Collateral Sensitivity of P-glycoprotein Expressing Multidrug Resistant Cells to Tamoxifen is Mediated Through Oxidative Stress

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Thesis Abstract

Multidrug resistance or MDR in cancer has been recognized as a major obstacle limiting efficient treatment – chemotherapy. MDR exists through various complex mechanisms; ATP binding cassette (or ABC) transporters such as P-glycoprotein 1 (P-gp1 or ABCB1), breast cancer resistance protein (BCRP2 or ABCG2), and multidrug resistance associated protein1 (MRP1 or ABCC1) are known to induce MDR due to the extracellular efflux of chemotherapeutic drugs in cancer cells. Increase in P-gp1 expression post-treatment has been demonstrated in different tumor patient's samples, including breast, ovarian, and myeloma. Efforts to overcome MDR in the clinic have led to various generation of reversing agents, which were used in combination with the conventional chemotherapy. Unfortunately, given the normal expression of P-gp1 at the blood brain barrier and other organs and tissues, the use of P-gp1-reversing drugs with anti-cancer drugs caused a significant toxicity to patients.

The results in Chapter two describe, for the first time, the effects of tamoxifen as a collateral sensitivity drug whereby P-gp1 expression is required. The results show that treatment of P-gp1-positive cells with clinically achievable concentrations of tamoxifen leads to the oxidative cell death of doxorubicin-selected triple negative breast cancer cells and colchicine-selected Chinese hamster ovarian tumor cells. These results are consistent with our hypothesis that tamoxifen stimulates P-gp1 ATPase, leading to increased ATP metabolism through oxidative phosphorylation and a consequent rise in mitochondria derived reactive oxygen species (ROS).

Résumé de thèse

La multi-résistance chez le cancer est reconnue comme un obstacle majeur, limitant l'efficacité du traitement chimiothérapeutique. La multi-résistance opère à travers des mécanismes variés et complexes: les transporteurs de type cassette de liaison à l'ATP comme la P-glycoprotéine 1 (ABCB1, MDR1), la protéine de résistance du cancer du sein (BCRP2, ABCG2) et la protéine associée à la multi-résistance (MRP1, ABCC1) sont toutes reconnues comme inductrices de la multi-résistance, dû à l'efflux des agents chimiothérapeutiques en dehors de la cellule cancéreuse. La présence ou la surexpression de P-gp1 à été démontrée en clinique dans plusieurs échantillons suite aux traitements chimiothérapeutiques chez des patients de cancer du sein, de l'ovaire et myélome. Les efforts pour surmonter la multi-résistance en clinique ont emmené la découverte de certains agents inversants, qui on été utilisés en combinaison avec la chimiothérapie pour augementer l'efficacité des traitements. Malheureusement, ces traitements on ellicité des effets secondaires inacceptables à cause de l'expression naturelle de la P-gp1 dans la barrier hémato-encéphalique et dans d'autres organes et les essais cliniques ont cessé.

Les résultats du deuxième chapitre démontrent, pour la première fois, que l'expression de la P-gp1 est essentielle pour induire l'hypersensibilité (ou sensibilité collatérale) envers le tamoxifen chez les cellules cancéreuses. Les résultats démontrent que le traitement des cellules surexprimant la P-gp1 avec le tamoxifen induit l'apoptose chez les cellules cancéreuses-provenant d'un cancer du sein triple-négatif et chez des cellules d'ovaire d'hamster chinois-. Ces resultants sont cohérants avec notre hypothèse que le tamoxifen stimule l'ATPase de la P-gp1, ce qui induit une hausse de production d'ATP par la phosphorylation

oxydative, et, par conséquence, une hausse en espèces réactives de l'oxygène produites dans la mitochondrie.

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List of abbreviations

ABC	ATP-binding cassette
P-gp1 (ABCB1)	P-glycoprotein-1
BCRP2 (ABCG2)	Breast Cancer Resistant Protein
MRP1 (ABCC1)	Mulridrug resistance associated protein1
MDR	Multidrug resistance
HPV	Human papillomavirus
HBV	Human hepatitis B virus
CS	Collateral sensitivity
DPPE	(N,N-Diethyl-2-[4-(phenylmethyl) phenoxy) ethanamine
TAM	Tamoxifen
SERMs	Selective estrogen receptor modulators
ROT	Rotenone
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
P_i	Inorganic phosphate
ETC	Electron transport chain
GSH	Glutathione (reduced)
GSSG	Glutathione (oxidized)
γ-GCS	γ-Glutamyl cysteine synthetase
GPX	Glutathione peroxidase
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
NBDs	Nucleotide binding domains

TMDs	Transmembrane domain
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
MPT	Mitochondria permeability transition pores
Apaf-1	Apoptotic protease-activating factor

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Statement of Contribution

The experimental work in this thesis was performed by the author, under the supervision of Dr. Elias Gorges.

General Introduction

Cancer is the most spread deadly disease in the developed countries. Chemotherapy is one of the main principle treatments for cancer (Christakis, 2011). However, multidrug resistance has been recognized as a major obstacle limiting cancer treatment with chemotherapy. Resistance to chemotherapy may be intrinsic or acquired. Tumors that show tolerance to anticancer drugs without prior exposure to drugs are labeled intrinsically resistant tumors; while tumors that respond initially but relapse following several treatment sessions are labeled as tumors with acquired resistance (Luqmani Y, 2005).

The most efficient multidrug resistance (or MDR) mechanisms against anti-cancer drugs have been those that are mediated by the activity of ATP binding cassette (ABC) transporters, which constituently pump anticancer drugs out of cancer cells. The large family ABC transporter consists of 48 members in humans, expressed in both normal and malignant cells. Studies to date have demonstrated that, of the 48 ABC transporters, only 10 have been shown to associate with cancer drug resistance. P-glycoprotein 1 (ABCB1 or/ and MDR1) is a 170 kDa well characterized protein that causes MDR in tumor cells by mediating the efflux of anticancer drugs in an ATP- dependent manner (Glavinas et al., 2004, Ozben, 2006).

In normal tissues and organs, P-gp1 mediates the secretion or absorption of xenobiotic and possibly other normal cell metabolites. The increase in P-gp1 expression in patients' tumors following chemotherapeutic treatment has been observed in breast, ovarian, and myeloma malignancies (Sharom, 2008).

Efforts to overcome clinical MDR has led to the development of various inhibitors, termed "chemo-reversing drugs"; however, the majority of these

Inhibitors failed to improve survival rates of cancer patients when combined with anti-cancer treatment. In principle, the use of chemo-reversing drugs should have led to increased patient survival, but the Inhibition of P-gp1 function in normal tissue with chemo-reversing drugs led to increased accumulation of toxic drugs in more tissues and organs, hence unacceptable patient toxicity. Paradoxically, P-gp1-positive cells were shown to be collaterally sensitive to certain drugs that were less toxic to P-gp1-negative cancer and normal cells. Two decades later, work in this lab described the mechanism behind P- gp1-mediated collateral sensitivity to such drugs. Hence, collateral sensitivity drugs are now considered as an alternative approach to inhibit MDR and P-gp1-positive cancer cells (Hall, Handley, & Gottesman, 2009).

The first chapter of this thesis is a summary of the literature focusing on (1) cancer biology and treatment, (2) drug resistance and the contribution of ABC transporters focusing on P-gp1, (3) the implication of reactive oxygen species (or ROS) modulation in cell death. The rational of the thesis is to identify a safe and clinically used drug that shows collateral sensitivity in P-gp1 positive cells. The second chapter describes for the first time the mechanism of tamoxifen as a collateral sensitivity correlates with P- gp1 expression.

Chapter One

Literature Review

Cancer and Drug Resistance

1.1.1 History of Cancer

Edwin Smith Papyrus was the first to describe tumors as a swelling in the breast(Breasted, 1930). Later, Hippocrates observed several other kinds of cancer (epithelial tumors) that he termed carcinos (crab in English, cancer in Latin) because of their unique shape (Garrison, 1926).

Cancer is a disease characterized by uncontrolled growth and spread of abnormal cells to other parts of the body via the lymphatic system and bloodstream, a process known as metastasis (terminal stage), which is responsible for the majority of cancer fatalities worldwide (Gabriel, 2007). It affects people of all ages; nevertheless, the risk of developing cancer increases with age (Hajdu, 2011). Cancer is the leading cause of death in developed countries, and the second leading cause of death in developing countries after infectious and parasitic diseases (WHO, 2008). The American Cancer Society predicts that in 2015 there will be 589,430 deaths and 1,658,370 new cases of cancer in the United States. Also, they report the lifetime probability of developing invasive cancer at 44% for men and 38% for women (R. Siegel, Ma, Zou, & Jemal, 2014; R. L. Siegel, Miller, & Jemal, 2015).

The disease can occur as a result of a variety of mutations in genes regulating DNA repair, cell cycle, as well as programed cell death, which cause a transformation of normal cells into cancer cells. Without the ability to repair the damaged DNA or control programmed cell death, more mutations are likely to occur and cells will replicate uncontrollably (Okada & Mak, 2004). Tumors can be either benign, which remain confined within the normal boundaries of a tissue, or malignant, which metastasize and invade other tissues.

Many factors increase the risk of developing cancer. Viral and bacterial infections can cause cancer; a phenomenon established when viruses were found to cause leukemia and lymphomas in chickens and mice, cows, cats, and gibbon apes (Chang & Jeang, 2014). In human liver cancer, malignancy was linked to human hepatitis B virus (HBV). Likewise, human papillomavirus (HPV) is a major cause cervical cancer among other forms of cancer such as head and neck cancer (Gabriel, 2007; Yarbro et al., 2010; Chang & Jeang, 2014). Environmental factors, chemical, and physical agents were also found to be dominant cancer-causing factors. In 1927, the discovery of the carcinogenesis mechanism was found to be associated with the exposure to the chemical and physical agents. For instance, mutations were induced in fruit flies and laboratory animals when they were exposed to X-ray and chemicals see table 1 (Weisburger, 1989; Gabriel, 2007).

Table (1): Chemicals linked to specific cancers. (Gabriel, 2007)

Chemical/Agent	Cancer
Asbestos	Mesothelioma
Pitch, soot, coal tar, oil	Squamous cell carcinoma of the skin, scrotum
Vinyl chloride	Liver
Arsenic	Sinuses, lung
Benzidine	Bladder
Wool/leather/wood dust	Nasal sinuses
Aniline dyes	Bladder

1.1.2 Cancer Therapy

The current treatment for cancer generally includes surgery, radiation therapy, chemotherapy, immunotherapy, among others. The choice of the treatment is based on several factors: the cancer type, the stage of the disease, the patient's age, health, gender, and wishes. Complete removal of the cancer is the primary objective of treatment regiments, which sometimes can be achieved by a combined treatment of surgery and radiation if metastasis has not occurred. Radiation therapy shrinks and

maintains the tumor size, but causes damage to normal tissues. The effectiveness of chemotherapy is limited by the toxicity to other tissues in the body.

1.1.2.1 Chemotherapy

Chemotherapy is a hallmark of cancer therapies, which emerged in the mid 20th century(Christakis, 2011). The main features of chemotherapeutic agents are either blocking the invasion of cancer cells into other normal tissues or reversing the progression of pre-malignant tissues (Umar, Dunn, & Greenwald, 2012).

The first chemotherapy was the remarkable discovery that nitrogen mustard caused a temporary regression in patients with advanced non-Hodgkin's lymphoma. Nitrogen mustard has the same behavior as alkylating agents on DNA purine bases. The DNA-damaging agents were found to be effective in any point of the cell cycle and thus promoted apoptosis. That led to the idea of developing new drugs capable of inducing apoptosis by targeting different mechanisms. Nowadays, chemotherapeutic drugs can target DNA synthesis, tubulin polymerization (inhibition or stimulation), vascularization factors, and hormone metabolism (Jorden & Wilson, 2004; Schally & Nagy, 2004; Helleday et al., 2008). Although chemotherapy has helped to extend life and cure millions, major problems have emerged over the years: drug resistance (Chabner & Roberts, 2005; DeVita & Chu, 2008).

1.1.3 Drug resistance in cancer

Drug resistance is the major cause of treatment failure in clinical oncology (Ambudkar et al., 2003). It is estimated that between 30-80 % of all cancer types can become resistant to chemotherapeutic drugs, leaving patients and doctors with few treatment options (Krishna &Mayer, 2001). This

failure frequently occurs as a result of intrinsic or acquired drug resistance of tumors to chemotherapeutic drugs. Multidrug-resistance (MDR) may develop, which is a process that involves cross-resistance to a range of unrelated agents. An example for the latter process is cisplatin resistant cancer cells, which, under drug pressure over time can lead to cross-resistance to many different hydrophilic anticancer agents and other small molecules as well (Ozben, 2006).

Numerous mechanisms can cause MDR including prevention of drug entry, increased drug efflux, attenuating drug activity, altered expression of the target, altered cell cycle checkpoints, and defective cell death pathways (McGrath & Center, 1987; Cole et al., 1992; Komarova & Wodarz, 2005)). The most extensively studied mechanism involved in MDR is related to the over-expression of specific ATP binding-cassette transporters (ABC). The ABC transporters associated with MDR are ABCB1, ABCC1, and ABCG2 (Glavinas et al., 2004). The over-expression of ABC transporters has been shown to correlate directly with the resistance level to anticancer drugs (Juliano & Ling, 1976). Attempts to inhibit ABC transporter functions by administering various chemo-reversing drugs that have been unsuccessful due to the changes in the pharmacokinetics of anticancer drugs leading to high toxicity in the body (Ozben, 2006).

1.1.4 ABC transporters

ABC proteins encompass one of the largest protein families, which are conserved from bacteria to humans (Higgins & Linton, 2003; Ozben, 2006). The human ABC transporters are classified into seven subfamilies (ABCA to ABCG) according to their phylogenetic origin, which are encoded by 48 genes (Dean et al., 2001). The transporters are localized in the plasma membrane as well as various

organelles (e.g, endoplasmic reticulum and mitochondria) within eukaryotic cells (Dean et al., 2001; Sheps et al., 2004).

ABC transporters play a physiological role in the excretion and absorption of a diverse array of substrates including sugars, amino acids, drugs, endogenous and exogenous metabolites, xenobiotics, peptides, nucleotides, bile salts, and lipids (Sharom, 2008). ABC transporters coordinate the uptake and distribution of drugs. A P-gp1 knock- out mice experiment was shown an increased in sensitivity in the blood- brain barrier to the therapeutic drugs, which led to neurotoxicity (Doran et al., 2005). Moreover, mutations in ABC transporters have been associated with various human diseases (Gottesman & Ambudkar, 2001; Glavinas et al., 2004; Sharom, 2008). The role of ABC transporters in MDR to cancer chemo-drugs has been documented in the last thirty years. The ABC transporters associated with MDR are different in regards to gene locus, amino acid sequence, structure, and substrates specificity (Choi, 2005).

1.1.5 ABC transporter general structure

ABC transporters in mammalian cells share in common their structure and basic mechanism of action. ABC transporters are composed of two similar halves; each half has a transmembrane domain (TMD), which is embedded in the lipid bilayer and forms the permeation pathway for substrate transportation, and nucleotide binding domains (NBDs), which are localized in the cytoplasm where they bind and hydrolyze ATP molecules (Ozben, 2006). These domains might be present within one

polypeptide chain (full transporter) such as P-gp1, or with two separate proteins (half transporter) such as ABCG2, which needs dimerization to be functional as illustrated in Figure 1 (Hyde et al., 1990; Ambudkar et al., 1992; Ozben, 2006).

The nucleotide binding domains are homologous throughout ABC transporters, allowing easy identification and classification of ABC transporters (Sharom, 2008). The NBDs in ABC transporters consist of three highly conserved sequence motifs that engage strongly in ATP binding and hydrolysis. These motifs include Walker A and Walker B motifs, which are common to many nucleotide binding proteins. Also, The LSGGQ/R/KQR motif C or link peptide a signature sequence, which locates in the N terminal upstream the site of Walker B. Walker C is considered as a hallmark in distinguishing between ABC proteins and non-transporting ABC proteins (Hyde et al., 1990; Jones & George, 1999).

1.1.6 ABC transporters mechanism of action

Extensive studies have been done on the biochemical characterization of ABC transporters, and several mechanisms for drug efflux have been elucidated. The ATP hydrolysis powers transportation of a variety of substrates by ABC transporters.

The ATP switch hypothesis offers an explanation for the mechanism of ABC transporters. The ligand binds to the TMDs with high affinity, and induces an open conformational change in the NBDs, resulting in high affinity for ATP.

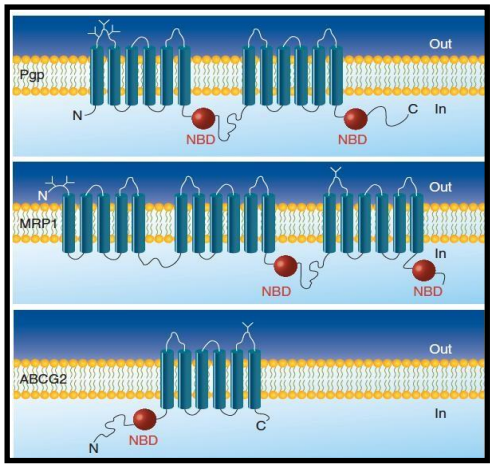

Figure 1: **A Schematic representation of anticipated membrane topology models of some ABC transporters**. The blue bars represent predicted transmembrane helices; the red circles represent the nucleotide-binding domains (NBDs), and the white trees are glycosylation site at the extracellular surface. N and C refer to the amino and carboxyl-terminal ends of the molecules (Sharom, 2008).

Two ATP molecules bind to the NBD dimers causing a conformational change in the TMDs. The energy released by the formation of the closed NBDs dimer causes conformational changes in the TMDs enough to translocate the ligand. The ATP hydrolysis initiates dissolution of the closed NBDs dimer. Finally, inorganic phosphate (P_i) and adenosine diphosphate (ADP) are released to complete the transportation cycle and restore the transporter to the open conformation to let the cycle begin again (figure 2) (Linton, 2007).

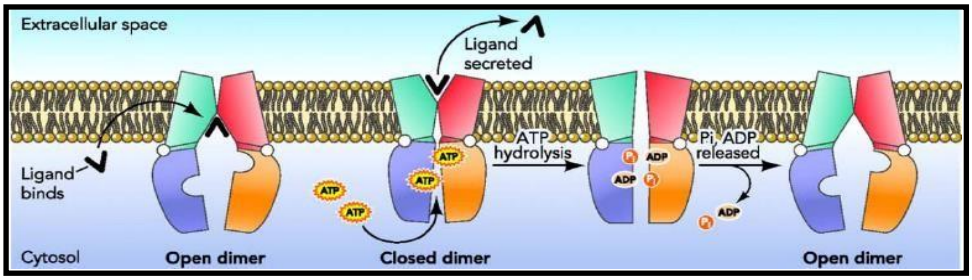

Figure 2: **A simple ATP-switch mechanism powers of ABC transporters** (Linton, 2007).

1.1.7 ABCB1 or P- glycoprotein 1 (P-gp1)

Human P-gp1 is encoded by the *MDR1* gene, whereas in rodents the protein is encoded by *mdr1* (*mdr1b*) and *mdr3* (*mdr1a*). The mouse *mdr3* gene has an 88% identical nucleotide sequence with human *mdr1* (Fardel et al., 1996; Aller et al., 2009). P-gp1 was purified and shown to express at the plasma membrane of Chinese hamster ovary cells (Juliano & Ling, 1976). It was the first identified human ABC transporter and was documented as a potential candidate involved in drug resistance when Chinese hamster ovarian cells showed resistant to colchicine due to the alteration of the membrane permeability. Fifty percent of clinical post-chemotherapeutic breast, ovarian, and myeloma patient samples have increased P-gp1 expression levels (Juliano & Ling, 1976; Leonard et al., 2003). Therefore, the correlation between P-gp1 overexpression and MDR has led to the current acceptance of P-gp1 as an energy-dependent efflux pump of various drugs that converts chemotherapeutically sensitive cancer cells to MDR phenotype (Polgar & Bates, 2005; Sharom, 2008).

Pgp1 is a 170 kDa single polypeptide composed of two similar halves, each half formed by a transmembrane domain (TMD) consisting of six α-helical membrane-spanning domains and a nucleotide-binding domain (NBD) (Gottesman et al., 1996). It fulfills a cellular detoxification function because of its ability to transport various substrates, which are often toxic to the cell (Sugawara, 1990). P-gp1 is ubiquitously expressed in many organs such as the liver, brain, kidney and gastrointestinal tract.

A higher concentration is seen in the apical membranes of epithelial cells lining the colon, small intestine, pancreatic bile ductules, kidney proximal tubule, placenta, brain capillary endothelium, and in the endothelial cells lining capillaries in the testis and inner ear (Figure3) (Sugawara, 1990; Doran et al., 2005).

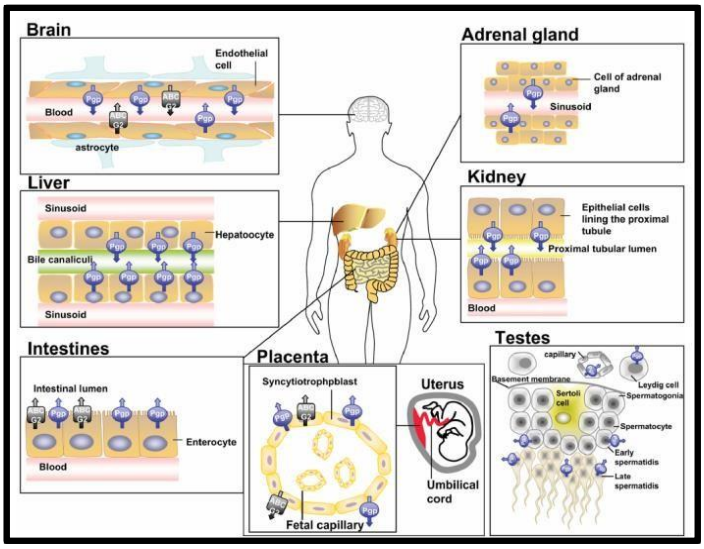

Figure 3: **ABCB1 or P-gp1 and ABCG2 localization in human organs.**

(Wu et al., 2011).

ATP hydrolysis provides energy for P-gp1 to accelerate drug effluxing by a direct interaction between the substrate and the recognition regions in the transporter. P-gp1 interacts with structurally diverse substrates, including natural products, anti-cancer drugs, steroids, fluorescent dyes, and linear or cyclic peptides (Velingkar and Dandekar, 2010). The direct interaction between many substrates and P-gp1 has been elucidated along with drug binding studies. A highly specific drug binding to the P-gp1 transporter is essential to initiate transporter activity, which was shown when Cornwell and colleagues found various calcium channel blockers, such as verapamil, desmethoxyverapamil, and diltiazem, bind specifically to the membrane vesicles isolated from resistant KB cells (Cornwell et al., 1987).

The high affinity of these calcium channel blockers for P-gp1 provides further evidence of the important role of P-gp1 in causing MDR. The discovery of P-gp1 generated many drugs known as MDR chemosensitizers, modulators, or inhibitors. Some of these compounds interact and stimulate P-gp1 basal ATPase activity (Garrigos et al., 1997). They considerably prevent drug resistance by either inhibiting the activity of P-gp1, which occurs when the modulator competes with the anti-cancer drug for the binding site, or blocking the action of P-gp1 and thus increase the accumulation of chemo- drugs in MDR cells (Ozben, 2006). As an example for the latter, cholesterol was found to stimulate P-gp1 ATPase, thus, cholesterol is recognized and transported by P-gp1(Bucher et al., 2007). It was also shown the reduction of cholesterol reduced the activity of P-gp1, which leads to an increase in the drug accumulation inside MDR cells. Cholesterol and verapamil were identified as P-gp1 chemosensitizers since the 1980s (Bucher et al., 2007). Many of the P-gp1 chemosensitizers cannot be clinically used because they change the pharmacokinetic of the conventional chemo-drugs, which result in a fatal toxicity (Ramu et al., 1984; Fardel et al., 1996; Lavie et al., 1997; Krishna & Mayer, 2001; Polgar & Bates, 2005).

P-gp1 is described as a 'hydrophobic vacuum cleaner' or 'flippase'. It detects and removes the hydrophobic substrates from the lipid bilayer into the outer environment of the cells. The idea was supported after finding that the MDR2 gene product (a close homologue of P-gp1) is a phosphatidylcholine translocase (flippase) fundamental for efflux of phosphatidyllcholine from the hepatic plasma membrane into the bile (Ruetz & Gros, 1994). When two ATP bind to an ATP-binding domains, the two domains bring together, initiate the ATP hydrolysis and release ADP and Pi.

The hydrolysis of one ATP enables to transport the substrate, then the transporter resets to its open confirmation to sequester another substrate. Highly lipophilic molecules such as verapamil and tamoxifen rapidly return to the lipid bilayer to re- bind with P-gp1, which eventually results in a cycle of constant efflux called "futile cycling". Consumption of ATP leads to an increase in reactive oxygen species (ROS) to the threshold levels and consequently apoptosis (Karwatsky et al., 2003). Alternative approaches are still needed due to poor responses to treatment as a result of MDR as well as the toxic side effects of reversing agents. Thus, there is great potential for exploiting the collateral sensitivity feature that elicits some MDR.

1.1.8 Collateral sensitivity

Collateral sensitivity (CS) is an increase in the sensitivity of resistant cancer cells to certain drugs but not to others (Hall et al., 2009). The concept of CS was first illustrated when hypersensitivity or CS was observed in drug- resistant *Escherichia coli* (Szybalski & Bryson, 1952). Following, in 1976, Bech- Hansen and coworkers selected the first MDR cell lines derivative from the Chinese hamster ovarian (CHO) line AuxB1, at increasing concentration of colchicine. The cell lines exhibited CS to various steroid hormones and detergents. The same group observed that the higher the resistance of CHO cells, the greater degree of CS, suggesting the increase in CS is directly linked with the over-expression of P-gp1 (Bech Hansen, Till, & Ling, 1976). Since then the phenomenon of CS has been studied intensively in an effort to understand the mechanism.

The CS concept has been demonstrated with drugs, including calcium channel blockers, steroids, and nonionic detergents. In 2003, Karwatsky *et al*. were able to illustrate the mechanism of verapamil inducing CS in CH^RC5 cells by oxidative stress due to increase P-gp1 ATPase and rise in ROS levels above the normal levels, which lead eventually to cell death (Figure 4) (Karwatsky et al., 2003). Moreover, the knockdown of P-gp1 gave more evidence of the direct correlation between P-gp1 overexpression and verapamil CS (Laberge et al., 2009). In spite of verapamil andother enhance hypersensitivity in MDR, the non-clinically relevant concentration needed to induce CS and the severe side effects were major barriers to apply in clinic (Ozols et al., 1987; Miller et al., 1991). Therefore, it would be ideal to find less toxic and clinically approved drugs that are able alone to induce CS in P-gp1 positive cancer cells. A N,N-Diethyl-2-[4-(phenylmethyl) phenoxy] ethanamine or (DPPE) tamoxifen derivative and a intracellular histamine antagonist was shown to potentiate the toxicity of some anticancer drugs (doxorubicin, cisplatinum) in breast cancer phase III clinical trials (Kudoh et al., 1997). Moreover, the ability of DPPE to induced CS in P-gp1 positive cells alone (Georges et al., 2014). CS has been seen with tamoxifen derivatives in P-gp1 positive cells, allowing us to closely consider tamoxifen, a widespread drug used in treating breast cancer, to be tested for it is ability to induce CS in P-gp1 positive cells at clinically relevant concentration without severe side effects.

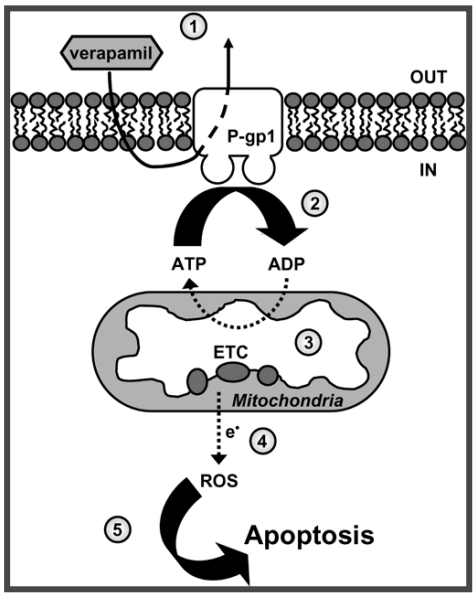

Figure 4: A proposed mechanism of verapamil collateral sensitivity in P-gp1 expressing cells. Verapamil crosses the cellular membrane, interacts and transports out of cells by P-gp1 (1). The interaction between P-gp1 and verapamil causes elevated levels of ATP hydrolysis (2). This creates a high demand for ATP, which is generated from oxidative phosphorylation in the mitochondria (3). A consequence of high ATP demand, electrons are lost from the electron transport chain (ETC), causing the production of higher than normal levels of ROS (4). The high concentration of ROS causes apoptosis by damaging lipids and DNA, or by initiating the cytochrome c apoptotic pathway (Karwatsky et al., 2003).

1.1.9 Tamoxifen

Tamoxifen is a widely used anti-estrogen agent for treatment and prevention of recurrent cancer in both pre- and post- menopausal women who are at high risk of breast cancer. High doses of tamoxifen tend to be safely tolerated in humans with minimal side effects (Trump et al., 1992). It is a member of the selective estrogen receptor modulators (SERMs), which function by binding to the estrogen receptor and prevent the binding to other coactivators and consequently inhibit cell proliferation (Lewis-Wambi & Jordan, 2009).

Tamoxifen was initially shown to reverse P-gp1-mediated MDR in doxorubicin-resistant P388 murine leukemia cells, breast cancer cells, and other P-gp1 expressing cells (Ramu et al., 1984; Saeki et al., 2005). The reversing mechanism of tamoxifen was thought to be due to its ability to compete for the substrate-binding site of P-gp1. Consequently, tamoxifen was shown to increase the accumulation of vinblastine and adriamycin in P-gp1 overexpressing cell lines. By contrast, other studies reported that tamoxifen is not transported by P-gp1 (Ramu et al., 1984; Berman et al., 1991; Wu et al., 2011). In addition, tamoxifen has shown to increase ROS production by disrupting the mitochondria electron transport chains (mETC) through its interaction with complex I (Moreira et al., 2006). In this proposal we highlight the CS mechanism of tamoxifen induced cell death of P-gp1-positive cancer cells, independently of its genomic functions.

2 Reactive oxygen species and programmed cell death

2.1 Reactive oxygen species (ROS)

Oxidative stress occurs when an imbalance between ROS levels, reactive nitrogen species (RNS) levels, and anti-oxidative defense mechanisms (Ozben, 2007). Several cellular sites can generate ROS, including cytosol, peroxisomes, mitochondria matrix and membrane, and endoplasmic reticulum membranes. Indeed, the mitochondria are considered the main source leading to ROS production (Andreyev et al., 2005; Jezek & Hlavata, 2005).

The mitochondrial ROS production differs based on cell physiological and pathological conditions. ROS are free radicals generated in cells such as hydroxyl, superoxide radicals, and non-radicals components like hydrogen peroxide and singlet oxygen (Ozben, 2007). Mitochondria contain various redox carriers such as NAD^+ and FAD that can give away electrons to oxygen to convert to superoxide anion O^-. The superoxide anion considers the primary ROS radical that lead to the formation of more secondary reactive ROS radicals (Venditti et al., 2004). The unreactive O- interact with superoxide dismutase (SOD) to produce hydrogen peroxide (H_2O_2). The H_2O_2 can be converted into hydroxyl radical in the presence of reduce transition metals (Fe_2^+ or Cu^+). It is highly reactive, which makes it a very toxic radical with a very short half life of approx. 10^{-9}s in vivo. The H_2O_2 can be deactivated by antioxidants such as vitamin E, vitamin C, coenzyme Q10, glutathione, and antioxidant enzyme systems, such as glutathione peroxidase (GPX), and catalase (CAT) (Radi et al., 1991; Ozben, 2007).

Cells can normally sustain and tolerate the increase in ROS levels; however, when the level heightens and the defense system is overwhelmed, cellular damage occurs (Deavall, Martin, Horner, & Roberts, 2012). This damage targets different cellular sites, including DNA, proteins, lipids, and modulate survival-signaling cascades (Figure 5). Drugs that target mitochondria lead to high oxidative stress and eventual cell death (Deavall et al., 2012). Generally, tumor cells have high ROS, and that was shown in human malignancies relative to normal cells (Waris & Ahsan, 2006). Moreover, ROS generating drugs induce oxidative stress as a targeting mechanism that leads to fatal damages to cancer cells (Figure 5) (Deavall et al., 2012).

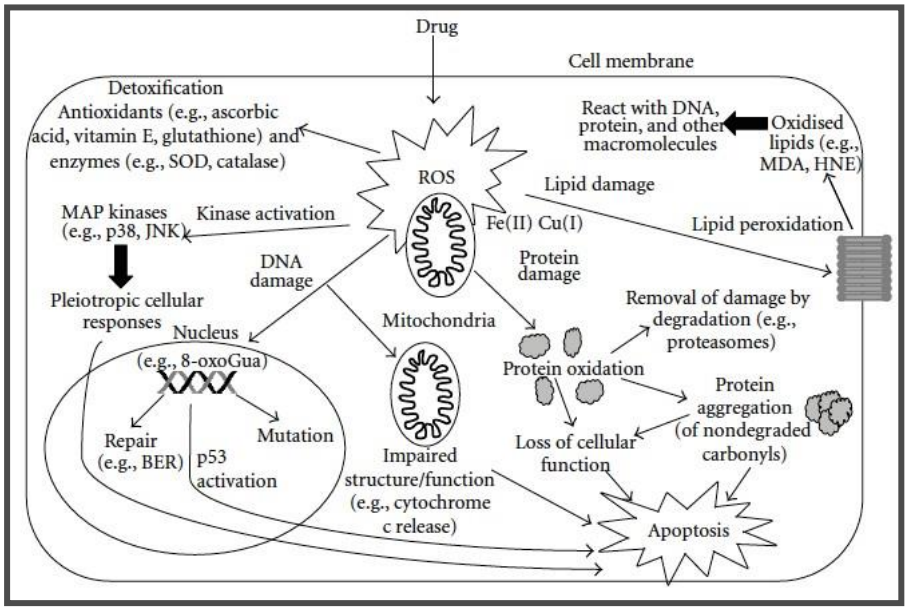

Figure 5: **The damaging effects of drug induced oxidative stress in cells**

(Deavall et al., 2012).

2.2 Glutathione (GSH)

GSH is a tripeptide, y-L-glutamyl-L-cysteinyl-glycine, which exists in all cells but has a particularly high concentration in the liver (5-10 mM) (Kaplowitz, 1981). GSH is a modulator of cell survival and cell death. Low GSH levels are associated with mitochondrial dysfunction and induction of apoptosis (Mérad-Saïdoune et al., 1999; Coppola & Ghibelli, 2000).

GSH synthesis requires two ATP-dependent enzymes: γ-glutamylcysteine synthetase (γ-GCS) and glutathione synthetase (GSS). Ninety-eight percent of intercellular glutathione exists in the thiol-reduced form (GSH) and a small percentage in the thiol-oxidized form GSH disulfide (GSSG). GSH/GSSG levels are maintained by the nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) dependent enzyme glutathione reductase, forming the redox cycle (Figure 6) (Haddad, 2002).

The primary function of the GSH is to maintain the thiol redox status of cells and subsequently protect cells against ROS, reactive metals and electrophiles (Meister & Anderson, 1983; Meister, 1984; Lu, 2009). The cellular GSH mostly resides in the cytosol and some in the mitochondria and endoplasmic reticulum (Meredith & Reed, 1982; Hwang et al., 1992).

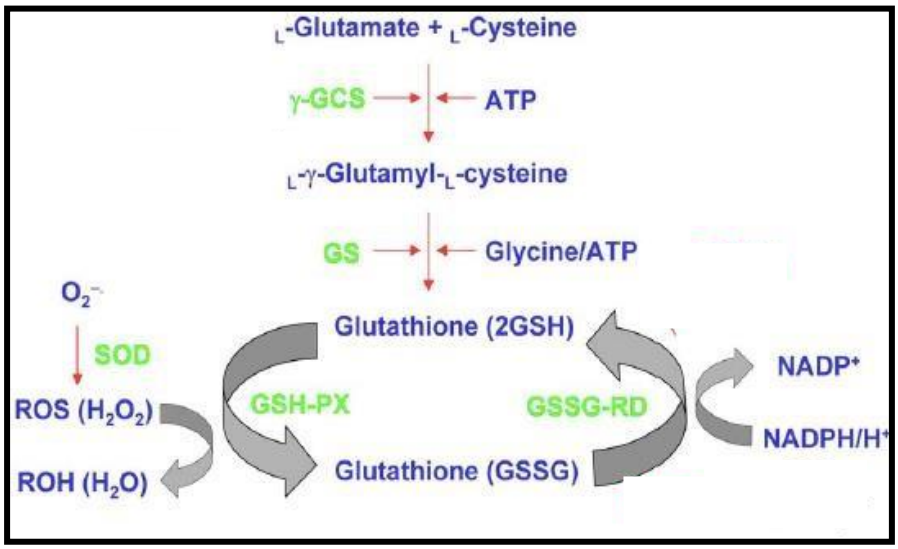

Figure 6**: The schematic for redox cycle**. Enzymes show in green; □- glutamylcysteine synthetase (□-GCS); glutamyl synthase (GS);superoxide dismutase (SOD); glutathione- peroxidase (GSH-PX); glutathione reductase (GSSG-RD). Modified from (Haddad, 2002).

2.3 Programed cell death

The three main categories of programmed cell death are apoptosis, autophagy, and necroptosis; can be differentiated by their morphological features (Bursch et al., 2000; Ouyang et al., 2012). Apoptosis is the most well-defined programmed cell death mechanism. It is a complex mechanism that involves several pathways and proteins such as the cysteine proteases, known as caspases (Alnemri et al., 1996).

It is characterized by distinctive morphological and biochemical changes, including cell shrinking, nuclear condensation and fragmentation, chromosomal DNA cleavage into inter-nucleosomal fragments, and phosphatidylserine externalization (Nishida et al., 2008).

The two major pathways that stimulate apoptosis in damaged cells are extrinsic and intrinsic pathways (Igney & Krammer, 2002; Okada & Mak, 2004). The extrinsic pathway involves specific external ligands, such as Fas-L and TNFα, binding to cell surface receptors or plasma membrane death receptors such as Fas and tumor necrosis receptor 1 respectively (Budihardjo et al., 1999). The binding of Fas-L to Fas results in Fas/Fas-L, which composes of death domain- containing protein (FAAD). TNFα to TNFR binding induces the formation of the death induced signaling complex (DISC) that leads to the activation of pro-caspase-8 and 10, which cleave pro-caspase-3, 6, and 7 to its active form. The process can be inhibited by a protein called c-FLIP binds to FADD and pro-caspase-8 and inactivates them (Elmore, 2007). Other protein called Toso also interrupts apoptosis process by blocking Fas- induced apoptosis in T-cells through the inhibition of caspase-8 processing (Budihardjo et al., 1999; Elmore, 2007).

In contrast, the apoptotic intrinsic pathway is controlled by mitochondria pro-enzymes and is triggered by various extracellular and intercellular stresses such as growth factor depletion, hypoxia, DNA damage, and oncogene induction. A series of biochemical events are induced as a result of intrinsic pathway induction. These biochemical stimuli result in changes in the mitochondria inner membrane which lead to an opening of the mitochondria permeability transition pores (MPT), loss of the mitochondria transmembrane potential, and release of cytochrome C that regulated by

Bcl-2 family members and other pro-apoptotic compounds (Smac/Diablo, serine protease Omi/HtrA2, endonuclease G, and apoptosis inducing factor (AIF)) (Wang and Youle, 2009; Ouyang et al., 2012). Once cytochrome C is released, it binds and activates apoptotic protease-activating factor (Apaf-1) as well as caspase-9, forming a large protein complex called an apoptosome, which promotes the cascade of caspase activations (Budihardjo et al., 1999). Moreover, the anti-apoptotic members, such as the Bcl-2 family, are regulator compounds of the outer mitochondria membrane permeability, which stop the cascade events that lead to apoptotic death; however, once the cytochromes are released, the cascade of caspase activation is irreversibly activated (Cory & Adams, 2002).

Rational and Objectives

Rational

The emergence of multidrug-resistance has reduced cancer treatment options. Therefore, finding an alternative approach to overcome this issue is necessary. MDR is mediated by various intercellular alterations; one of these mechanisms is the reduction of chemo-drugs accumulation by overexpression of ATP-binding cassette (ABC) efflux transporters. Analyses of clinical samples from cancer patients (breast, ovarian, myeloma, and AML), post-chemotherapeutic treatment showed a significant increase in the expression of P-gp1. Thus, blocking or inhibiting its function is required, but use of these inhibitors in combination with chemotherapeutic drugs was abandoned due to changes in the pharmacokinetics of anticancer drugs leading to unacceptably high toxicity (Ozben, 2006). The exploitation of P-gp1 function is a novel notion that increases the CS of P-gp1-positive cancer cells to various non-toxic compounds. Earlier studies have shown that P-gp-positive cells exhibit CS to non-toxic compounds. Moreover, we have previously shown that verapamil and other steroids drive Pg-positive MDR cells to apoptosis through oxidative stress (Karwatsky et al., 2003; Georges et al., 2014). However, because of the cardio-toxicity that verapamil cause, it would be ideal to find drugs with known safety indices, clinically used, and able to induce alone CS at a clinically relevant concentration. Tamoxifen is an antagonist of the estrogen receptor, used to treat and prevent recurrence of breast cancer. We hypothesize that tamoxifen could elicit CS in P-gp1 overexpressing cancer cells through an increase in oxidative stress levels to the threshold level, which consequently leads to cell death.

Objectives

- Determine the collateral sensitivity of tamoxifen at low concentrations in overexpressing P-gp1 CHO cells and MDA-MB-231/ doxorubicin cancer cells, relative to the parental cells.
- Determine the collateral sensitivity of tamoxifen to P-gp1 positive cells is due to not only the overexpression of P-gp1 transporter, but also the ATPase activity of P-gp1.
- Determine if apoptosis, which occurs after the treatment with tamoxifen, is due to intracellular decrease in GSH accompanied by dramatic increase in ROS.

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Chapter Two

Manuscript I

P-glycoprotein (ABCB1) Expression Intensify the Sensitivity of Triple Negative Breast Cancer Cells to Tamoxifen

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Running Title: P-glycoprotein increases sensitivity of cancer cells to tamoxifen

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Key words: P-glycoprotein; Collateral Sensitivity; Multidrug Resistance; ABCB1; Tamoxifen.

The authors disclose no potential conflicts of interest

Abstract

P-glycoprotein1 (P-gp1) has been shown to confer resistance to clinically relevant anticancer drugs, including targeted chemotherapy. Paradoxically, P-gp1 expression has been shown to confer hypersensitivity or collateral sensitivity to non-toxic drugs. In this report, we examined the role of tamoxifen as a collateral sensitivity drug in P- gp1 expressing Chinese hamster ovary and triple negative human breast cancer cells. Our results showed that resistant cells ($CHO^{R}C5$ and MDA-$Doxo^{400}$) were more sensitive to tamoxifen than drug sensitive parental cells (AuxB1 and MDA-MB-231). Moreover, the collateral sensitivity to tamoxifen correlated with P-gp1 expression levels and was reversed with specific inhibitor of P-gp1 ATPase, PSC-833. Furthermore, tamoxifen induced apoptosis in P-gp1-expressing cells that was partially reversed in $CHO^{R}C5$ cells stably overexpressing Bcl-2. Consistent with current understanding of P-gp1-mediated collateral sensitivity, the presence of tamoxifen caused a significant increase in reactive oxygen species in $CHO^{R}C5$ and MDA-$Doxo^{400}$ cells and a drop in total cellular thiols, of which glutathione is a major component. Consistent with the latter, we show that addition of 0.5-1 mM N-acetyl cysteine completely reversed the effect of tamoxifen on the proliferation of $CHO^{R}C5$ and MDA-$Doxo^{400}$ cells. Taken together, this is the first demonstration of tamoxifen as a collateral sensitivity drug that preferentially targets P-gp1 expressing drug resistant cells. Moreover, our findings support the use of tamoxifen in the treatment of triple negative breast cancer patients following chemotherapy.

Introduction

Clinical drug resistance, intrinsic or acquired, remains an obstacle in the treatment of cancer patients with chemotherapeutic drugs [1-3]. P-glycoprotein1 (P-gp1, or ABCB1), member of a large family of ATP Binding Cassette (ABC) transporters, mediates the efflux of anticancer drugs from cancer cell in an ATP-dependent manner [4]. P-gp1 expression has been detected in normal tissues and organs, where it mediates the secretion of xenobiotics and other cell metabolites [5]. Moreover, increased P-gp1 expression post-treatment has been demonstrated in several cancers [6] and its expression in tumors from breast, lung and neuroblastoma cancers have been associated with poor prognosis and patient outcome [7, 8]. Clinical trials using increasingly more potent inhibitors of P-gp1 drug efflux function (1st to 3rd generation MDR-reversing drugs) have been largely unsuccessful due to changes in the pharmacokinetics and unacceptable toxicity [9, 10]. Remarkably, P-gp1 expressing cells are also hypersensitive or collaterally sensitive to certain drugs and initially included calcium channel blockers and other membrane active agents [11]. We have recently shown that the collateral sensitivity of drug resistant cells to such drugs is dependent on P-gp1 expression, whereby stimulation of it ATPase activity triggers a rise in reactive oxygen species (ROS) leading to selective oxidative cell death of resistance cells [12-15]. Consistent with this mechanism, non-P-gp1 substrates have also been shown to selectively target drug resistant cells and trigger P-gp1-dependent oxidative cell death through increased ROS levels [16, 17].

In an effort to validate the possibility of using collateral sensitivity drugs in the treatment of drug resistant cancers, it was of interest to identify clinically approved drugs that can trigger P-gp1-dependent collateral sensitivity. Based on our working hypothesis of P-gp1-dependent collateral sensitivity [12-15], it was of

interest to test the ability of tamoxifen, a widely used drug for the adjuvant treatment of breast cancer [18], as a collateral sensitivity drug. Tamoxifen has been previously shown to activate P-gp1 ATPase at clinically achievable concentration, but is not a substrate for P-gp1 drug efflux mechanism [19, 20]. In addition, tamoxifen has been shown to induced apoptosis in both estrogen receptor-α positive and –negative breast cancer cells by increasing intracellular ROS levels [21]. Hence, tamoxifen combines two favorable properties associated with compounds that elicit P-gp1-dependent collateral sensitivity of cancer cells. In this study, we show for the first time that an anti-estrogen receptor drug, tamoxifen, preferentially targets P-gp1 expressing cells, including triple negative doxorubicin selected human breast cancer cells. The potential clinical applications of tamoxifen in the treatment of drug resistant triple negative breast cancer are discussed.

Material and Methods

Tissue culture and cell growth assays

Drug-sensitive (AuxB1, MDA-MB-231) and -resistant (CHO^{R}C5, CHO^{R}C5/Bcl-2, MDA-$Doxo^{400}$) cells were grown in α-minimal essential medium (α-MEM), dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco, Life technologies) at 37°C in the presence of 5% CO_2, without or with selective concentrations of colchicine or doxorubicin (5 μg/ml for CHO^{R}C5 and 400 nM for MDA-$Doxo^{400}$ cells, Sigma-Aldrich, Ont., CA). For cell survival assays, drug sensitive and resistant cells were plated in 200 μl α-MEM, DMEM containing 10% FBS (Gibco, Life technologies) in 48-well plates. Cells were incubated for 24 hours at 37^{o}C prior the addition of 200 μl media containing tamoxifen, hydrogen peroxide or rotenone alone or in combination (Sigma-Aldrich, Ont., CA). Cell clones were allowed to proliferate for 7-8 days at 37^{o}C without or with drugs prior to the addition of cell staining dye methylene blue (0.1 - 1% methylene blue in ethanol/H_2O). The dye solution was removed and plates washed gently in cold water and air-dried. Fixed and dye stained cells were extracted with 0.1% SDS/PBS from each well and the absorbance quantified at 660 nm (Synergy H4, generation 5, BioTek, Canada). The effect of drugs on cell growth was determined by comparing the absorbance of cells grown in the presence of drugs to solvent control without added drugs (100% cell survival). All graphs shown represent the mean ± SD of two independent experiments done in triplicate.

Protein extraction and immuno-detection

Total cells lysate (20µg) from drug sensitive and drug resistant cells was resolved on 6% SDS-PAGE and transferred onto a PVDF membrane. The membrane was blocked in 5% (w/v) milk in phosphate buffered saline with tween 20 (PBST) and probed with specific P-gp1 monoclonal antibody (0.1 µg/ml of C-494 mAb; [22]) in 5% milk/PBST, followed by several washes in PBST and incubation with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:5000 v/v, BioRad, Ont., CA). The signals were developed using WesternBreeze Chemiluminescent Kit and captured according to the manufacture's protocol (Thermo-Fisher Scientific, Ont., CA) and captured using ECL-imager from Thermo-Fisher Scientific. Tubulin expression was detected on the same PVDF membrane using monoclonal anti-tubulin (1 µg/ml Sigma-Aldrich, Ont., CA), followed and HRP-conjugated goat anti-mouse IgG (1:5000 (v/v) dilution, BioRad, Ont., CA).

Plasma membrane and ATPase assay

Plasma membranes from AuxB1 and CHO^{R}C5 cells were prepared as previously described [13]. P-gp1 ATPase activity was measured in a reaction mix containing drugs together with ATPase inhibitors and plasma membranes in ice-cold Buffer I (100 mM Tris-HCl, pH 8.0, 4 mM DTT, 100 mM KCl, 10 mM MgCl2, 10 mM NaN3, 4 mM EGTA, 2 mM Ouabain and 3 mM ATP). Na-orthovanadate (500µM) was added to 96-well assay plates on ice. Membranes from AuxB1 and CHO^{R}C5 cells were added to the wells (30 µl at 1 µg/well) and incubated at 37^{O}C for 30 min. Tamoxifen, at increasing concentrations (100-0), was added to the wells and plates were incubated for one hour at 37^{O}C. The reaction was stopped with the addition of 200 ml of buffer S containing [0.2% (w/v) ammonium molybdate, 1.3% (v/v) sulfuric acid, 0.9% (w/v)

SDS, 2.3% (w/v) TCA and 1% (w/v) L-ascorbic acid]. Plates were incubated at room temperature for 75 min and absorbance read at 620 nm.

ROS measurements with H_2DCFDA

Drug sensitive (MDA-MB-231, AuxB1) and –resistant (MDA-Doxo^{400}, CHO^{R}C5) cells were plated at density of 1x10^{5} cells in 100ml of media per well in 96-black well plates and incubated at 37^{O}C for 24hr prior the addition of tamoxifen (0.5 - 10μM final concentration). Cells were incubated without and with increasing concentrations of tamoxifen for 24hr, prior to the addition of 100μM of 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) for 30 min at 37^{O}C. AuxB1 and CHO^{R}C5 cells were co-incubated with 2μM of PSC-833 and H2DCFDA. Cells were washed with ice-cold PBS and the resulting fluorescent signals, measured at excitation and emission wavelengths of 495 excitation – 527 emission nm, were used to calculate intracellular ROS values relative to control untreated cells. The results shown represent two independent experiments done in triplicate.

Total thiol measurement

Cells were plated in 48 well plates and incubated for 24hr, prior to the addition of tamoxifen (25 μM). Plates were incubated at 37^{O}C for 24hr, then washed with PBS and extracted with RIPA buffer (50 mM Tris, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP40 and 0.1% SDS, pH 8.0). Aliquots of cell lysates were used for protein quantification, to measure cellular thiols using 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB). Briefly, 100ml of DTNB was added to 150ml of cell lysates and incubated for 10 minutes at room temperature prior to reading the absorbance at 410 nm (Synergy H4, generation 5, BioTek). The effect of tamoxifen

on cellular thiols was determined by comparing the absorbance of cells grown in the presence of drugs to solvent control without added drugs (set at 100%). The results represent the mean ± SD of two independent experiments done in triplicates.

Apoptosis assays

For annexin-V staining of apoptotic cells, drug sensitive and resistant cells (AuxB1, $CHO^{R}C5$ respectively) were seeded in six-well plates at 1-2 x 10^5 density per well and incubated for 24 hrs prior the addition of tamoxifen for 24 hours. Cells ($1x10^6$) were lifted and washed with ice cold PBS and resuspended in 100ml of 1x binding buffer. Five µl of Annexin V- FITC solution and 5µL propidium iodide (PI) were added to cells and allowed to incubate for 15 minute in the dark according to the manufacturer protocol (BD, FITC Annexin V Apoptosis Detection kit I). 400ml of the 1xbinding buffer was added prior analysis with (BD FACSDiva). Percent apoptosis was determined by flow cytometry (BD FACSDiva) measuring the relative fluorescence in drug treated versus control untreated cells.

Statistical analysis

All graphs and statistics were performed using GraphPad prism version 6.

Statistics represent the student *t* and one-way ANOVA test.

Results

We have previously demonstrated a correlation between P-gp1 ATPase stimulation and collateral sensitivity of MDR cells (Georges et al., 2014; Karwatsky et al., 2003; Laberge et al., 2009; Laberge et al., 2014). In this study we examined the ability of the anti-estrogen drug tamoxifen, shown previously to activate P-gp1 ATPase (Callaghan and Higgins, 1995; Rao et al., 1994), to induce collateral sensitivity in two multidrug resistant cell lines (colchicine-selected Chinese hamster ovary cells ($CHO^{R}C5$) and doxorubicin-selected triple negative human breast cancer cells (MDA-$Doxo^{400}$)). A Western blot in figure 1A shows the relative expression of P-gp1 in total cell extracts from $CHO^{R}C5$ and MDA-$Doxo^{400}$ cells. Both drug resistant cell lines (e.g., $CHO^{R}C5$ and MDA-$Doxo^{400}$) show P-gp1 overexpression relative to their parental drug sensitive cells (e.g., AuxB1 and MDA-MB-231), with $CHO^{R}C5$ cells showing higher levels of P-gp1 expression (Fig. 1A). Figure 1B shows the growth of drug sensitive and resistant cells in the presence of increasing concentrations of tamoxifen. The results in figure 1B show that P-gp1-overexpressing cells ($CHO^{R}C5$, MDA-$Doxo^{400}$) are more sensitive to tamoxifen than their respective parental drug sensitive cells (AuxB1 and MDA-MB-231). Moreover, the more drug resistant $CHO^{R}C5$ cells with higher P-gp1 expression reveal greater sensitivity to tamoxifen than MDA-$Doxo^{400}$ cells (IC_{50} values of 0.900 ± 0.0900 μM and 2.998± 0.0714 μM, respectively; Table I). These results are consistent with results from earlier studies demonstrating a correlation between P-gp1 expression levels and the sensitivity of drug resistant cells to collateral sensitivity drugs (Karwatsky et al., 2003; Laberge et al., 2009). It is noteworthy that the observed collateral sensitivity of MDA-$Doxo^{400}$ cells to tamoxifen is ER-independent as their parental MDA-MB-231 cells are ER-negative (Pedro et al., 2006). The differential

effect of tamoxifen on the proliferation of AuxB1 and MDA-MB-231 drug sensitive cells is not clear, but may be due to several factors including ER-expression in CHO cells (Thomas et al., 2003).

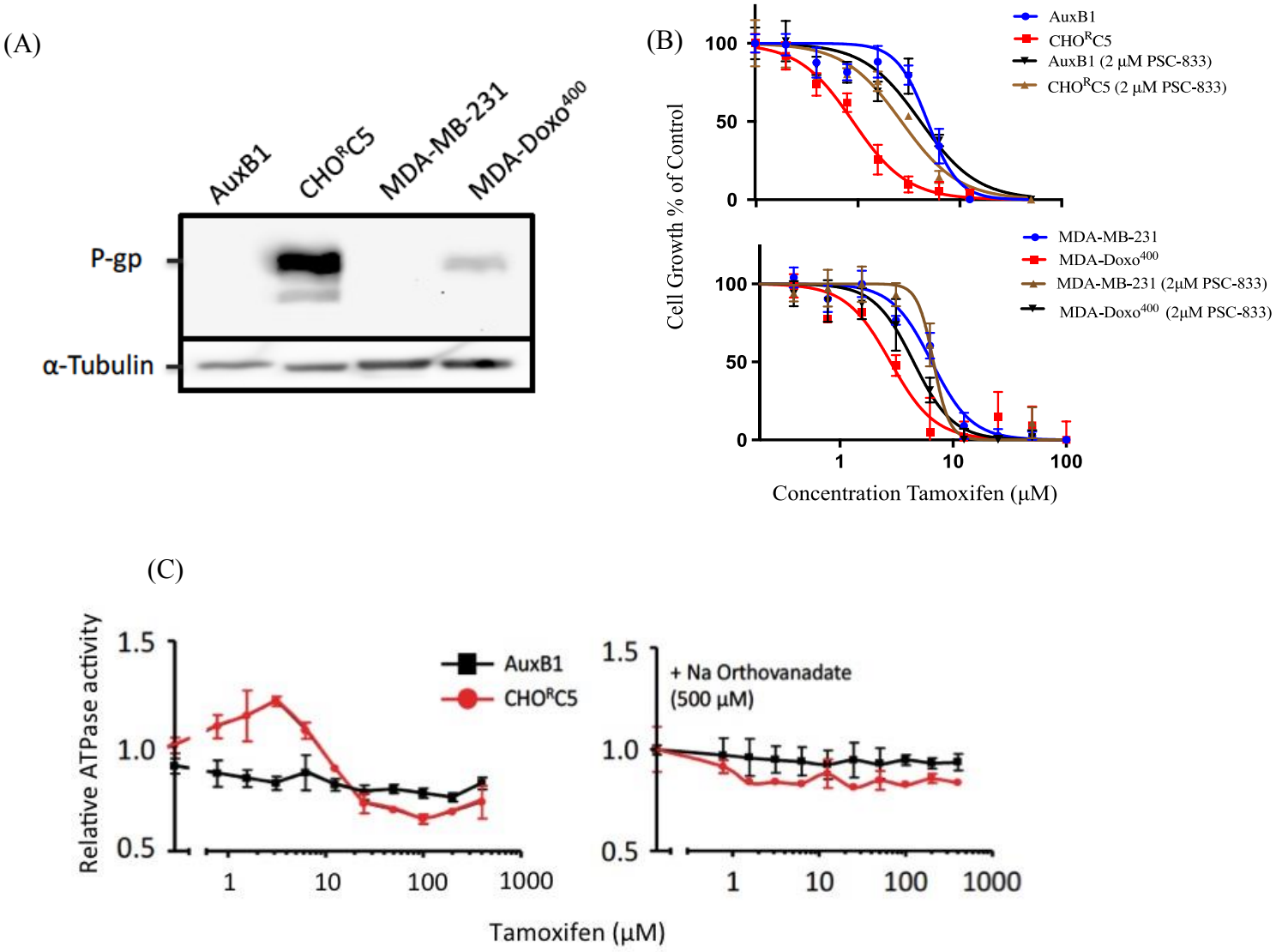

Figure 1. Sensitivity to tamoxifen correlates with P-glycoprotein1 expression and ATPase – Panel (A) shows P-glycoprotein1 expression in total cell extracts from drug-sensitive (AuxB1 or MDA-MB-231) and -resistant (CHO^{R}C5 and MDA-Doxo^{400}) cells by Western blots probed with anti-C494 monoclonal antibody. Panel (B) shows the effect of increasing tamoxifen concentrations on the growth of drug sensitive (AuxB1, MDA-MB-231) and resistant (CHO^{R}C5, MDA-Doxo^{400}) cells in the absence or presence of 2 μM PSC-833. The effect of tamoxifen on cell growth was plotted as a percent of control relative to cells treated with carrier solvent in the absence or presence of 2 μM PSC-833 alone. Panel (C) shows the ATPase activity of AuxB1 and CHO^{R}C5 that measured using purified plasma membranes exposed to tamoxifen with or without Na-orthovanadate. Graphs represent the mean ± SD of three

independent experiments done in triplicates

Table 1. The IC_{50}s of tamoxifen drug sensitive (AuxB1, MDA-MB-231) and drug resistance ($CHO^{R}C5$, MDA-$Doxo^{400}$) cells.

Cell Lines	Tamoxifen IC50±SD (μM)
AuxB1	4.714 ± 0.056
$CH^{R}OC5$	0.900 ± 0.0900
MDA-MB-231	6.499± 0.712
MDA-MB $^{400\ nM\ DOXO}$	2.998± 0.0714
AuxB1(2 μM PSC-833)	2.4835 ± 0.137
$CH^{R}OC5$(2 μM PSC-833)	3.582± 0.0480
MDA-MB-231(2 μMPSC-833)	6.394± 0.210
MDA-$MB^{400\ nM\ DOXO}$ (2μM PSC-833)	4.336± 0.895

To determine if tamoxifen-induced stimulation of P-gp1 ATPase modulates the collateral sensitivity of drug resistant cells, the results in figure 1B show the growth of drug sensitive and resistant cells with increasing concentrations of tamoxifen but in the presence of PSC-833, a specific inhibitor of P-gp1 ATPase (Atadja et al., 1998). The presence of 2 μM PSC-833 reversed the collateral sensitivity of $CHO^{R}C5$ and MDA-$Doxo^{400}$ cells to tamoxifen, with shift in IC_{50} values from 0.900 ± 0.0900 and 3.582 ± 0.0480 μM to 2.998 ± 0.0714 and 4.336 ± 0.895 μM with PSC-833 for $CHO^{R}C5$ and MDA-$Doxo^{400}$, respectively. Figure 1C shows significant increase in P-gp1 ATPase between 1 and 5 μM tamoxifen and drops off at higher concentrations, the activation of ATPase was inhibited with Na-orthovanadate is consistent with earlier observation. Similar results were obtained using increasing concentrations of 4-hydroxy-tamoxifen, a metabolite of tamoxifen (Taylor et al., 1984),without and with 2 μM PSC-833(Fig .2).

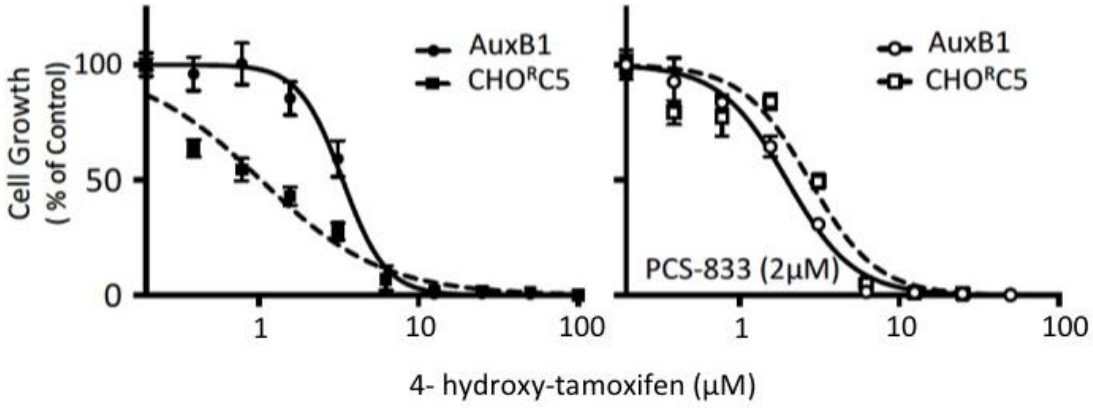

Figure 2. The sensitivity to 4-hydroxy-tamoxifen correlates with P-glycoprotein1 expression and ATPase. The graph shows the effect of increasing 4-hydroxy-tamoxifen concentrations on the growth of drug sensitive (AuxB1, MDA-MB-231) and resistant ($CHO^{R}C5$, $MDA\text{-}Doxo^{400}$) cells in the absence or presence of 2 μM PSC-833. The effect of 4-hydroxy-tamoxifen on cell growth was plotted as a percent of control relative to cells treated with carrier solvent in the absence or presence of 2μM PSC-833 alone. Graphs represent the mean ± SD of three independent experiments done in triplicates.

Collateral sensitivity of P-gp1 overexpressing cells was shown to be due to oxidative cell death (Karwatsky et al., 2003). To determine if tamoxifen-induced collateral sensitivity of drug resistant cell is caused by enhanced apoptosis, drug sensitive and resistant cells were stained with FITC-modified annexin V following tamoxifen treatment. The results in figure 3A show higher percentage of drug resistant cells ($CHO^{R}C5$ and $MDA\text{-}Doxo^{400}$) stained with FITC-annexin V than drug sensitive (AuxB1 and MDA- MB-231) cells at different concentrations of tamoxifen (i.e., 2.5 μM to 10 μM). These results are consistent with earlier studies using different collateral sensitivity drugs (Georges et al., 2014; Laberge et al., 2014; Karwatsky et al., 2003). Given the latter results, it was of interest to determine if $CHO^{R}C5$ cells stably transfected with Bcl-2 ($CHO^{R}C5$/BCl-2) are less sensitive to tamoxifen collateral sensitivity (Karwatsky et al., 2003).

Figure 3B shows the effects of increasing tamoxifen on the growth of AuxB1, CHO^{R}C5 and CHO^{R}C5/Bcl-2 cells in the absence or presence of PSC-833. The results in figure 3B show that the over-expression of Bcl-2 in CHO^{R}C5/Bcl-2 cells is protective of tamoxifen toxicity relative to CHO^{R}C5 cells (IC50 0.593±0.28 µM versus 1.49±0.23 µM, respectively; Table II). Moreover, inhibiting P-gp1 ATPase with PSC-833 reversed the sensitivity of CHO^{R}C5 and CHO^{R}C5/Bcl-2 cells to tamoxifen (Fig. 3B and Table II).

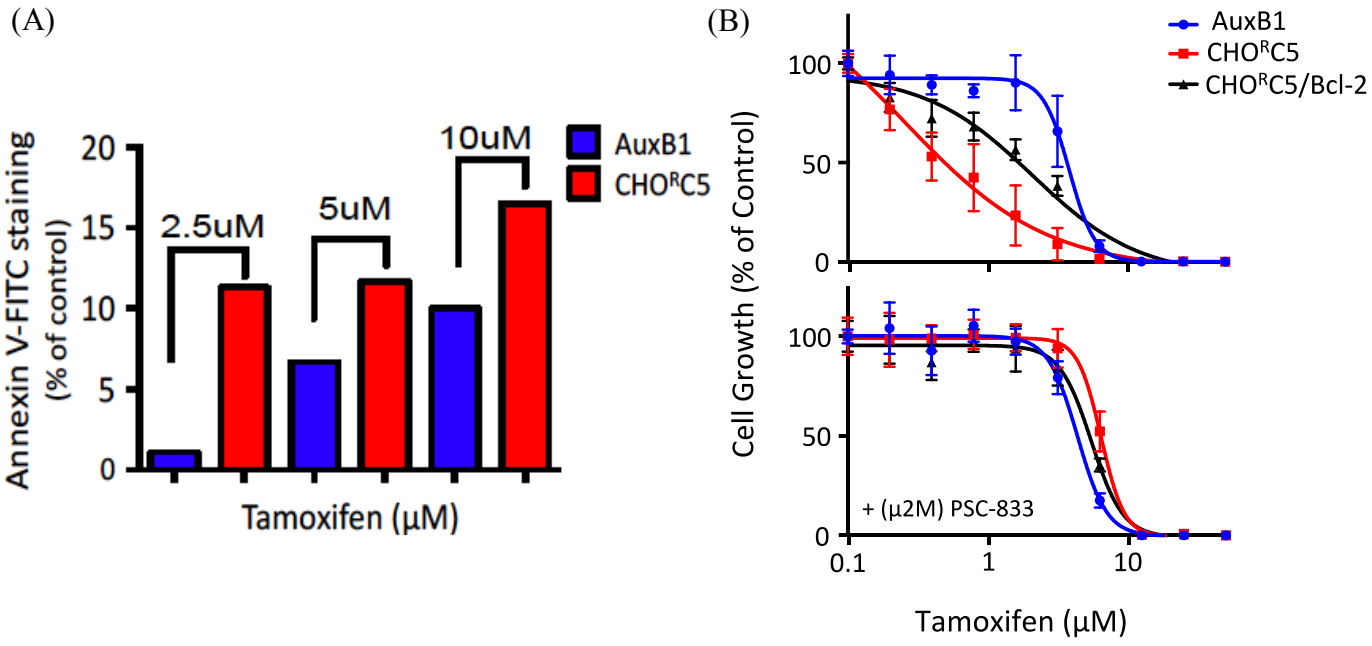

Figure 3. Tamoxifen induces apoptosis in P-glycoprotein1 expressing cells – Panel (A) shows drug sensitive (AuxB1 and MDA-MB-231) and P-gp1 overexpressing drug resistant cells (CHO^{R}C5 and MDA-Doxo^{400}) were incubated without and with tamoxifen (0.5 - 10 µM) for 24hrs. The percent of apoptotic cells was determined by FACS analysis using FITC-labeled annexin V. Panel (B) shows the effects of increasing concentrations of tamoxifen alone or in the presence of 2 µM PSC-833 on the growth of AuxB1, CHO^{R}C5 and CHO^{R}C5/Bcl-2 (CHO^{R}C5 cells stably transfected with human Bcl-2 cDNA, [13]) relative to solvent control set at 100%. Graphs represent the mean ± SD of two independent experiments done in triplicates.

Table 2. $IC_{50}S$ of tamoxifen alone or in the presence of 2 μM PSC-833 on the growth of AuxB1, $CHO^{R}C5$ and $CHO^{R}C5$/Bcl-2 ($CHO^{R}C5$ cells stably transfected with human Bcl-2 cDNA.

Cell Lines	Tamoxifen IC50±SD (μM)
AuxB1	3.607 ± 0.257
$CH^{R}OC5$	0.593 ± 0.28
$CH^{R}OC5$/Bcl-2	1.49 ±0.228
AuxB1 (2μM PSC-833)	4.35±0.307
$CH^{R}OC5$(2μM PSC-833)	6.412 ± 0.361
$CH^{R}OC5$/Bcl-2 (2μM PSC-833)	5.0405 ±0.506

Earlier reports have shown that heightened P-gp1 ATPase leads to selective decrease in cellular ATP level and a rise in reactive oxygen species (ROS), likely due to enhanced electron leak from mETC though enhanced oxidative phosphorylation (Laberge et al., 2009; Karwatsky et al., 2003). Tamoxifen has been shown by several investigators to increase P-gp1 ATPase at clinically relevant concentrations (<5 μM; (Litman et al., 1997; Scarborough, 1995)) To determine if the presence of low concentrations of tamoxifen causes a rise in ROS, cells were exposed for 24 hours to increasing concentrations of tamoxifen (1-10 μM) and cellular ROS measured using 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA). Figure 4A shows significant increase in ROS in MDA-$Doxo^{400}$ relative to MDA-MB-231 cells in the presence of low concentrations of tamoxifen. Moreover, consistent with tamoxifen biphasic effect on P-gp1 ATPase, lower concentration of tamoxifen (1-5 μM) shows greater increase ROS while 10 μM tamoxifen does not activate P-gp1 ATPase which shows no significant increase in ROS (Fig. 4A and Fig. 3). Similar selective increase in cellular ROS was observed in $CHO^{R}C5$ cells incubated with increasing concentrations of tamoxifen (0.5 - 1 μM) relative to AuxB1 cells (Fig. 4B).

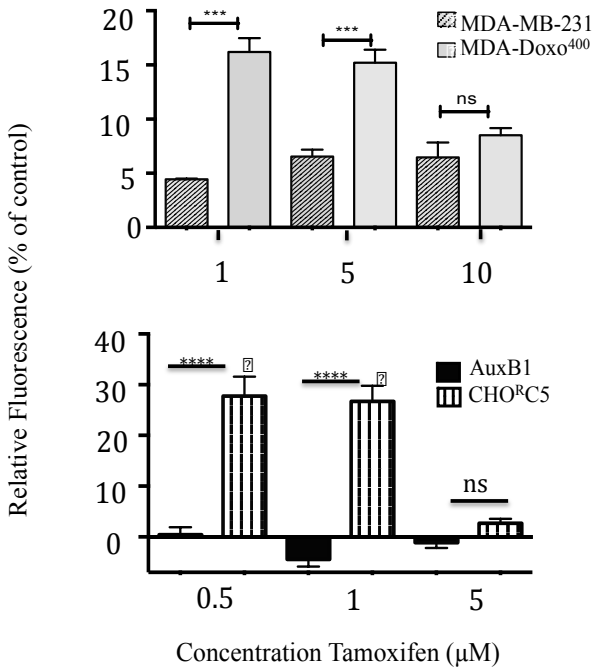

Figure 4. Effects of tamoxifen treatment on cellular ROS levels – Drug sensitive (AuxB1 and MDA-MB-231) and P-gp1 overexpressing drug resistant cells ($CHO^{R}C5$ and MDA-$Doxo^{400}$) were incubated without and with tamoxifen (0.5 - 10 μM) for 24hrs. Cells were then incubated with 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA) for 30 min at $37^{O}C$ prior to measuring fluorescence at excitation and emission wavelengths of 495–527nm. The change in cellular ROS levels, at different concentrations of tamoxifen, is described as the change in fluorescence relative to control, whereby the fluorescence of control (solvent treated cells) is set at 0% for each of the four different cell lines. Graphs represent the mean ± SD of three independent experiments done in triplicates. Stared bars show a significant increase ($P< 0.0001$) relative to negative controls.

Given these results it was of interest to determine if exogenously added H_2O_2 synergizes with tamoxifen. The results in figure 5 show that H_2O_2 alone showed similar inhibition of drug sensitive (AuxB1 and MDA-MB-231) and resistant ($CHO^{R}C5$ and MDA-$Doxo^{400}$) cells, but the presence of tamoxifen synergized with H_2O_2 showing greater inhibition of $CHO^{R}C5$ and MDA-$Doxo^{400}$ cell growth relative AuxB1 and MDA-MB-231 cells. Together, the results suggest that collateral sensitivity of P-gp1-positive cells to tamoxifen is likely due to increased ROS levels.

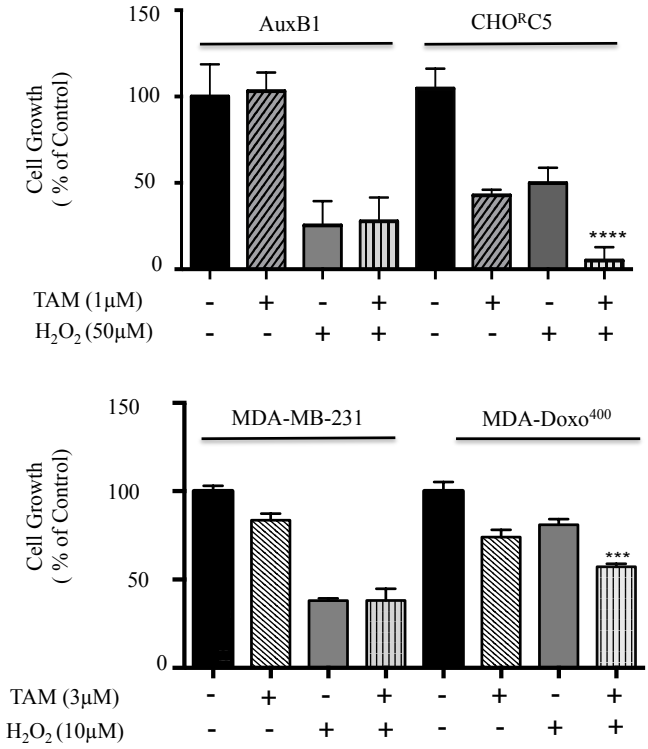

Figure 5. Hydrogen peroxide synergizes with tamoxifen - Drug sensitive (AuxB1 and MDA-MB-231) and P-gp1 overexpressing drug resistant cells ($CHO^{R}C5$ and MDA- $Doxo^{400}$) were incubated without and with tamoxifen (1 μM and 3 μM, respectively) for 24hrs in the absence or presence of H_2O_2 (50 μM and 10 μM, respectively). The effects of tamoxifen or H_2O_2 alone and combined on cell growth is shown relative to control with cell growth for each cell line.

It is well established that perturbing the redox homeostasis of tumor cells can lead to inhibition of cells growth and apoptosis (Wang et al., 2016). Given the results in figure 5, we measured the total thiol contend of cells in the absence and presence of tamoxifen. Figure 6 shows total thiol levels in untreated and tamoxifen treated drug sensitive (AuxB1 and MDA-MB-231) or – resistant ($CHO^{R}C5$ and $MDA\text{-}Doxo^{400}$) cells. The results of figure 6 show that tamoxifen treatment caused a significant drop in total thiol levels in $CHO^{R}C5$ and $MDA\text{-}Doxo^{400}$ cells relative to AuxB1 and MDA-MB- 231 cells.

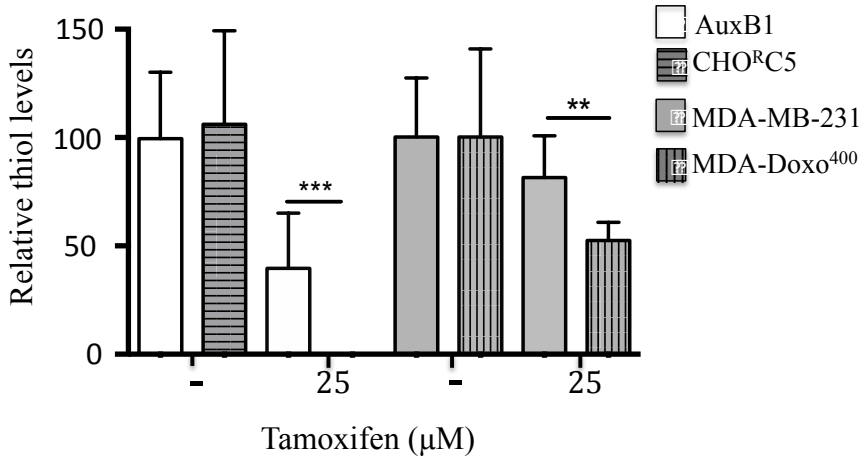

Figure 6. Effect of tamoxifen on intracellular thiol levels – Drug sensitive (AuxB1 and MDA-MB-231) and P-gp1 overexpressing drug resistant cells ($CHO^{R}C5$ and MDA-$Doxo^{400}$) were incubated without and with tamoxifen (25 μM) for 24hrs. Cells were extracted and equal proteins were used to measure cellular thiols using 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB). Results are expressed percent of cellular thiols in tamoxifen treated cells relative to control untreated cells. Error bars represent standard deviation from at least two representative experiments done in triplicate. Stars indicates, $P < 0.0003$ and $P < 0.0071$ respectively, statistically significantly different using unpaired t test.

Based on the above findings, we examined the possibility of reversing the effects of tamoxifen on the growth of drug resistant cells by restoring their reductive capacity with the addition of N-acetylcysteine (Samuni et al., 2013). Figure 7 shows that the presence of N-acetylcysteine (0.5, 1 mM) reversed the collateral sensitivity of $CHO^{R}C5$ and MDA- $Doxo^{400}$ cells to tamoxifen.

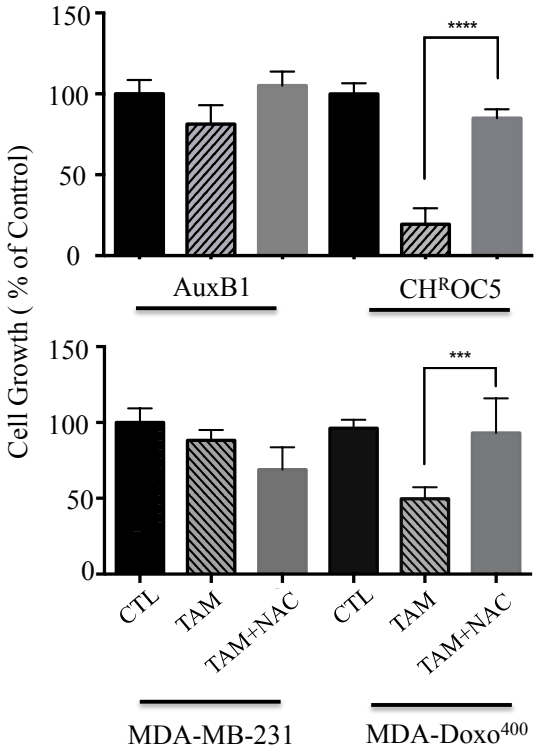

Figure 7. N-acetyl cysteine reverses collateral sensitivity to tamoxifen - Drug sensitive (AuxB1, MAD-MB-231) and -resistant ($CHO^{R}C5$, $MDA\text{-}Doxo^{400}$) cells were treated with tamoxifen without or with 0.5 mM and 1 mM N-acetly cysteine (NAC). Graphs represent the mean ± SD of two independent experiments done in triplicates. The $P < 0.0001$, statistically significant difference.

Discussion

Tamoxifen, a non-steroidal anti-estrogen, has been used for decades as chemotherapeutic drug for the treatment of estrogen-receptor positive breast cancer. Its anti-proliferative effect as a transcription inhibitor of estrogen-responsive genes is well established (Osborne and Schiff, 2005). However, tamoxifen has non-genomic effects that include the promotion of protein kinase C activity, intracellular calcium, mitochondrial stress, and P-gp1 ATPase (Radin and Patel, 2016; Li et al., 2012; Zheng et al., 2007). In this report we demonstrated for the first time the preferential targeting of P-gp-positive ($CHO^{R}C5$ and $MDA\text{-}Doxo^{400}$), relative to their drug sensitive P-gp1-negative cells (AuxB1 and MDA-MB-231), with clinically achievable concentrations of tamoxifen. Moreover, the sensitivity of resistant cells to tamoxifen correlated with P-gp1 expression level and was reversed with specific inhibitor of P-gp1 ATPase (e.g. PSC-833). Importantly, our results show that low levels of P-gp1 expression, as seen in $MDA\text{-}Doxo^{400}$ cells (Fig. 1A), are sufficient to confer collateral sensitivity onto drug resistant cells. Together these results are consistent with earlier reports demonstrating a link between stimulation of P-gp1 ATPase and collateral sensitivity of drug resistant cells (Laberge et al., 2009; Karwatsky et al., 2003).

The tamoxifen-induced mitochondria stress has been attributed to its inhibition of complex I and III of the mETC (Theodossiou et al., 2012; Moreira et al., 2006) and a consequent increase of intracellular ROS in both ER-α+ and ER-α- cells (Bekele et al., 2016). The rise of ROS in ER-α- cells is consistent with our findings in this study, as observed with MDA-MB-231 cells. However, tamoxifen shows significantly higher ROS levels in P-gp1-positive cells, likely due to its stimulation of P-gp1 ATPase, beyond its effect on mETC. In support of the latter, it is noteworthy that the

addition of rotenone, potent inhibitors of complex I of mETC (Vinogradov and Grivennikova, 2016), to CHO and MDA cells showed modest collateral sensitivity, significantly less than tamoxifen alone (Fig. S1-supplementry). The modest increase in the sensitivity of $CHO^{R}C5$ and MDA-$Doxo^{400}$ cells to rotenone, relative to AuxB1 and MDA-MB-231, is likely due to basal or unstimulated P-gp1 ATPase activity that was reversible with PSC-833 (Laberge et al., 2009).

Tamoxifen and its metabolites have been shown to cause a concentration-dependent biphasic stimulation of P-gp1 ATPase (Rao et al., 1994). This biphasic stimulation effect on P-gp1 ATPase activity has been observed with other drugs (e.g., verapamil, progesterone, and deoxycorticosterone (figure 2; (Karwatsky et al., 2003)), whereby 1-5 μM of tamoxifen stimulate P-gp1 ATPase, while ≥10 μM of tamoxifen did not stimulate P-gp1 ATPase. These results are consistent with the observed increase in ROS at lower concentrations of tamoxifen, as higher concentrations did not lead to higher ROS levels. Further support for tamoxifen-induced increase in ROS is provided by the decrease in total thiol levels in P-gp1-positive cells and the reversal of tamoxifen induced apoptosis with N-acetyl cysteine. Together these results suggest that P-gp1 expression enhances tamoxifen induced oxidative cell death drug resistant cells. A similar mechanism for the selective targeting of P-gp1-positive cells was proposed for thiosemicarbazone derivative (NSC73306) (Pluchino et al., 2012). However, unlike tamoxifen, NSC73306 did not interact nor stimulate P-gp1 ATPase but is thought to act as redox cycling agent in the presence of metal ions (Pluchino et al., 2012). Interestingly, NSC73306 induced collateral sensitivity was reversed by inhibitors of P-gp1 ATPase (Ludwig et al., 2006). The latter observation suggests P-gp1-basal or unstimulated ATPase activity could be the trigger for oxidative stress threshold.

In line with the above speculation, exogenously was added H_2O_2 synergized with tamoxifen in P-gp1 expressing cells to inhibit the proliferation of $CHO^{R}C5$ and MDA-$Doxo^{400}$ cells. It has been suggested that chemotherapeutic treatment is believed to enrich for resistant cells, which may express higher P-gp1 (Leonard et al., 2003). Hence, it is tempting to speculate that tamoxifen efficacy in delaying the reoccurrence of breast cancer is due, in part, to its collateral sensitivity effect to selectively target P-gp1-positive breast cancer cells (Figure 8). In addition, the findings of this study may provide a rationale for increasing the efficacy of tamoxifen treatment through drug combinations that independently increase ROS and synergize to: a) better target P-gp1 expressing cells; b) reduce tamoxifen concentrations below clinically achievable levels; and c) reduce cellular toxicity of collateral sensitivity drugs due to off-target effects.

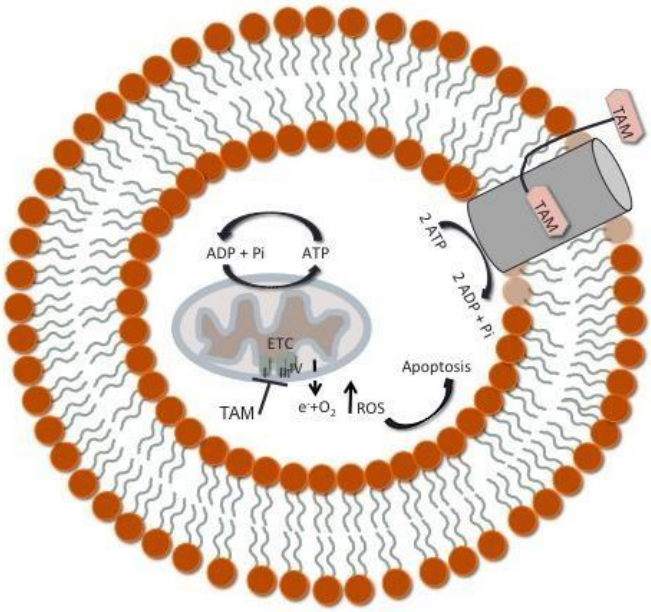

Figure 8. Proposed tamoxifen collateral sensitivity mechanism. Tamoxifen interacts with P-gp1, the interaction elevates ATP hydrolysis level. The usage and production of ATP generate from oxidative phosphorylation in the mitochondria put high demand on ATP from the mitochondria which cause electrons release from the electron transport chain (or ETC), lead to high ROS level and consequently death.

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Conflicts of Interest – There are no conflicts of interest from both authors.

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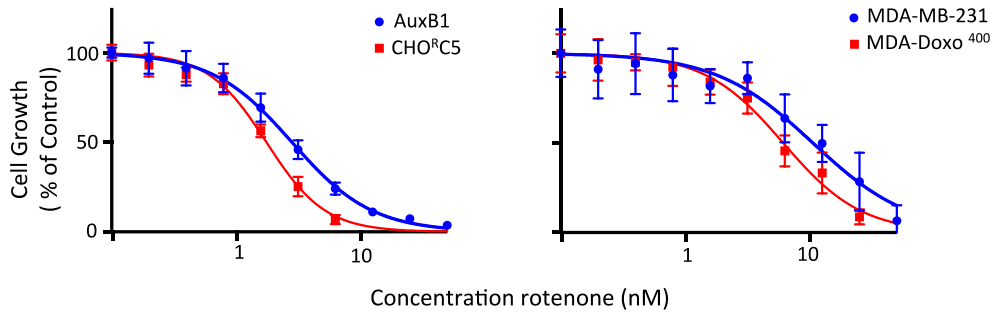
Appendix



Chapter 2. Supplemental figure. Rotenone Effects on the growth of drug sensitive (AuxB1, MDA-MB-231) and drug resistance (CHO^{R}C5, MDA-Doxo ^{400}) cells. It was plotted as a percent of control relative to cells treated with carrier solvent. Graphs represent the mean ± SD of two independent experiments done in triplicates.

Chapter2. supplemental Table 2. IC_{50}s of rotenone alone on the growth of drug sensitive (AuxB1, MDA-MB-231) and resistance (CHO^{R}C5, MDA-Doxo^{400}) cells.

Cell Lines	Rotenone IC_{50} ±SD (nM)
AuxB1	2.80 ± 0.544
CHORC5	1.743±0.127
MDA-MB-231	12.470±6.912
MDA-DOXO ^{400}	5.791±0.369