR mix, probes and housekeeping genes (HKGs) were purchased from Applied Biosystems (Foster, CA, USA). MEGM media with bulletkit and MycoAlert mycoplasma detection kit were purchased from Lonza Rockland Inc (Rockland, ME, USA). Ebastine, hydroxy ebastine, carebastine, $^{2}H_{5}$-hydroxyebastine, $^{2}H_{5}$-carebastine and $^{2}H_{6}$-hydroxybupropion and 7-ethoxyresorufin were purchased from Toronto Research Chemicals (North York, Ontario, Canada). Resorufin sodium salt, cholera toxin, glucose-6-phoshate, magnesium chloride, β-nicotinamide adenine dinucleotide phosphate sodium salt hydrate and glucose-6-phosphate dehydrogenase were purchased from Sigma (St-Louis, MO, USA). Pierce BCA protein assay kit was purchased from Thermo Scientific (Rockford, IL, USA). Recombinant supersomes, rCYP1A1, 1B1 and 2J2 were obtained from BS Scientific (Mississauga, ON, Canada). Acetonitrile and formic acid were obtained from EMD Chemicals (Gibbstown, NJ, USA). Other chemicals, including methanol were purchased from Fisher Scientific (Fair Lawn, NJ,

USA). Water was deoinized using a Nanopure Barnstead/Thermolyne system (Dubuque, IA, USA).

Cell Culture. MCF-7, SKBR3, Hs578T, MDA-MB-231 and MDA-MB-468 were cultured in DMEM media supplemented with 10% fetal bovine serum (FBS). T47D and ZR-75-1 were cultured in RPMI supplemented with 10% FBS and MCF-10A were grown in MEGM media with supplements and 0.05 ng/mL cholera toxin. All cells were cultured at 37°C with 5% CO_2 and were tested negative for mycoplama.

RNA Extraction. Cell lines were grown to 70% confluency before RNA isolation. Briefly, cells were washed with PBS, trypsinized, and harvested at 100 x g. Under RNase free conditions, and using the QIAGEN RNA extraction kit, RNA was harvested and quantified by UV absorption at 260 and 280nm. RNA was stored at -80°C until used.

Reverse Transcriptase. Isolated RNA was used to synthesize cDNA, where each 20 µL mixture contained 1 µg of RNA, 40 units of RNaseOUT inhibitor, 200 units of Superscript II reverse transcriptase, 3 µg of random primers, 1.25 mM dNTP, 10 mM DTT in first-strand buffer (at a final concentration of 50 mM Tris-HCl, pH 8.3; 75 mM KCl; 3 mM $MgCl_2$). Reverse transcriptase cycle was performed as per manufacturer's protocol. Briefly, a mix containing RNA, random primers and water was heated at 65°C for 5 minutes. Contents were then placed on ice. A second mixture containing buffer, DTT, RNaseOUT and Superscript was added to the RNA mix, which was then placed back in the thermal cycler (2720 Thermal Cycler, Applied Biosystems) and heated at 42°C

for 50 minutes. The mixture was then inactivated by heating at 70°C for 15 minutes. cDNA was then aliquoted and stored at -80°C.

Real-Time-PCR Standard Curves. All Real-Time PCR runs were performed using the RotorGene RG6000 (Corbett Research, Mortlake, Australia). Standard curves were prepared for each CYP450 using the RNA isolated from various cell lines known to highly express the RNA of interest. Samples contained 1 µL of 20X probes, 10 µL of 2X TaqMan Universal Mix and 4 µL of cDNA (final concentration ranging from 0.156-40 ng), where the final assay volume was completed to 20 µL with water. All samples were prepared on ice. Thermal cycling conditions were as follows: 95°C for 10 minutes, followed by 45 cycles of 95°C for 10 seconds and 60°C for 45 seconds. Every standard curve was validated using the technique described by previously, using Ct/log of cDNA concentration curve. [18]

CYP450 mRNA determination. Expression levels of 19 CYP450 isoenzymes were determined by Real-Time PCR. Samples were prepared in triplicate using the TaqMan universal mix and specific CYP450 probes from Applied Biosystems, along with 10 ng of cDNA in a final assay volume of 20 µL. The thermal cycling was as described for standard curves. Cycle thresholds (Ct) were determined and the deviation from average was determined. NUP-214 was used as the housekeeping gene (HKG) because it was previously shown to be the most stable.[19] Comparative quantitative relative expression levels were determined using the following calculations:

ΔCt_{CYP450}: $Ct_{CYP450} - Ct_{NUP-214}$

$\Delta\Delta Ct_{CYP450}$: $\Delta Ct_{CYP450} - \Delta Ct_{CYP450\ median}$

Relative Expression: $2^{-\Delta\Delta Ct\ CYP450}$

Total CYP450 mRNA determination. Expression levels of total CYP450 mRNA was determined by making a master mix containing all specific probes for the 19 CYP450 isoenzymes. To a total volume of 20 µL, 0.2 µL of each probe was added to 10 µL of 2X Taqman and 10 ng of cDNA. The expression levels were determined using the same conditions as described for the housekeeping gene selection. NUP-214 was used as the HKG. Comparative quantitative relative expression levels were determined using the following above calculations.

Ebastine Metabolism in Breast Cancer Cell Lines. Cells were plated in 24 well plates and allowed to grow to confluence for 3 days. Cells were then incubated with 500 µL of various concentrations of ebastine (0.1 to 5 µM) prepared in DMEM without phenol red or serum. After 1 hour at 37°C, the reaction was stopped by adding 500 µL of 1 M formic acid in acetonitrile. The reaction mixture was collected and centrifuged at 13 rpm for 10 minutes. 100 µL of the supernatant was then transferred to a borosilicate tube, where 100 µL of internal standard (25 µg/mL $^{2}H_{5}$-hydroxyebastine, $^{2}H_{5}$-carebastine) was added prior to evaporation. Samples and standards were resolubilized in 200 µL of 1 M formic acid in acetonitrile and analyzed by LC-MS-MS. Cell proteins were quantified using the BCA Pierce Kit.

7-Ethoxyresorufin Metabolism in Breast Cancer Cell Lines. Cells were plated in 12 well plates and allowed to grow to confluence for 3 days. Cells were then incubated with 500 µL of various concentrations of 7-ethoxyresorufin (0.01 to 1 µM) prepared in DMEM without phenol red or serum. After 5 hour at 37°C, the reaction was stopped by adding 500 µL of internal standard (10 µg/mL $^{2}H_{6}$-hydroxybupropion) prepared in 1 M formic acid in Acetonitrile. The reaction mixture was collected and centrifuged at 13 rpm for 10 minutes. Samples were analyzed by LC-MS-MS. Cell proteins were quantified using the BCA Pierce Kit.

Ebastine and 7-ethoxyresorufin metabolism in recombinant supersomes. Ebastine was incubated in the presence of recombinant CYP2J2 supersomes while 7-ethoxyresorufin was incubated in the presence of recombinant CYP1B1 supersomes. Incubations containing supersomes, buffer and an NADPH regenerating system (3.3 mM $NADP^{+}$, 3.3 mM Glucose-6-phophate, 5 mM $MgCl_2$ and 0.2 Units of glucose-6-phosphate dehydrogenase) were pre-incubated at 37°C for 10 minutes. To initiate the reaction, various concentrations of ebastine (0.0 to 100 µM), and 7-ethoxyresorufin (0.01 to 1 µM) were added and incubated at 37°C for 20 and 10 minutes respectively. Reactions were terminated by adding two times the volume of internal standard prepared in methanol, and placed on ice. Cells were centrifuged and analyzed by LC-MS-MS.

LC-MS-MS Analysis:

Standard solutions Hydroxy ebastine and carebastine stock solutions were prepared in methanol at 2.059 mM and 2.001 mM respectively. Resorufin stock solution was prepared in type 1 water at

4.69 mM. A series of standard working solutions containing hydroxy ebastine, carebastine and resorufin were obtained by diluting the standard stock solutions with methanol. Calibration standards were prepared by fortifying incubation media with the standard working solutions at 2% (v/v) to enable concentrations spanning the following analytical ranges 4.0 – 2000 nM for hydroxy ebastine, 60-30,000 pM for carebastine and 2.0 – 1000 nM for resorufin. The internal standard working solution was prepared at 5.0 ng/mL for $^{2}H_{5}$-hydroxyebastine, $^{2}H_{5}$-carebastine and $^{2}H_{6}$-hydroxy bupropion in methanol.

Instrumentation The HPLC system consisted of a Shimadzu Prominence series UFLC pump and auto sampler (Kyoto, Japan). The tandem MS system used was a Thermo TSQ Quantum Ultra (San Jose, CA, USA). Data were acquired on a Dell Precision desktop computer (Round Rock, TX, USA) equipped with operation Windows XP professional. Data acquisition and analysis were performed using Xcalibur 2.0.7 (San Jose, CA, USA). Calibration curves were calculated from the equation y = ax + b, as determined by weighted (1/x) linear regression of the calibration line constructed from the peak-area ratios of the drug to the internal standard.

An isocratic mobile phase was used with a Thermo Scientific Aquasil C18 column (100 x 2.1 mm I.D., 5 μm) operating at 40°C. The mobile phase conditions consisted of 10 mM ammonium formate pH 3.0 and acetonitrile at a ratio of 40:60, respectively. The flow rate was fixed at 0.30 mL/min and resorufin, carebastine and ebastine eluted at 1.6, 7.3 and 7.4 min, respectively. Five microliters of the extracted sample was injected and the total run time was set at 10.0 min.

The mass spectrometer was interfaced with the UPLC system using a pneumatic assisted heated electrospray ion source. MS detection was performed in positive ion mode, using selected reaction monitoring (SRM). The precursor-ion reactions were set at 214.0 → 103.0, 486.1 → 167.0 and 500.1 → 167.0 for resorufin, hydroxy ebastine and carebastine respectively. The precursor-ion reactions for the internal standards were set at 256.0 → 138.9, 491.1 → 171.9 and 505.1 → 172.0 for the internal standards $^{2}H_{6}$-hydroxybupropion, $^{2}H_{5}$-hydroxyebastine and $^{2}H_{5}$-carebastine. In order to optimize the MS/MS parameters, a standard solution of each analyte was infused into the mass spectrometer. The following parameters were obtained. Nitrogen was used for the sheath and auxiliary gases and was set at 35 and 20 arbitrary units. The HESI electrode was set to 3000 V. The capillary temperature was set at 300°C and its voltage offset was 35 V. Argon was used as collision gas at a pressure of 1.5 mTorr. The collision energy was set at 29 eV for all compounds. Scan width for SRM was 0.5 m/z; and scan time 0,1s. Peak width of Q1 and Q3 were both set at 0.7 FWHM.

Data Analysis The values obtained in the metabolism assays were an average of triplicate determination. The data was fit to the following Michaelis-Menten equation in order to determine its kinetic parameters (V_{max} and K_m) using GraphPad Prism 5 (GraphPad Software, Inc. CA, USA);

$$y = \frac{V_{max} \times x}{(K_m + x)}$$

where x is the substrate concentration, y is the enzyme velocity, Vmax is the maximum reaction velocity and Km is the Michaelis-Menten constant.

$$Cl_{int} = \frac{V_{max}}{K_m + [S]}$$

Where the Vmax is expressed in pmol/mg protein/min and the Km in µM (or pmole/µL), and results in a Clint in µL/mg protein/min.

Genomic DNA extraction: Cells were harvested and their genomic DNA was extracted using the kit GenElute Blood Genomic DNA kit, Miniprep (Sigma) following manufacturer's instructions. Extracted DNA was then stored at -20°C.

CYP1B1 Sequencing. Using genomic DNA extracted from the breast cancer cell lines, CYP1B1 exons 2 and 3 were cloned and sequenced. The CYP1B1 gene is encoded across 3 exons total, however, the protein is encoded only on exon 2 and 3. The primers used for cloning, and sequencing can be found in Table 3. For exon 2 and 3, the following concentrations were used in a total volume of 25 µL and 50 µL, respectively : 1.25U Taq polymerase, 2 mM $MgCl_2$, 0.2 mM dNTP, 4% DMSO and 0.4 µM of each primer. The thermal cycling was as follows: exon 2, an initial denaturation of 95°C for 4 minutes, following by 35 cycles of 95°C for 1 minutes, 58°C for 45 seconds and 72°C for 4 minutes, and finished with a final elongation of 10 minutes at 72°C. For exon 3, an initial denaturation of 95°C for 4 minutes, following by 35 cycles of 95°C for 45 seconds, 54°C for 45 seconds and 72°C for 2 minutes, and finished with a final elongation of 10 minutes at 72°C. Bands were run on a 0.7% agarose gel, and the DNA extracted using the EZ-10 spin column DNA gel extraction kit from Bio Basic Inc following manufacturer's instructions. Extracted DNA was sequenced using the sequencing primers found in Table 3.

RESULTS

Determination of relative expression of CYP450s in breast cancer cell lines. The relative expression of CYP450 mRNAs was evaluated in seven breast cancer, and one benign breast cell line. Table 1 lists the relative expression of each CYP450 enzyme mRNAs by calculating the ΔCt of the isoenzyme to NUP-214, followed by the ΔΔCt to the Ct value of the median cell line. For each isoenzyme, the cell line which expresses the mRNA the most is highlighted, where the greater the value, the greater the expression level. This data can also be visualized in Figure 1, 2 and 3. It can be seen that certain mRNAs are expressed mainly in one cell line, while others are more evenly expressed. The data shows that CYP450s mRNAs of family 3 (Figure 3) are more strongly expressed in MCF-10A cells than in the cancer cell lines. When comparing the expression of the CYP450 family 3 mRNAs in MCF-10A cells to the cell line with the next greatest expression, the following is observed. For CYP3A4, this expression proves to be 4.1 fold higher in MCF-10A compared to MDA-MB-231 ($p=0.085$). For CYP3A5, the expression is 4.8 fold higher in MCF-10A compared to SKBR3 cells ($p=0.0184$), and finally for CYP3A7, a 26 fold increase is observed compared to SKBR3 cells ($p=0.041$). CYP1A2 mRNA is another interesting observation where T47D cells greatly express this mRNA, while the other cell lines show minimal expression ($p=0.0003$). The selective expression of the CYP3A subfamily in the benign cell line, MCF-10A is of great interest. Since CYP3A4 is the most important enzyme involved in the metabolism of drugs, the overexpression of this subfamily in MCF-10A cells would signify a greater clearance capacity of many medications from cancer free cells. [20]

Figure 4 graphically represents the expression levels of the various CYP450 mRNAs in each cell line. The difference in the Ct values between the values obtained in Table 1 and Figure 4 is in the

threshold used to extract the Ct values. In Table 1, the values were obtained by using the individual standard curves for each CYP450 mRNAs. However, in order to properly compare the expression levels for each cell line, the analyses were redone using the standard curve generated for the housekeeping gene NUP-214. Therefore, Figure 4 truly indicates the relative amount of each CYP450 mRNAs in each cell line. Overall, CYP1B1 and 2J2 mRNAs appear to be the most important in these cancer cell lines, while CYP1A1 and 1B1 mRNAs are the most important in the malignant cell line (MCF-10A), as well as in the cancer cell line Hs578T.

Figure 5 was generated to demonstrate the total CYP450 mRNA expression in each cell line. Values were calculated by adding the total relative expressions each cell line and calculating them as a percentage. This data shows that not only does the overall make-up of CYP450 mRNA expression change from each cell line, but so does the total CYP450 mRNA expression. For example, MDA-MB-231 and MCF-7 have a much higher total expression of CYP450 mRNAs than the other cell lines, whereas Hs578T has very little CYP450 mRNA expression.

Determination CYP450 metabolic potential in breast cancer cell lines. The metabolic activity of the breast cancer cell lines were determined using ebastine as a specific susbtrate of CYP2J2, and 7-ethoxyresorufin as a substrate of CYP1A1 and 1B1. The metabolism of these two substrates were also evaluated using recombinant supersomes (BD-Canada), where ebastine was incubated in the presence of rCYP2J2 and 7-ethoxyresorufin in the presence of rCYP1A1 and 1B1. The results were fit using the Michaelis-Menten equation, and the kinetic parameters were extracted. Table 2 lists the kinetic data for both whole cell metabolism and recombinant microsome metabolism

whereas the Michaelis-Menten curves and be visualized in Figures 6 and 7 for whole cells only. Overall, ebastine metabolism was measurable in all 7 cell lines, where ZR-75-1 showed the greatest metabolism and little metabolism was observed in Hs578T, MDA-MB-231 and MCF-10A cells. There Km of ebastine in whole cells has been determined to be around 0.5-1 µM, a value that is slightly lower than what is observed in recombinant supersomes (~5 µM). While these values are slightly different, they are within the same low range suggesting that CYP2J2 has a great affinity towards ebastine. The small differences obtained may simply be due to the difference in assay conditions, meaning, whole cell incubations compared to supersomes incubations.

When evaluating the metabolism of 7-ethoxyresorufin, measurable activity was only observed in 4 cell lines. The Kms obtained were between 0.05 and 0.1 µM in whole cells compared to a Km of 0.16 µM observed in rCYP1B1 supersomes. This shows that the affinity obtained in whole cell incubations is within the same range as what is observed using CYP1B1 supersomes. Surprisingly, ZR-75-1 and MDA-MB-231 cell lines which express CYP1B1 at a very high level, showed no metabolism for this CYP1B1 substrate. Not even the sequencing data of CYP1B1 for these two cell lines could explain the lack of functional activity.

Correlation between mRNA expression and metabolic activity. The correlation between mRNA expression and metabolic activity was examined (see Figure 8). An excellent correlation of 0.9909 was obtained between the mRNA expression of CYP2J2 and the metabolism potential of ebastine. Therefore, this indicates that the large majority of ebastine metabolism is produced by CYP2J2.

The correlation of mRNA expression and 7-ethoxyresorfin metabolism was also examined. Since 7-ethoxyresorufin is a substrate of CYP1A1, 1A2 and 1B1, all three isoforms were considered. However, since CYP1A2 is barely expressed compared to CYP1A1 and 1B1 in these cell lines, the metabolism of 7-ethoxyresorufin observed would not be due to CYP1A2. While both CYP1A1 and 1B1 are present, the cell lines analysed preferentially express CYP1B1 compared to 1A1 (See Figure 4). However, a lack correlation was observed for CYP1A1 and 7-ethoxyresorufin (data not shown, with and without MDA-MB-231 and ZR-75-1). When using the mRNA expression of CYP1B1 and 7-ethoxyresorufin metabolism, an excellent correlation of 0.9832 was obtained which excluding the cell lines MDA-MB-231 and ZR-75-1. Therefore, this shows that the impact of CYP1A1 in these cell lines on the metabolism of 7-ethoxyresorufin is negligible and that the metabolism observed in these breast cancer cell lines is due to the presence of CYP1B1.

Determination of CYP1B1 mutations The genotyping of CYP1B1 was then evaluated to determine if the lack of 7-ethoxyresorufin metabolism in MDA-MB-231 and ZR-75-1 cell lines was due to polymorphisms. Many mutations of CYPB1 have been documented, both in the intro and exon. Genotyping studies were focussed on mutations in the exons since mutations in this region can result in amino acid sequence. Therefore, the portions of the exons encoding for the protein were cloned, sequenced and compared to wild type DNA. Table 4 lists the mutations observed for CYP1B1 for all cell lines. As can be seen, the majority of the cell lines have mutations present which include the genotype known as *2 (Arg48Gly, Ala119Ser), *3 (Leu432Val) and *4 (Asn453Ser).[21] According to the literature, these three genotypes have been associated with no loss in function compared to the wild type protein, and therefore should have no effect on the metabolism potential of CYP1B1.

DISCUSSION

Results obtained in this study demonstrate that each cell line has a unique make-up of CYP450 mRNA expression, which could explain the differential survival of one cell line to another in the presence of anti-cancer agents. We found that the expression of CYP3A4/5/7 mRNAs are greatly reduced in the cancer cell lines compared to the benign control, MCF-10A cells. Therefore new active anti-cancer agents that could be metabolised by CYP3 family would be locally detoxified in cancer free cells, and remain active longer in breast cancer cells. We have also shown that mRNA expression correlates well to the metabolic activity using two drug probes. Therefore mRNA determination allows for an excellent prediction of enzyme presence.

This study was the first to evaluate the local expression of several CYP450 mRNAs in breast cancer cell lines. The results of this study demonstrate that a large inter-cell line variability was present, and that the isoforms abundantly found in breast cancer cell lines were different from the isoforms abundantly found in the liver. These results suggest that the same may be true in patients and that local metabolism may be implicated in the inter-subject variability observed in chemotherapy response.

The CYP3A subfamily is of particular interest because of its selective expression, and its large involvement in drug metabolism.[20] In MCF-10A cells, the expression of CYP3A4, 3A5 and 3A7 mRNAs are greater than in any of the breast cancer cell lines evaluated (41.5, 13.9 and 43.4 times more expressed than the median cell line, respectively). This result shows that the CYP3A subfamily is downregulated in breast cancer cell lines, and could be a potential target for new

chemotherapy agents. By synthesizing a chemotherapy agent that is metabolized by CYP3As, the administered dose would be cleared from healthy cells faster than from cancerous cells. CYP19A1 is another isoform which appears to have preferential expression in the non-malignant cell line. CYP19A1 is 13.8 fold more expressed in MCF-10A cells compared to the median cell line (MCF-7) and is 7.2 fold more expressed than in MDA-MB-468 cells, the cell line which has the second largest expression of this isoform ($p<0.0001$).

As for the other CYP450 isoenzyme mRNAs, there is no clear difference between the cancer cell line and the control, meaning that there is no over- or under-expression in the cancer cell lines compared to the control. However, in some cases, we observed a very large variability in mRNA expression levels of enzymes between cell lines. For example, CYP1A2 mRNA is 46.9 times more expressed in T47D cells compared to the median cell line, whereas CYP2J2 mRNA is 14.3 fold more expressed in ZR-75-1 compared to the median. This data therefore shows that there is variability in expression from one cell line to the next, and would suggest that this same variability may exist between individuals. Gene variability or inter-individual variability is a phenomenon that is commonly observed and can be due to many factors including genetic and environmental. [22] Environmental factors are of particular importance in cancer patients because they are often exposed to various anti-cancer agents, and potentially to natural products, which are known to affect RNA levels through induction and inhibition. Genetics affect RNA levels in the sense that some patients may naturally produce an enzyme more than another (including CYP450s). This could cause toxicity or loss of efficacy of an anticancer agent, through slower or faster metabolic rates, respectively. Overall, there are many sources that can contribute to inter-individual variability, which can also be seen in the results obtained in the cell lines.

Metabolic studies using ebastine and 7-ethoxyresorufin also displayed an inter-subject variability in terms of maximum activity as well as the enzymes affinity to the substrate. This same variability has been observed in human liver microsomes from individual donors for a variety of substrates,[23,24] Therefore, the inter-cell line variability observed is a common phenomenon. Overall, the variability in metabolism follows the same variability observed in mRNA expression (see Figure 8) and therefore suggests that mRNA expression is an excellent method for predicting inter-subject variability. However, a lack of metabolism of 7-ethoxyresorufin was observed in MDA-MB-231 and ZR-75-1 despite the very high level of CYP1B1 mRNA present, compared to the other cell lines analyzed. We attempted to explain this finding by sequencing the exons of CYP1B1 for all cell lines. Sequencing data revealed that these two cell lines contain known mutations, MDA-MB-231 being genotyped as *2/*2, while ZR-75-1 was genotyped as *1/*4. Unfortunately, previous reports have shown that these genotypes are not expected to lead to a decrease in CYP1B1 metabolic activities.[20] Therefore, the CYP1B1 genotype does not seem to explain the lack of activities observed in these two cell lines. The reason for this discrepancy remains unknown at this time.

Cytochrome P450 activity is of great importance in chemotherapy treatment because many agents are substrates of at least one of the isoenzymes.[25-28] Since the expression of CYP450 mRNAs, and metabolic activities have been displayed in this study for CYP1B1 and 2J2, the local metabolism of anti-cancer agents is possible. Consequently the inter-subject variability in the local expression of these isoforms may further contribute to the inter-subject variability observed in drug response, particularly of anti-cancer agents. This suggests that the local metabolism must be taken into account.

In summary, future anti-cancer agent studies using breast cancer cell lines need to consider these results before choosing a cell line for metabolic purposes. Choosing several cell lines with different CYP450 profiles will help determine the efficacy and/or toxicity of new chemotherapy agents. Not only does the overall profile of CYP450 expression vary from one cell line to the next, but so does the total metabolism potential of each cell line (Figure 6). It is important to evaluate the total CYP450 mRNA expression because many compounds or medications can be metabolised by several CYP450 isoenzymes. Therefore, a cell line such as ZR-75-1 which expresses a lot of CYP450s might metabolise a medication more efficiently than the cell line Hs578T which has very little CYP450 expression. Therefore, several cell lines with different characteristics should be evaluated during chemotherapy agent studies.

Overall, this study demonstrates that an important inter-cell line variability in CYP450 mRNAs is present in breast cancer cell lines and suggests that this same inter-subject variability may be present in breast cancer patients. The inter-subject differences observed could cause major variability in local drug metabolism in breast cancer tumours and therefore explain the large variability in drug toxicity and efficacy that is observed. This study also shows the importance of testing potential chemotherapy agents in several breast cancer cell lines in order to determine drug efficacy and toxicity. We propose that soectific CYP450s may serve as a new target for future anti-cancer agents, since the mRNA expression was downregulated in breast cancer cell lines. These new active chemotherapy agents would be locally metabolised and cleared more slowly from breast cancer cells and prove to be more efficacious.

CONCLUSION

In conclusion, we performed a thorough determination of the expression level of 19 CYP450 isoforms in 8 breast cell lines. This study serves to evaluate the role of CYP450s in the local metabolism of medications in breast cancer. Overall, the results demonstrate that a large inter-cell line variability is present, both in mRNA expression, and metabolic activities, which would suggest that an inter-subject variability may also be present.

Table 1: Relative expression of CYP450 mRNAs.

Table showing the relative expression of each CYP450 mRNA compared to the various cell lines using the Standard Curves for each individual enzyme. The cell line which expresses the most mRNA of each isoenzyme is highlighted. Values represent the relative mRNAs expression of each isoform, compared to the median expressed cell line. ND: Not detected in cell line.

CYP450	MCF-10A	MCF-7	MDA-MB-468	Hs578T	SKBR3	MDA-MB-231	T47D	ZR-75-1
1A1	0.20	0.14	1.00	1.33	0.19	1.75	1.35	0.23
1A2	0.35	1.00	1.64	0.34	0.17	0.00	46.85	1.48
1B1	0.20	0.95	2.82	0.03	0.03	4.13	1.00	9.04
2A6	10.13	1.76	0.57	0.19	0.50	7.94	1.00	0.00
2B6	0.00	27.35	1.00	0.00	2.02	0.00	89.68	0.38
2C19	1.93	0.41	0.82	4.97	21.21	0.67	0.06	1.00
2C8	0.66	76.11	1.27	0.00	0.92	1.00	0.06	2.35
2C9	ND	ND	ND	ND	ND	ND	ND	ND
2D6	1.00	0.00	0.78	1.01	1.84	0.04	0.04	2.87
2E1	0.16	1.00	0.03	0.27	5.38	8.13	0.29	12.30
2J2	0.05	0.57	1.86	0.01	1.84	0.04	1.00	14.29
2W1	0.00	1.00	2.61	0.00	0.00	5.23	0.71	3.00
3A4	41.45	0.00	1.00	0.00	0.23	10.06	0.00	7.40
3A5	13.86	1.00	2.53	0.95	2.87	0.00	0.23	0.00
3A7	43.26	0.00	1.00	1.06	1.64	0.36	0.00	0.00
4A11	0.87	1.38	16.04	0.46	1.00	0.00	1.90	0.62
4Z1	0.73	1.00	176.48	0.08	0.04	0.01	33.44	55.72
17A1	1.00	0.59	0.49	0.00	2.49	0.42	1.00	3.44
19A1	13.83	1.00	1.91	1.32	0.00	0.09	0.13	0.04

Table 2: Kinetic data for ebastine and 7-ethoxresorufin metabolism in breast cancer cell lines.

In order to compare whole cell kinetic data, ebastine was incubated with CYP2J2 supersomes, while 7-ethoxyresorufin was incubated in the presence of CYP 1B1 supersomes. BLQ: below level of quantitation.

Cell Line	Ebastine Metabolism			7-Ethoxyresorufin Metabolism		
	Km (μM)	Vmax (pmol/mg protein/min)	Cl int (μL/mg protein/min)	Km (μM)	Vmax (pmol/mg protein/min)	Cl int (μL/mg protein/min)
Hs578T	3.1 ± 0.6	0.13 ± 0.64	0.041	BLQ	BLQ	BLQ
MCF-7	1.4 ± 0.2	0.92 ± 0.05	0.642	0.049 ± 0.016	1.456 ± 0.128	29.714
MCF-10A	0.7 ± 0.2	0.25 ± 0.02	0.339	BLQ	BLQ	BLQ
MDA-MB-231	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
MDA-MB-468	0.7 ± 0.1	1.50 ± 0.06	2.286	0.097 ± 0.008	3.709 ± 0.075	38.237
SKBR3	0.5 ± 0.0	2.45 ± 0.07	4.571	0.055 ± 0.028	0.041 ± 0.006	0.745
T47D	0.2 ± 0.0	0.58 + 0.015	2.391	0.061 ± 0.027	1.609 ± 0.208	26.377
ZR-75-1	0.4 ± 0.0	11.49 ± 0.33	30.397	BLQ	BLQ	BLQ
rCYP2J2	4.9 ± 0.8	5435 ± 229.6	1100			
rCYP1A1				0.321 ± 0.044	18.120 ± 0.947	56.449
rCYP1B1				0.157 ± 0.025	4.596 ± 0.217	29.274

Table 3: *CYP1B1* primers.

Table lists the primers used for the cloning of exon 2 and 3, as well as the primers used to sequence the complete coding regions of these exons. Also listed are the expected band sizes.

Exon	Cloning Primers	Sequencing Primers	Band Size
2	F- 5’ TCT TCG GCC ATT TCT CCA GAG AGT CAG CT 3’ R- 5’ACC CCA AAC CCG GGG CCC TGC TT 3’	5’ GCG TGG GGC GCC CGC TCC TG 3’ 5’ CCC GGT GCG CAC CGT TTT CC 3’	1287 bp
3	F 5’ CAG GTA TCC TGA TGT GCA GAC T 3’ R- 5’ GAG AAG CAG CAC AAA AGA GGA A 3’	5’ GAT CAG GTC GTG GGG AGG G 3’ 5’ TGG TCT AAC CAT TAA ACC CA 3’	690 bp

Table 4: CYP1B1 observed mutations

Cell Line	Mutations Base Pairs	Mutations Amino Acids	Variant Name
Hs578T	T 1347 C Heterozogeous	Silent Mutation Heterozogeous	*1/*1
MCF-7	T 1347 C Homozygeous A 1358 G Homozygeous	Silent Mutation Asn 453 Ser Homozygeous	*4/*4
MCF-10A	C 142 G Heterozygeous G 355 T Heterozygeous T 1347 C Heterozygeous A 1358 G Heterozygeous	Arg 48 Gly Heterozygeous Ala 119 Ser Heterozygeous Silent Mutation Asn 453 Ser Heterozygeous	*2/*4
MDA-MB-231	C 142 G Homozygeous G 355 T Homozygeous T 1347 C Homozygeous	Arg 48 Gly Homozygeous Ala 119 Ser Homozygeous Silent Mutation	*2/*2
MDA-MB-468	C 1294 G Homozygeous	Leu 432 Val Homozygeous	*3/*3
SKBR3	C 1294 G Homozygeous	Leu 432 Val Homozygeous	*3/*3
T47D	C 142 G Homozygeous G 355 T Homozygeous T 1347 C Homozygeous	Arg 48 Gly Homozygeous Ala 119 Ser Homozygeous Silent Mutation	*2/*2
ZR-75-1	T 1347 C Heterozygeous A 1358 G Heterozygeous	Silent Mutation Asn 453 Ser Heterozygeous	*1/*4

Figure 1. Relative expression of various CYP450 Families 1, 17 and 19 mRNAs in various breast cancer cell lines.

The cell line with the median ΔCt was set at a relative expression of 1, and used to compare the expression levels of all other cell lines.

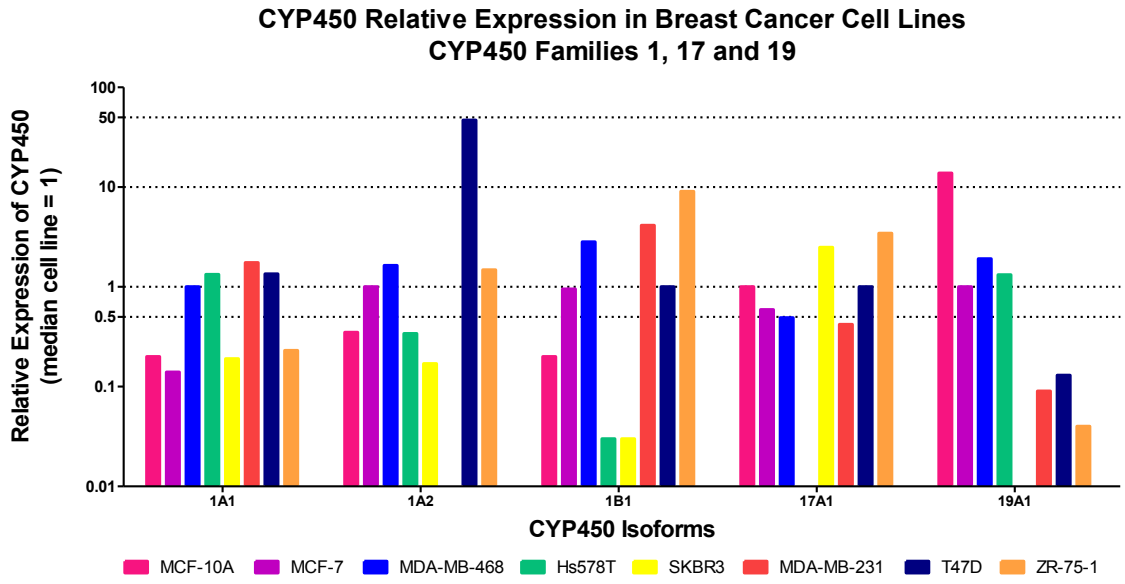


Figure 2. Relative expression of various CYP450 Family 2 mRNAs in various breast cancer cell lines.

The cell line with the median ΔCt was set at a relative expression of 1, and used to compare the expression levels of all other cell lines. CYP2C9 mRNAs is not shown here because only MDA-MB-231 cells express this enzyme.

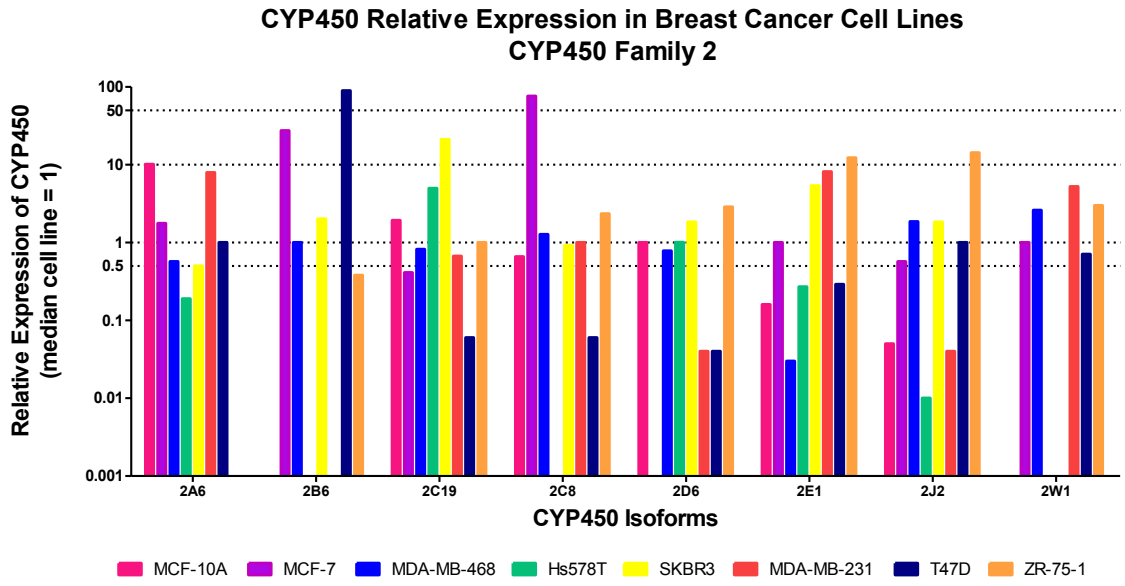


Figure 3. Relative expression of various CYP450 Family 3 and 4 mRNAs in various breast cancer cell lines.

The cell line with the median ΔCt was set at a relative expression of 1, and used to compare the expression levels of all other cell lines.

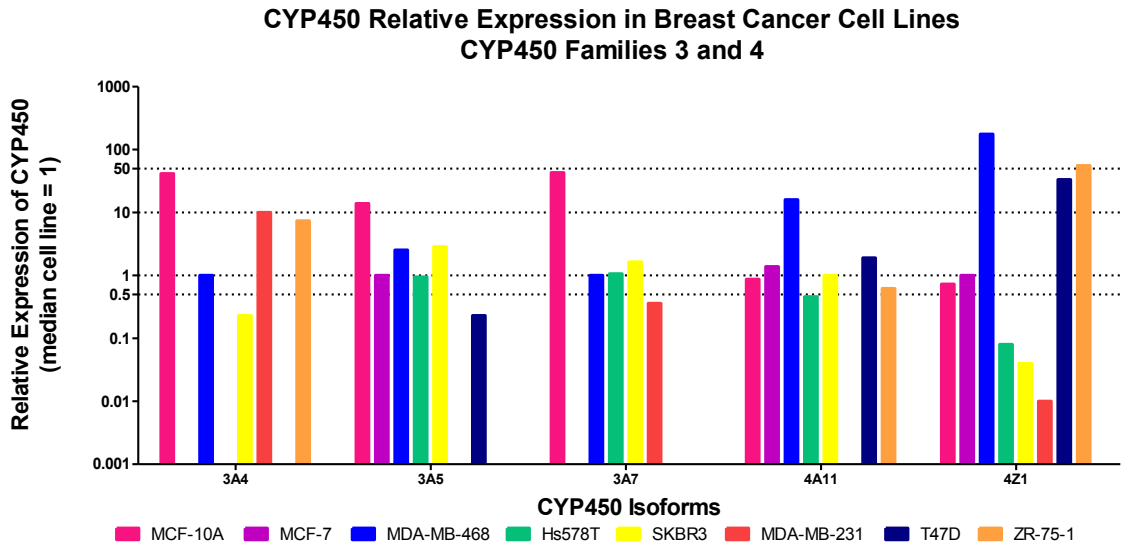


Figure 4. CYP450 profile of each cell line.

Indicates the relative expression of CYP450 mRNAs in each cell line, where the Ct values were determined for each enzyme using the Standard Curve of NUP-214. The use of one threshold cut-off (one standard curve) permits a comparison of the different isoenzymes in each cell line.

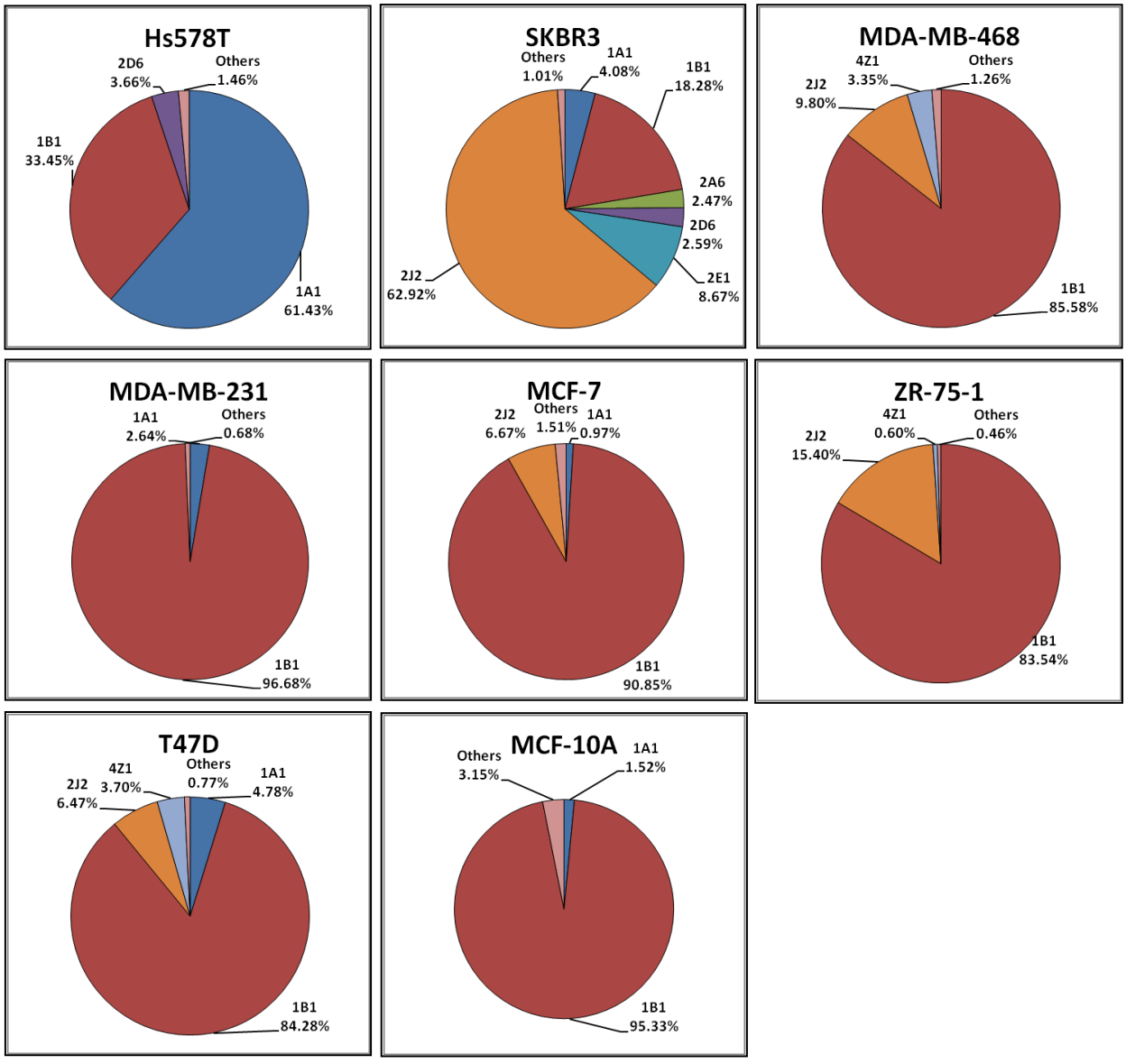


Figure 5. Expression of total CYP450 mRNA in each cell line.

This indicates which cell like has the most CYP450 total mRNA expression. ZR-75-1 proves to be the cell line with the greatest expression of CYP450 mRNAs, while Hs578T has very little CYP450 mRNA expression. Values were calculated by adding the total relative expressions each cell line and calculating them as a percentage.

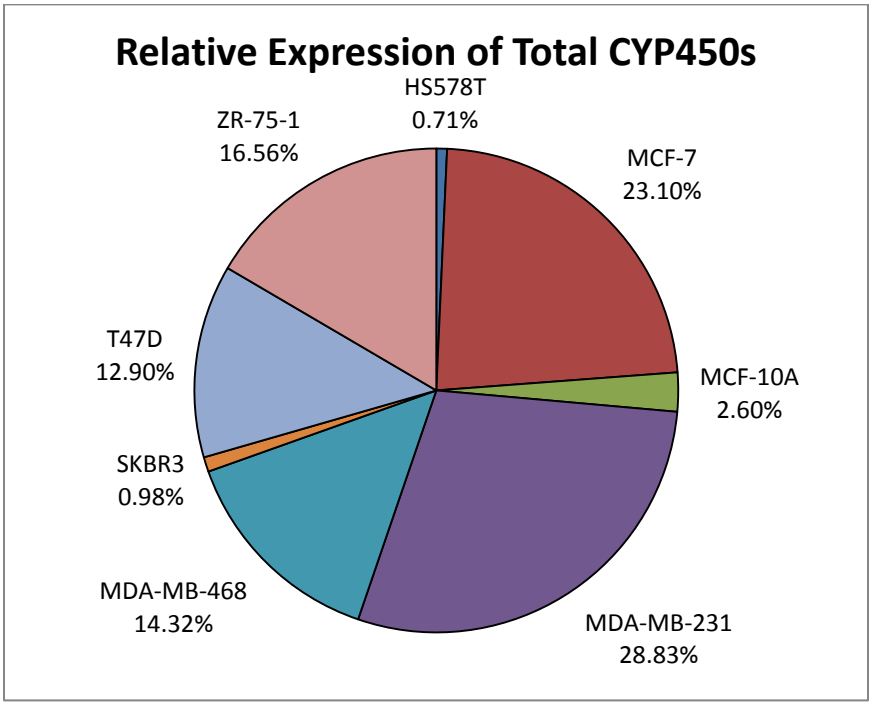


Figure 6. Ebastine metabolism in breast cancer cell lines.

Metabolism rates were corrected for protein concentration and time (1 hour incubations). The metabolism of Ebastine is a measure of CYP2J2 activity. SKBR3 shows the greatest metabolism of Ebastine of the cell lines analyzed.

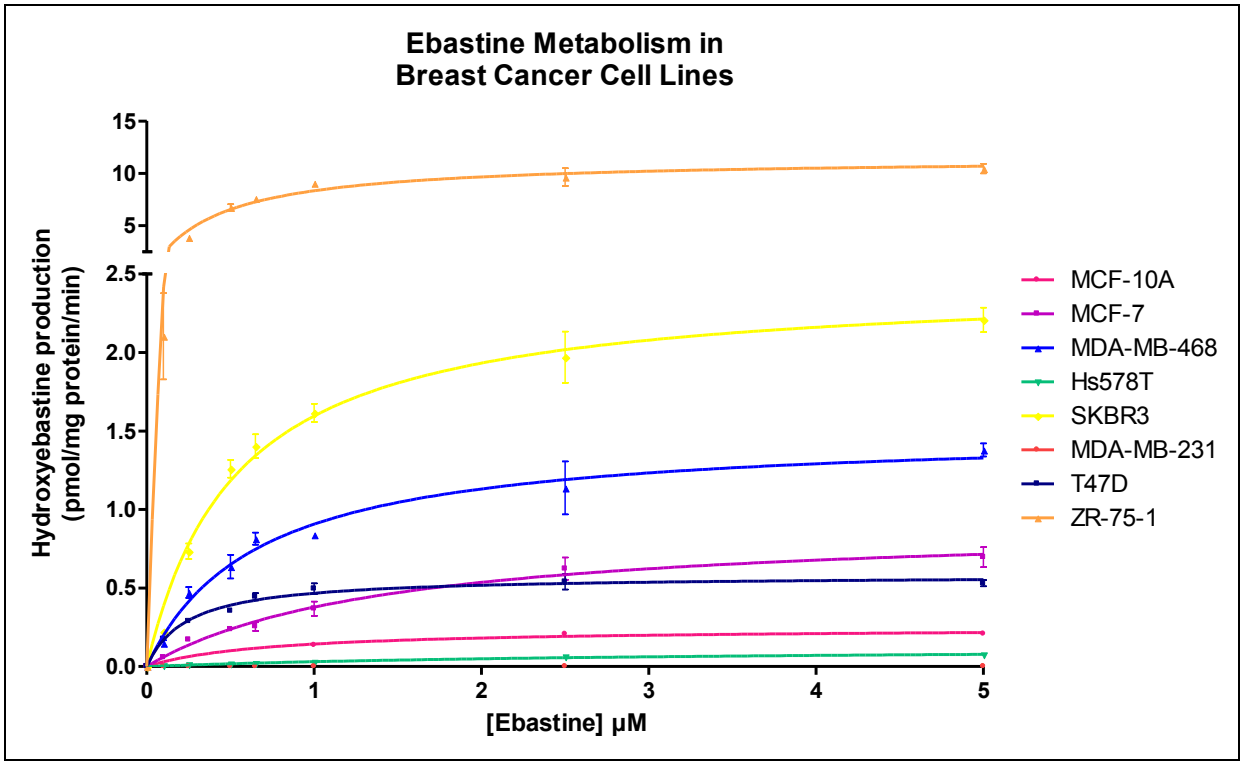


Figure 7. 7-Ethoxyresorufin metabolism in breast cancer cell lines.

Metabolism rates were corrected for protein concentration and time (5 hour incubations). MDA-MB-468 demonstrates the greatest 7-ethoxyresorufin metabolism with no auto-inhibition from 0-1 µM. Measurable activity was observed for T47D and MCF-7 cells, however, above 0.5 µM, an auto-inhibition is observed, and therefore concentrations above this concentration were removed prior to kinetic calculations.

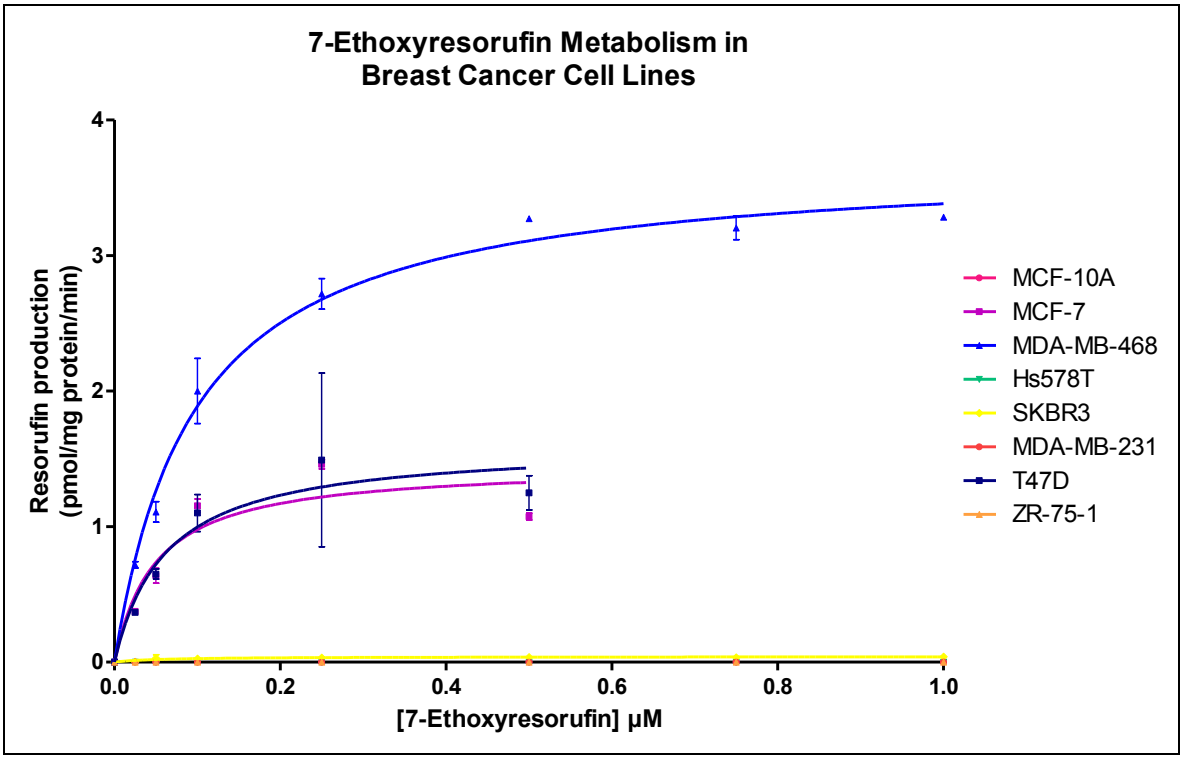
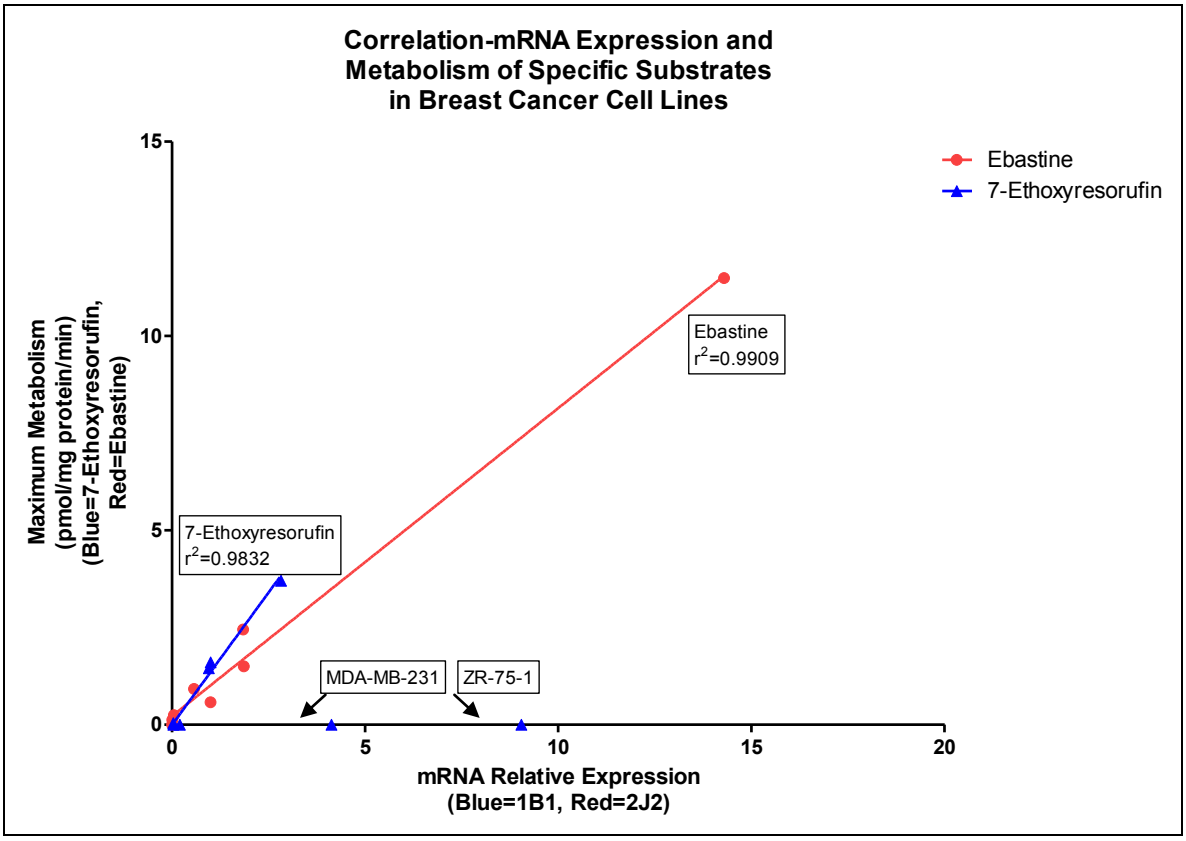


Figure 8. Correlation studies of mRNA expression and metabolic activity.

The correlation between CYP2J2 mRNA expression and metabolism of ebastine is displayed in red, and shows an excellent correlation of 0.9909. In blue is the correlation between CYP1B1 mRNA expression and the metabolism of 7-ethoxyresorufin. This correlation of 0.9832 was calculated excluding ZR-75-1 and MDA-MB-231 because they were outliers.



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CHAPTER 5

GENERAL DISCUSSION AND PERSPECTIVES

GENERAL DISCUSSION AND PERSPECTIVES

The response and efficacy of anti-cancer agents in breast cancer have been known to vary greatly from individual to individual. Since many of the anti-cancer agents used to treat breast cancer are substrates of various Cytochrome P450s (CYP450), the variability in expression of these enzymes could greatly impact the plasmatic concentrations of the anti-cancer agents. Consequently, the response and efficacy of these medications could be affected. Therefore, the main objective of these studies was to evaluate the expression of nineteen CYP450 mRNAs, in order to determine which isoforms are greatly expressed, and if the expression between different cell lines shows a large inter-subject variability.

In order to evaluate the expression of CYP450 mRNAs, RT-PCR studies were completed using the delta-delta Ct method. In order to correct for RNA integrity and cDNA concentration differences between samples, a housekeeping gene (HKG) is used as a calibrator. Consequently, the key to good quality RT-PCR data is choosing a HKG that is ubiquitously expressed despite tissue source, pathology or inter-subject variability. Traditionally, *GAPDH* and *β-actin* have served as, and continue to serve as HKGs. However, preliminary studies completed in our laboratory showed that the expression of these two commonly used HKGs were highly variable within the breast cancer cell lines of interest. These results lead to the screening of six different genes, *GAPDH*, *β-Actin*, *RPLPO*, *TBP*, *NUP-214* and *PPIG* across the breast cancer cell lines of interest as well as in 15 other cell lines, which were isolated from a variety of tissue sources.

After analyzing the expression level, the stability of the six HKGs was determined by calculating the standard deviation and the deviation from average for each gene. In order to confirm our results, an

online database specific for HKG analyses was also used. The database permitted our results to be imputed and analyzed by a variety of techniques.[1] Each analysis method lead to the same conclusion: *NUP-214* was the most stable HKG analyzed.

These results were the first study to demonstrate that one gene, namely *NUP-214,* was not only the most stable within the same tissue source, but also across various tissue sources. Analyses using *NUP-214* as the HKG will lead to more reliable and convincing results, and will permit cross-organ studies. Therefore, these results should have a significant impact in RT-PCR studies, where future analyses using this technique should greatly consider switching from the traditional HKGs, *GAPDH* and *β-actin* to *NUP-214.*

Our next goal was to evaluate the mRNA expression level of 19 CYP450 isoforms in 7 breast cancer cell lines and 1 benign breast cell line. By evaluating which isoforms are highly expressed in the breast cancer cell lines, we could better understand if these enzymes may play a key role in the clearance of anti-cancer agents, and therefore be causing inter-subject variability. By comparing the expression level of CYP450 isoforms in the benign cell line compared to the breast cancer cell lines, we were able to evaluate if any isoforms are up- or down-regulated in breast cancer.

The results obtained in this study demonstrate that the CYP450 mRNA make-up is unique to each cell line, and that the overall expression of total CYP450s varied greatly between cell lines. Therefore each cell line would interact with xenobiotics in very a different way, where certain cell lines may be more or less sensitive to CYP450 metabolized xenobiotics. This would therefore explain the

differential survival of cell lines when in the presence of anti-cancer agents. These results would suggest that new anti-cancer agents need to be tested on several breast cancer cell lines in order to determine if a medication is efficacious or not. Currently several cell lines may be evaluated during this process. However, their selection may be chosen based on cell receptor presence (ER, PR and HER2) as opposed to the CYP450 make-up. A more concrete cell line selection should take into account all of these factors.

When evaluating the expression of CYP450 isoforms between the benign cell line, MCF-10A and the breast cancer cell lines, one subfamily, the CYP3A, showed a significantly lower expression in the breast cancer cell lines compared to the benign cell line. This selective expression is of great interest because the CYP3A family, specifically the CYP3A4 isoform, is largely involved in the metabolism of many different medications.[2] With this differential metabolism in mind, the development of new anti-cancer agents that are primarily metabolized by CYP3A4/5/7 would be ideal because a high clearance in normal breast cells would be present, and therefore would lead to the targeting of breast cancer cells.

Another isoform that shows differential expression between malignant and cancer cell lines is CYP19A1. This isoform was significantly more expressed in the control cell line, MCF-10A, then in the breast cancer cell lines. This result is quite interesting because CYP19A1, otherwise known for its aromatase activity, has been a target of interest for breast cancer treatment. Aromatase is one of the key enzymes involved in the biosynthesis of estrogen, and has been shown to be greatly expressed in breast cells. [3-4] Since previous reports have shown an elevated expression of CYP19A1 in breast cells, aromatase inhibitors (Anastrozole, Exemestane and Letrozole) are used to treat breast tumors in post-menopausal, ER+ tumors. [5-6] If the same expression pattern of CYP19A1 is also observed in breast cancer patients, it would mean that the aromatase inhibitors would be more significantly decreasing the

production of estrogen in the normal breast cells than in the cancer cells. If the cancer cells are ER+, they would require estrogen to stimulate their growth, and therefore by decreasing the surrounding concentration of estrogen would inhibit the growth of the cancer cells. These results would suggest that the targeted cells of these inhibitors would not directly be the cancer cells, but the normal breast cells.

This study also revealed that the two most abundant isoforms present in the breast cancer cell lines are CYP1B1 and CYP2J2. These two isoforms are important to evaluate because of their different roles. The high expression of CYP1B1 is not too surprising because of its involvement in the metabolisms of hormone, specifically the hydroxylation of 17β-estradiol and testosterone.[7] Furthermore, CYP1B1 expression has been linked to the production of pre-carcinogenic molecules (benzanthracene, benzo(a)pyrine, DMBA, 1-ethynyl-pyrene, 3-methyl-cholantrene and oestradiol), and is believed to be a source of steroid hormone-mediated cancers. [7-9] Therefore, a significant expression of CYP1B1 is to be expected in breast cancer cells. Since CYP1B1 is capable of metabolizing hormones, anti-cancer agents that resemble hormones, such as SERMs, could be locally metabolized by this isoform. Future metabolic studies of this class of anti-cancer agents using CYP1B1 supersomes would be very interesting.

The role of CYP2J2 is substantially different from that of CYP1B1. Its role is quite diverse, because not only is this enzyme involved in the metabolism of endogenous molecules, such as fatty acids, arachidonic and linoleic acid, but is also involved in the metabolism of a great number of xenobiotics.[2, 10-13] Since the active site of CYP2J2 is so similar to that of CYP3A4 (just slightly narrower), many of the xenobiotics metabolized by CYP3A4 are also substrates of CYP2J2. [10, 14] Since there is such a lack of CYP3A4 in the breast cancer cell lines analyzed, CYP2J2 may be a replacement for the isoform, and therefore the role of CYP2J2 in the local metabolism of xenobiotics needs to be

evaluated. Since CYP3A4 is involved in the metabolism of many anti-cancer agents, such as cyclophosphamide, paclitaxel, docetaxel, tamoxifen and exemestane, metabolism studies of these medications using recombinant CYP2J2 supersomes would be extremely interesting. [10, 15-31] Understanding if these treatments can be locally metabolized by CYP450s will allow for better adjusted dosing for breast cancer patients and should ultimately lead to better patient treatment and survival.

Finally, metabolism studies to demonstrate the activity of CYP2J2 and CYP1B1 were completed using two probe drugs, ebastine and 7-ethoxyresorufin, respectively. While a lack of metabolism of 7-ethoxyresorufin was observed in two cell lines (MDA-MD-231 and ZR-75-1) despite an elevated expression of CYP1B1, a strong correlation remains present between this isoform and its substrate. The lack of metabolism for these two cell lines could not be explained through the genotyping of the coding region, and therefore the reason is unknown at this time. The mRNA expression of CYP2J2 and ebastine metabolism showed an excellent correlation where a substantial amount of metabolism was observed after only a one hour incubation. These metabolic studies revealed that an inter-subject variability is present for the cell lines, when evaluating their maximum activity, as well as their substrate affinities. Therefore, this study not only insinuates that substantial local metabolism is possible, but also that local metabolism may impact the inter-subject response to medications.

Overall, this project was comprised of several interesting and important results. The first analysis revealed a new and more stably expressed HKG for RT-PCR analyses, *NUP-214.* This HKG is more ubiquitously expressed than other genes, such as *GAPDH,* despite tissue source or pathology. Therefore this study demonstrates the need to change from traditional HKGs to *NUP-214.* The second study demonstrated that an important inter-cell line variability in CYP450 mRNAs is present in breast

cancer cell lines. This study suggests that inter-subject variability may also be present in breast cancer patients and that the local metabolism could therefore be an additional cause to the toxicity and response differences currently observed in patients. This research suggests that CYP1B1 and CYP2J2 may be significantly impacting the local concentration of anti-cancer agents in breast cancer cells, and therefore decreasing the efficacy of certain medications, specifically in patients with a high expression of CYP1B or 2J2. Therefore, future metabolism studies of these isoforms with various chemotherapy agents could be extremely interesting and lead to important results. This study also suggests that during the development of future chemotherapy agents, several breast cancer lines should be screened to determine drug efficacy and toxicity since inter-cell line variability was so high. We propose that CYP450s that are downregulated in breast cancer cell lines, could serve as new targets for anti-cancer agents. This would lead to anti-cancer agents that are cleared more slowly from breast cancer cell lines, leading to higher intracellular concentrations and therefore more efficacious medications.

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CHAPTER 6

GENERAL CONCLUSIONS

GENERAL CONCLUSIONS

Breast cancer is a disease which affects thousands of women and men in Canada yearly. Some breast cancer patients have shown a de novo and acquired resistance to anti-cancer agents, resulting in a treatment failure. Identifying potential causes for this resistance is required in order to develop better treatment options which could avoid these causes. Some studies have shown that factors affecting drug disposition, such as membrane transporters could affect the local concentrations of anti-cancer agents in the targeted cells. Other enzymes which could influence the cellular concentrations of these agents are the metabolizing enzymes, Cytochrome P450s. Many chemotherapy agents are known substrates of one or many CYP450 isoforms, and therefore CYP450s locally expressed in breast cancer cells could greatly decrease the cellular concentration of these treatments. Therefore, the objective of this work was to evaluate the expression of CYP450 mRNAs locally expressed in breast cancer cell lines and to determine if their expressions could be significant enough to affect the intracellular drug concentrations.

Prior to determining CYP450 mRNA expression by RT-PCR, a screening of potential housekeeping genes was completed in order to determine which gene was the most stably expressed. In order to complete this study, the stability of six candidate HKGs (*β-Actin*, *GAPDH*, *NUP-214*, *PPIG*, *RPLPO*, *TBP*) was determined in twenty-three different cell lines (coming from different pathologies and isolated from different tissues). Overall, the results demonstrate that *NUP-214* is the most stable HKG candidate, and was expressed in all cell lines tested at a very similar level. Therefore *NUP-214* can be considered a universal HKG.

Using *NUP-214* as the HKG, the expression level of 19 CYP450 isoforms in 8 breast cancer cell lines (1 of which is a benign breast cell line) was determined. The results showed that many CYP450s are expressed locally in these cell lines, and that the isoforms found in these cells are not the same

isoforms expressed hepatically. Specifically, CYP1B1 and 2J2, two isoforms which are considered extra-hepatic isoforms are very strongly expressed in these cell lines. Furthermore, a large inter-cell line variability in CYP450 expression was observed in these cell lines. Using specific metabolic substrates of CYP1B1 and 2J2, namely, 7-ethoxyresorufin and ebastine, respectively, metabolism studies were completed. These studies demonstrated that not only are these cell lines capable of locally metabolizing substrates, but also that the metabolism of these two substrates correlates very well with mRNA expression.

This is the first time that whole cell metabolism studies were completed using breast cancer cell lines. This study was therefore the first to demonstrate the potential that local metabolism may play in the chemoresistance in breast cancer cells. In specific, CYP1B1 and 2J2 may play a significant role in the metabolism of chemotherapy agents. Since these two isoforms are not highly expressed in the liver, they are not evaluated during the *in vitro* drug metabolism process. Future studies need to be completed in order to evaluate the potential metabolism of various anti-cancer agents by these two isoforms.

Overall, these results would suggest that an inter-subject variability in CYP450 mRNA expression may be present in breast cancer patients, and that their local expression could be significant enough to modulate the local cellular concentrations of anti-cancer agents. In conclusion, this study demonstrates, for the first time, that CYP450s are significantly present in breast cancer cells, and that their local expressions need to be considered during anti-cancer drug development.

ANNEX 1

DECLARATION OF CO-AUTHORS