EFFICACY OF GAP JUNCTION ENHANCERS AND ANTINEOPLASTIC DRUGS IN MAMMARY CARCINOMA MODELS

by

STEPHANIE NICOLE SHISHIDO

B.S., Pennsylvania State University, 2008

M.S., California State University, 2010

AN ABSTRACT OF A DISSERTATION

Submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine/Pathobiology
College of Veterinary Medicine

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Abstract

Preclinical animal models of mammary carcinoma formation are vital for the advancement of cancer research, specifically in drug development. Two different types of animal models were utilized to determine the efficacy of combinational treatment of common antineoplastic drugs and the new class of primaquines that act as gap junction enhancers (PQs) at attenuating mammary tumor growth. The classic xenograft mouse model was used to show that PQs could increase the efficacy of cisplatin and paclitaxel. Combinational treatment induced an upregulation of connexin and caspase expression in the isolated tumor. Next the transgenic PyVT mouse model was characterized by multiple factors, including hormone receptor status, molecular markers for survival and proliferation, tissue histopathology, and secondary metastases during multiple stages of tumor development. This model showed limited therapeutic response to the antineoplastic drugs tested. PQ1 effectively attenuated tumor growth at all stages of tumorigenesis in the PyVT model, while PQ7 was determined to be an effective chemopreventive compound rather than chemotherapeutic. The PQs altered the expression profiles of connexins during tumorigenesis. Together the results indicate that PQs have an anticancer effect that is more efficient at attenuating tumor growth than the common antineoplastic compounds. Lastly the PyVT mouse model was used to determine the efficacy of antineoplastic compounds on male mammary carcinoma development. Interestingly, the antineoplastic compound that attenuated female mammary carcinoma growth did not produce a therapeutic response in the males and vice versa, suggesting a need for further studies into the male response to therapy.

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Preclinical animal models of mammary carcinoma formation are vital for the advancement of cancer research, specifically in drug development. Two different types of animal models were utilized to determine the efficacy of combinational treatment of common antineoplastic drugs and the new class of primaquines that act as gap junction enhancers (PQs) at attenuating mammary tumor growth. The classic xenograft mouse model was used to show that PQs could increase the efficacy of cisplatin and paclitaxel. Combinational treatment induced an upregulation of connexin and caspase expression in the isolated tumor. Next the transgenic PyVT mouse model was characterized by multiple factors, including hormone receptor status, molecular markers for survival and proliferation, tissue histopathology, and secondary metastases during multiple stages of tumor development. This model showed limited therapeutic response to the antineoplastic drugs tested. PQ1 effectively attenuated tumor growth at all stages of tumorigenesis in the PyVT model, while PQ7 was determined to be an effective chemopreventive compound rather than chemotherapeutic. The PQs altered the expression profiles of connexins during tumorigenesis. Together the results indicate that PQs have an anticancer effect that is more efficient at attenuating tumor growth than the common antineoplastic compounds. Lastly the PyVT mouse model was used to determine the efficacy of antineoplastic compounds on male mammary carcinoma development. Interestingly, the antineoplastic compound that attenuated female mammary carcinoma growth did not produce a therapeutic response in the males and vice versa, suggesting a need for further studies into the male response to therapy.

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Dedication

I would like to dedicate this dissertation to my parents. Mom, Dad: I couldn't have made it this far without you.

Chapter 1 - Review of Literature

1.1 Intercellular communication

Multicellular organisms are composed of complex networks of cells that form tissues and organs to perform specific functions in maintaining homeostasis. Optimal cell, tissue, and organ functions depend on maintaining the limits of the internal environment, which involves a variety of regulatory mechanisms and the coordination of a vast number of physiological activities, such as intermediary metabolism, cellular communication, cell growth, and cell differentiation. Cells are able to communicate with one another by releasing soluble factors that affect other cells either locally or distantly. There are several modes of transmitting information between cells: direct communication between adjacent cells, autocrine and paracrine signaling, and neurotransmitters and hormones produced by nerve and endocrine cells respectively. Here the focus is on the direct communication between neighboring cells.

Adjacent cells may be connected together through tight junction, desmosomes, gap junctions and adherens to facilitate passage of signaling molecules from cell to cell. Tight junctions, or zonula occludens, form a virtually impermeable barrier to fluid between closely associated cells by joining the membranes together. These are typically more apically located. Desmosomes, or macula adherens, support cell-cell adhesion by attaching to intermediate filaments of keratin in the cytosol. Adherens junctions, or zonula adherens, form strong mechanical attachments by forming a bridge connecting the actin cytoskeleton of neighboring cells. These protein complexes are composed of cadherins and catenins, and are usually more basally located. Gap junctions form direct intracellular connections between adjacent cells allowing for intercellular communication via the transfer of low molecular weight molecules.

The integrity of epithelial cell layers is maintained by tight junctions, adherens junctions, and desmosomes, while gap junctions provide a route for intercellular communication, allowing a single cell to directly influence the behavior of neighboring cells in a specific manner. Intercellular communication between groups of specialized cells within organs/tissues regulates proliferation, apoptosis, and differentiation to maintain tissue homeostasis.

1.2 Gap junctions

Gap junctions consist of aggregated transmembrane proteins that form channels between adjacent cells directly connecting their cytoplasms. Unlike other modes of intercellular communication, gap junctions are present in all animals and provide direct exchange of molecular signals between cells. Gap junctions are present in all vertebrate cell types, with a few exceptions: red blood cell, platelets, some neurons, mature skeletal muscle fibers, and spermatozoids [1]. The diffusion of molecules between adjacent cells through gap junctions is termed gap junctional intercellular communication (GJIC). This process is vital in maintaining homeostasis, synchronizing cellular activities, and regulating proliferation and apoptosis [2]. GJIC is critical for normal embryogenesis and development, neural activity, gamete production, endocrine function, immune function, smooth muscle function, and cardiovascular function. Defects in GJIC can lead to teratogenesis, neuropathy, infertility, diabetes, autoimmunity, atherosclerosis, cancer [3] [4].

Adjacent cells are able to communicate directly with each other through gap junctions. Gap junctions are protein channels made of the protein connexin (Cx). Connexins have four hydrophobic membrane spanning domains; two conserved, extracellular domains involved in paired hemichannel docking; and three cytoplasmic domains **(Figure 1.1)** [4]. Intercellular channels are formed through oligomerization of six connexins into a hexameric hemichannel

called a connexon, which is trafficked to the plasma membrane. Hemichannels allow communication between cytoplasm and extracellular space. On the membrane the connexon floats laterally until it docks with a second connexon on the adjacent cell to form an intact gap junction channel. Groups of these channels form gap junctional plaques, allowing the flow of small molecules between the cytosol of neighboring cells.

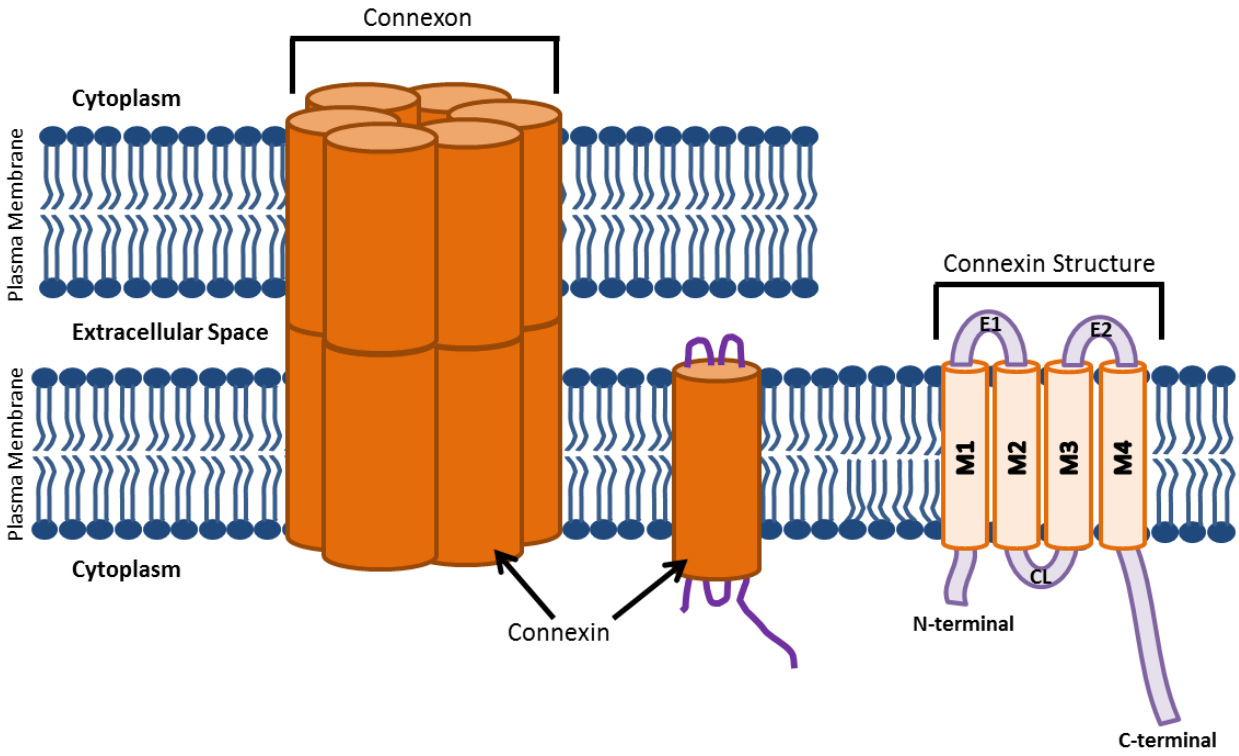

Figure 1.1 Structure of the gap junction. Connexins have four transmembrane domains (M1-M4) with the C and N cytoplasmic termini, a cytoplasmic loop (CL) and two extracellular loops (E1 and E2). The connexon is formed from the assembly of six connexins. Two connexons come together to form the gap junction.

The cytoplasmic regions of the connexin have variable amino acid sequences, leading to different connexin types [5]. Vertebrates have approximately 20 different connexins [1, 4, 6], commonly designated with numerical suffixes identifying the molecular weight of the sequence

in kilodaltons (kDa) [4]. The following studies will discuss Cx26, Cx32, Cx43 and Cx46. Cx46 is a novel gap junction protein in mammary tissue that is hypoxia-specific. Vertebrate gap junctions have a permeability size of 1.2 kDa, allowing amino acids, sugars, nucleotides, most secondary messengers, water, and other small molecules to diffuse between cells [1, 4, 7]. This allows adjacent cells to share a common intracellular environment through the formation of gap junctions. GJIC provides cells a route to share information to function as a single metabolic unit, creating a local environment leading to regional functionality [8].

Gap junction channels are not typically formed exclusively of one connexin subtype (homotypic), but rather consist of multiple distinct connexin isoforms forming a heteromeric channel [9]. The formation of heteromeric connexons increases the complexity of regulation. GJIC is regulated by activation of protein kinases [10-12] and protein phosphatases [13]. Phosphorylation induces channel gating: the molecular transition leading to gap junction channel opening or closing [14, 15]. Channels are regulated by various stimuli, including changes in pH, voltage, and phosphorylation stage of the c-terminal tail [1, 4, 7, 16]. The connexin proteins are regulated by protein kinases that phosphorylate serine and threonine residues of the connexin carboxyl terminal region [17, 18]. Studies have shown that the mitogen-activated protein kinase (MAPK) and protein kinase C (PKC) mediate phosphorylation of the C-terminal end to close Cx43 hemichannels [6]. Phosphorylation of gap junctions occurs after membrane insertion and deposition into gap junction plaques [19].

There are three kinetic routes of gap junctional control: fast, intermediate, and long-term. The fast gap junctional control involves gating responses that result in an effect within milliseconds of the initial response. These immediate effects may be the result of rapid changes in ion concentration, pH, protein kinases, phosphatases, or lipid composition of the membrane

[20-22]. Intermediate control is conducted by vesicular withdrawal or insertion of connexins in the membrane, which takes minutes. This is possible due to a perimembrane pool of connexins that is present within cytoplasmic vesicles, determined by the observation that connexins can be inserted into the plasma membrane despite the presence of inhibitors of protein synthesis [23]. Lastly, long-term control is regulated at a transcriptional level, where connexin mRNA is altered, adjusting the connexin protein pools within the cell. This takes hours from the initial stimuli. Gap junctions are dynamic plasma membrane structures with rapid turnover rates [24-26]. The half-life of the connexin protein is between 2-5 hours in cultured cells and tissue [22, 27-30]. There is controversy as to whether all the connexin proteins are conventionally synthesized in the endoplasmic reticulum, transported through the Golgi and exported to the plasma membrane. This is true for Cx43.

Gap junctions provide regional functions. First they buffer the harmful effects of xenobiotic metabolites by dispersing them outward from the exposure point into the tissue. Second, healthy cells can provide nourishment for deprived or sick cells through shared metabolites. This gives the tissue plasticity as long as there is functional GJIC. Third, they function in the rapid exchange of electrical signals and regulators. Fourth, they distribute metabolites vital for cellular proliferation/health (i.e. cAMP). This is important in that not all cells within a tissue have the same metabolic capacity, requiring that essential metabolites are shared between cells [31]. Lastly gap junctions function to eliminate waste or unwanted byproducts.

1.3 Cancer and gap junctions

Cx46 is a novel gap junction protein that is hypothesized to have pro-tumor effects due to its ability to prevent hypoxic death [32]. Due to this it will not be discussed in association with an observed GJIC deficiency as part of the cancer phenotype.

1.3.1 Gap junctions/connexins in initiation and promotion

Defects in critical signaling pathways that regulate cellular properties, such as proliferation, differentiation, and apoptosis result in the formation of cancer. Tumorigenesis may be affected by secondary mechanisms not related to direct gene damage [33-35]. This is a category of carcinogens classified as non-genotoxic which indirectly stimulate hyperplastic growth, without altering DNA sequence or structure. Non-genotoxic carcinogens elicit a mechanism of cancer induction that includes receptor- and nonreceptor-mediated endocrine modulation, tumor promoters, toxicity responses, inflammatory responses, or deficiencies in GJIC. Many non-genotoxic carcinogens inhibit or reduce GJIC, including chlordane [36], TCDD [37], DDT [38], phenobarbital [38], and acetamide [39]. The absence of genotoxicity and the tendency of being tissue specific make non-genotoxic mechanisms challenging to identify and characterize.

A deficiency in GJIC affects tumorigenesis in two main ways: 1) the loss of GJIC leads to a lack of homeostasis resulting in cellular damage and 2) excessive proliferation may occur due to hormone, metabolite, or secondary messengers not being effectively distributed to adjacent cells. Loewenstein and Kanno first reported a lack of electrical coupling in rat hepatomas in 1966 [40]. This was observed in chemically-induced and transplanted hepatomas [40, 41], which differed significantly from the normally well-coupled liver cells. Similar results were obtained in transplanted thyroid tumors [42] and carcinomas of the stomach [43]. The lack

of electrical coupling soon became a common characteristic found in solid tumors, whether chemically-induced, transplanted, or spontaneously formed. This was true for various species across an array of tissue types.

Over 40 years, later multiple studies confirmed that a deficiency in gap junctions, and thus GJIC, is associated with the cancer phenotype [44-46]. There is a clear correlation between reduced GJIC and the promotion and progression stages of cancer formation [47-53]. Inhibition of GJIC in initiated tissue, in which there are I+ cells, leads to tumor promotion of a chemically induced carcinogenesis [47, 54-56]. Many tumor promoting agents have been found to be inhibitors of GJIC [54, 55, 57], reinforcing the hypothesis that a deficiency of GJIC leads to tumorigenesis. Most known oncogenes (i.e. *ras, raf, neu, src*) downregulate GJIC, while tumor suppressor genes upregulate GJIC [2, 58, 59]. In primary cells isolated from human breast cancer [60], rat mammary carcinomas [61], and transformed breast cancer cell lines [61, 62] a deficiency in Cx26 and Cx43 has been observed. In fact cell lines are known to have less gap junctions than their corresponding normal tissue cells [63, 64]. There is a distinct inverse relationship between cell growth and GJIC in transformed cell lines, where the induction of GJIC leads to the inhibition of growth, while the inhibition of GJIC promotes cellular proliferation [65].

Loss of connexin is an early event in cancer development, though researchers have been unable to clearly identify at which stage of tumorigenesis the decreased expression of connexins occurs. There are two routes to explain this early development: 1) clonal expansion of adult stem cells that do not express connexins, therefore resulting in a lack of GJIC at very early stages; or 2) differential expression of connexins after initiation, in that the expression level and/or function is reduced by the onset of oncogenic activities as malignancy progresses [66]. This second

explanation covers why only some cancers express connexins at early stages. In many cancer cases, loss of connexin expression is observed during dysplasia of the precancerous lesion, such as a reduction in Cx43 in cervical dysplasia [67]. Additionally in lobular or ductal mammary carcinomas, Cx43 is not detectable, indicating that Cx43 is a marker for early oncogenesis [61]. Reduced connexin expression may also be observed in the later stages of tumorigenesis. As an example there is a decrease in Cx43 expression during the late stages of prostate cancer, but not in the benign stages [68]. This observation indicates that loss of connexin expression in not required for initiation of prostate cancer [68]. In support of the hypothesis that loss of GJIC is important in carcinogenesis, there are studies showing that re-expression of connexins in cancer cells causes normalization of cell growth control and reduced tumor growth. In normal mammary tissue, Cx26 is not detected, but it is upregulated in invasive breast carcinoma lesions [69].

The observable lack of GJIC in neoplastic cells is not only due to a lack of connexin expression, but also aberrant localization of connexin proteins. Many studies demonstrate expression of connexins in neoplastic cells, but abnormally located in the cytoplasm *in vitro* and *in vivo*. In more than 50% of invasive breast carcinomas Cx26 is located in the cytoplasm [69], while about 90% of advanced grade tumors have cytoplasmic expression of Cx43 [70]. Cx32 has been shown to be cytoplasmic in liver cancer cell lines and tumors [71]. This is also true for chemically-induced tumors, in which Cx32 and Cx26 have been identified to be cytoplasmically located in 7,12-dimethylbenz(a)anthracene (DMBA)-induced rat hepatomas [72]. Aberrant connexin localization may be due to impaired trafficking of connexins to the membrane. This is seen with human colon cancer cells, which express Cx43 cytoplasmically, and the overexpression of Cx43 via transfection of cDNA does not improve localization at the membrane or restore GJIC [73]. Other reports have suggested it may be due to a lack of cell-cell

recognition. Neoplasms often show a decrease in E-cadherin, a cell-cell recognition transmembrane protein, which is lost with a transition in phenotype. Overexpression of E-cadherin produces a more epithelial phenotype with restoration of GJIC [74]. In addition nuclear localization of connexins has been reported, but no reasonable explanation has been provided. Specifically Cx43 is localized in the nucleus in rat liver cells that have been transformed by the oncogenes *src* or *neu* [75]. Additionally in HeLa cells the Cx43 carboxyl terminus is anchored in the nucleus, inhibiting cellular proliferation [76].

The alterations in the expression levels of connexin proteins may be due to genetic mutations in the neoplastic cells. Such as a series of mutations in a tumor suppressor gene, or a specific gene mutation that affects connexin production [46]. Mutations in tumor suppressor genes may lead to a lack of connexin expression directly, by altering the gene sequence, or indirectly via changes in regulatory factors. An example of the indirect route is in liver tumors where altered expression of the transcription factor hepatocyte nuclear factor 1α (HNF-1α) downregulated Cx32 [77-79]. Alterations in the expression of connexins may also be due to mutations in the non-coding sequence of the genes, thus modulating the regulation of expression.

1.3.2 Gap junctions/connexins in progression

Metastasis is a complex process involving cellular dissociation, tissue invasion, transport of metastatic cells via blood or lymph, extravasation to a distant site, and formation of a secondary tumor. Loss of GJIC has been observed in metastatic disease [47, 80-84]. There are two steps in which gap junctions play a role in metastasis: 1) cellular dissociation and invasion, and 2) extravasation at the secondary site. Evidence indicates that a loss of GJIC correlates with metastatic potential of the primary tumor. In a metastatic breast cancer cell line, transfection with the breast metastasis suppressor 1 (BRMS1) cDNA restores GJIC by increasing Cx43 expression

and reducing Cx32, resulting in a more normal phenotype [85]. E-cadherin expression also positively correlates with GJIC [74, 86]. A reduction in E-cadherin indicates a loss of cooperation between neighboring cells and reduced GJIC, leading to cellular dissociation in invasive tumors.

Interestingly low metastatic potential cells do not establish functional GJIC with the surrounding cells, but highly malignant mammary carcinoma or melanoma rat cells have been shown to readily transfer dye to endothelial cells at a secondary site [87]. Extravasation of malignant cells involves diapedesis across the endothelial barrier into the tissue prior to secondary tumor formation. This has been observed in breast cancer cells, which require GJIC between tumor cells and endothelial cells at a secondary site [70, 88]. El-Sabban and Pauli showed an increase in cellular adhesion and communication by highly metastatic lung carcinoma cells, presumably to facilitate extravasation during the metastatic process [89]. These studies exemplify the importance of GJIC in the establishment of metastatic foci, though more research is needed into the role of gap junctions in extravasation. During the process of metastasis, data suggests a change in connexin profile from a loss of GJIC during tissue invasion to functional GJIC during extravasation.

1.4 Breast cancer

Breast cancer is the most common cancer in women worldwide and the leading cause of premature death. Breast cancer is the formation of neoplastic cells in the tissue of the breast, usually the ducts and lobules. It occurs in both men and women, although male breast cancer is very rare. In the US, breast cancer is the second most commonly diagnosed cancer (skin cancers being the first) and the second leading cause of cancer related deaths [90].

Several factors have been associated with an increased risk for developing breast cancer, such as family and personal history, nulliparity, early menarche, and age. Age is the strongest risk factor associated with breast cancer in women. Diagnosis at a younger age correlates with a cancer phenotype that is more aggressive and less responsive to treatment [91]. Incidence of breast cancer increases dramatically with an increase in age among premenopausal women (≤50 years) and then slows among postmenopausal women (>50 years). This pattern reflects the influence of reproductive hormones on the occurrence of breast cancer [92]. There is a large variation in incidence and mortality rates across racial and socioeconomic groups. The risk of developing breast cancer is positively associated with socioeconomic status, measured by either income or education [93, 94]. Reproductive risk factors include less parity and later age at first child birth [95]. Of female breast cancer patients, 5-10% have a germ-line mutation in the *BRCA1* and *BRCA2* genes [96]. The lifetime risk of developing cancer in women with mutations in *BRCA1* and *BRCA2* is 40-85%.

The following studies characterize and discuss mammary carcinomas at different stages of tumorigenesis. Tumor formation is a multistage process in which epithelial cells go through a series of changes forming multiple premalignant phenotypes, ultimately developing into an invasive cancer (**Figure 1.2**). The process of tumorigenesis is divided into three stages: initiation, promotion, and progression. Initiation is the irreversible alteration of a normal cell in a cancer related gene (i.e. mutations). Promotion immediately follows initiation and involves clonal expansion of the initiated cell resulting in the development of benign tumors. This stage is facilitated by the stimulation of proliferation by mitogenic growth factors, hormones, and inhibition of apoptosis. The last stage, progression, is the stable alteration of the initiated cell to

confer the malignant phenotypes of invasiveness and metastasis. Progression may be considered the terminal stage of cancer development [97].

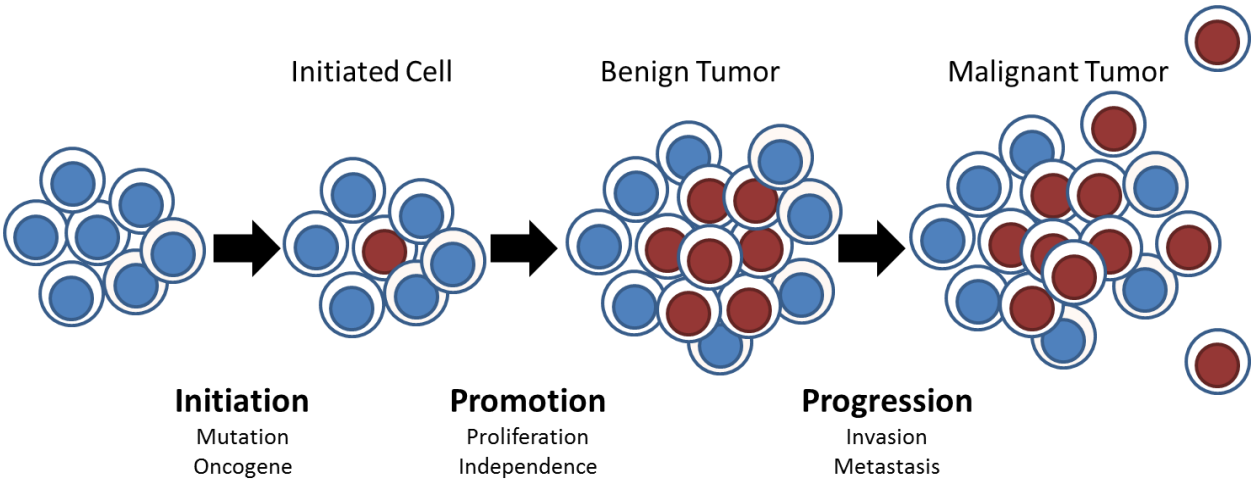

Figure 1.2 The stages of tumorigenesis. A tumor is generated by a three-step process: initiation, promotion, and progression. Blue represents the normal healthy cell. Red indicates a cell that has been transformed through either genomic instability or oncogenic activation. The latency period between the formation of the initiated cell and a malignant tumor may be 20 years or more.

Carcinoma cells undergo a type of epithelial-mesenchymal transition (EMT) that leads to a manifestation of cancer progression through the generation of invasive cells and metastatic lesions. EMT is a biological process that allows epithelial cells to undergo biochemical changes that enable it to assume a mesenchymal phenotype. This includes enhanced migration, invasiveness, resistance to apoptosis, and an increase in the production of extracellular matrix (ECM) components [98]. This process involves a variety of distinct molecular processes, including the activation of transcription factors, expression of surface proteins, and cytoskeletal protein reorganization. Cancer cells may go through different extents of EMT; some cells may

retain epithelial traits while acquiring some mesenchymal ones, while other cells may become fully mesenchymal.

Activation of EMT is a critical mechanism for the formation of malignant phenotypes by epithelial cancer cells [98]. Cell culture and mouse studies demonstrated that carcinoma cells can express mesenchymal markers such as vimentin and desmin when acquiring a mesenchymal phenotype [99]. These cells are observed at the invasive front of the primary tumor, and eventually enter into an invasion-metastasis cascade [100, 101]. The process of EMT is facilitated by the disruption of cell-cell adherens junctions and cell-ECM adhesions via integrins [99, 102, 103]. Many studies have established a connection between EMT and the loss of E-cadherin expression in carcinoma cells [104-106]. Cells with a decrease in surface E-cadherin expression are more responsive to EMT-induction by growth factors [107], and show an increase in tumorigenicity and metastasis in xenograft models [108]. Additionally patient survival is inversely related to E-cadherin expression levels [109]. Interestingly it has been observed that the EMT-derived mesenchymal cancer cells establish a secondary colony at a distant site that resembles histologically the primary tumor it originated from. This involves metastasizing neoplastic cells to convert back to epithelial phenotype through a process of mesenchymal-epithelial transition (MET) during the formation of a secondary tumor [110], possibly due to encountering a different local microenvironment in a distant organ [101, 111, 112]. It is unclear what signals induce EMT, and its reverse MET in carcinoma cells.

Hormone receptor expression status represents the best predictive marker for breast cancer currently in clinical use. Expression of the estrogen receptor (ER) and progesterone receptor (PR) are routinely profiled to determine if a patient would benefit from endocrine therapy. Hormonal treatments are only effective when hormone receptors are present. The

predictive value of these hormone receptors is not clear. Not all ER+ tumors benefit from hormone treatment [113]. Human epidermal growth factor receptor 2 (HER2) status is also standard for breast cancer evaluation [114]. HER2 gene amplification is seen in 15-25% of invasive breast cancers and is associated with a more aggressive phenotype and poor clinical outcome [115-118].

There are five distinct subtypes of breast cancer based on cDNA studies and molecular profiling [119, 120]. These were first categorized by estrogen receptor expression. There are two ER+ subtypes distinguished as Luminal A and B, named for the presence of luminal epithelial cytokeratin. The other three subtypes are ER-: basal-like, ERB2 (HER/neu) positive, and normal-like. The basal-like subtype has cytokeratin expression in basal epithelial cells. The observation of luminal and basal subtypes indicates differentiation from two different progenitor cell types. This hypothesis is supported by the observation that tumors with the breast cancer type 1 gene (BRCA1) mutations are commonly ER, PR, and HER2/neu negative tumors with basal cytokeratin and lymphocyte infiltrate [121-123], while those with breast cancer type 2 (BRCA2) mutations have luminal ER+ expression [124]. Interestingly despite sharing gene expression similarities the Luminal A tumors show the best prognosis of the five breast cancer subtypes, while Luminal B tumors correlate with poor patient survival, possibly due to overexpression of HER2 and presence of tumor protein 53 (P53) mutations. The basal-like and HER2+ subtypes have the poorest prognosis of all the subtypes.

There are five clinical stages of breast cancer development determined by the size of the primary tumor and by how far the cancer cells have spread; these are summarized in **Table 1.1**. Stage 0 represents three types of early breast cancer, carcinoma in situ, defined by the lack of invasion of tumor cells: Ductal carcinoma in situ (DCIS), lobular carcinoma in situ (LCIS), and

paget disease of the nipple. DCIS and LCIS are noninvasive condition with abnormal cell growth found in either the lining of the duct or the lobules, respectively, which may become invasive. Paget disease involves the formation of abnormal cells in the nipple only. Stage I is divided into stages IA and IB. Stage IA consists of a 2 cm or smaller tumor, with cancer restricted to the breast tissue. Stage IB is more advanced due to the inclusion of small clusters of neoplastic cells found in the lymph nodes. Stage II is divided into stages IIA and IIB. Stage IIA consists of either a tumor with a maximum diameter of 2 cm with neoplastic cells found in 1-3 axillary lymph nodes, or a tumor that is larger than 2cm, but smaller than 5cm in diameter without neoplastic cells found in the lymph nodes. Stage IIB is represented by a tumor that is either between 2-5 cm in diameter with neoplastic cells clustered in the lymph nodes (1-3 axillary LN), or a tumor larger than 5cm in diameter without spread of neoplastic cells to the lymph nodes.

Stage III is divided into three further stages: A, B, and C. Stage IIIA involves either 4-9 axillary lymph nodes and neoplastic cells attaching to the breast bone, or a tumor larger than 5cm in diameter with 1-3 clusters of neoplastic cells found in the lymph nodes. Stage IIIB consists of a tumor of any size that has spread to the chest wall or skin, possibly forming an ulcer, and up to 9 axillary lymph nodes or neoplastic cells attaching near the breast bone. Stage IIIC includes the spread of cancer cells near the breast bone and to more than 10 axillary lymph nodes or lymph nodes above the collar bone. Stage IV breast cancer has metastasized to other organs, typically to the bones, lungs, liver, or brain. Another form of advanced breast cancer involves the spread of neoplastic cells to the skin, causing the breast to look red, swollen, and warm. This is termed inflammatory breast cancer. In this situation the neoplastic cells block the lymphatic vessels in the skin, causing an inflammatory response, sometimes accompanied by dimpling of the skin called peau d` orange. Inflammatory breast cancer may be present during stages IIIB-C or IV.

Table 1.1 Summary of the clinical stages of breast cancer development. DCIS: Ductal carcinoma in situ, LCIS: lobular carcinoma in situ, LN: lymph node, BB: breast bone.

Stage		**Description**	
0		DCIS (abnormal cells in ducts)	
		LCIS(abnormal cells in lobules)	
		Paget disease (abnormal cells in nipple)	
I	IA	Tumor ≤ 2cm, breast tissue only	
	IB	Tumor ≤ 2cm, neoplastic cells found in LN	
II	IIA	Tumor ≤ 2cm, 1-3 axillary LN	
		Tumor 2- 5cm, breast tissue only	
	IIB	Tumor 2- 5cm, , 1-3 axillary LN	
		Tumor > 5cm, breast tissue only	
III	IIIA	4-9 axillary LN, near BB	
		Tumor > 5 cm, 1-3 LN	
	IIIB	Tumor spread to chest wall and skin, ≤ 9 axillary LN, near BB	**Inflammatory Breast Cancer**
	IIIC	> 10 LN	
IV		Metastasis	

1.5 Breast cancer treatment

There are six types of standard treatment options for breast cancer. These are surgery, sentinel lymph node biopsy, radiation, chemotherapy, hormone therapy, or targeted therapy. Most breast cancer patients have surgery to remove the tumor from the breast tissue. During

surgery some of the lymph nodes may be removed to determine the extent of lymph node metastasis [125, 126]. There are two types of surgical procedures: breast-conserving surgery or mastectomy. The types of breast-conserving surgery are lumpectomy, which involves removing the tumor and a small amount of normal tissue around it, and a partial mastectomy, which removes the region of the breast with cancer and possibly part of the lining of the pectoralis muscle below the tumor. There are also two types of mastectomy, either a total mastectomy or a modified radical mastectomy. A total mastectomy removes the whole breast that has developed cancer. The modified radical mastectomy involves removal of the whole breast, many of the nearby lymph nodes, and part of the pectoralis major muscle.

Often patients undergo a procedure called a sentinel lymph node biopsy prior to surgery. The sentinel lymph node is the first lymph node to receive lymphatic drainage from the tumor, thus it is the first lymph node that the neoplastic cells may spread to. A radioactive substance or dye is injected into the tissue near the tumor. The substance is allowed to flow through the lymphatic ducts to the sentinel lymph node, which is then removed and histologically observed for cancer cells. Additional lymph nodes may be removed depending on the presence of cancer cells found in the biopsy [127, 128].

Treatment that is given along with or after a surgical procedure to reduce the chances of recurrence is termed adjuvant therapy. This includes radiation, chemotherapy, hormonal therapy, and targeted therapy. Treatment decisions related to the use of adjuvant systemic therapies rely on clinicopatholgic staging, histological appearance, subtyping, grading, lymph node status, and the presence of metastasis. Lymph node negative and positive breast cancer patients are commonly recommended for adjuvant systemic treatment [129], though only about 2-15% respond to treatment [130].

Radiation therapy uses high-energy X-rays, or possibly other types of radiation, to prevent cellular proliferation and kill the cancer cells. There are two types of radiation therapy: external or internal. External radiation involves the use of a machine to send radiation towards the cancer. Internal radiation uses a radioactive substance that is placed directly into or near the tumor. Chemotherapy is a general term for any drug that inhibits the growth of rapidly dividing cells. This may be given systemically via the blood or regionally by direct injection into the cancerous tissue. The type of radiation or chemotherapy depends on the stage of cancer.

Hormone therapy involves the manipulation of the endocrine system through exogenous administration of compounds that block the production of hormone or prevents their activity. This type of treatment option is used in hormone-dependent cancers. The most common type of hormonal therapy is tamoxifen (sold under the trade names Nolvadex, Soltamox, Istubal, and Valodex) a selective estrogen receptor modulators used to treat patients with early stage breast cancer or metastatic breast cancer. Another type of hormone therapy is the use of aromatase inhibitors which prevent the conversion of androgen to estrogen in postmenopausal patients. Lastly targeted therapies use drugs or substances to attack cancer cells by interfering with specific targeted molecules required for tumorigenesis. This treatment option is less harmful to normal cells since it does not affect all rapidly dividing cells, but has specificity to the neoplastic tissue. Examples of targeted therapies are monoclonal antibodies, tyrosine kinase inhibitors, or poly (ADP-ribose) polymerase (PARP) inhibitors. Immunotherapy treatment is a subcategory of targeted treatments, which uses the patient's own immune system to fight cancer by either enhancing the immune system in general ways or training the immune system to attack cancer cells specifically. There are three types of immunotherapy: monoclonal antibodies, cancer vaccines, and non-specific immunotherapies such as cytokines or interleukins that are used to

boost immunity. Less common treatment options include hyperthermia and stem cell transplants into the peripheral blood or bone marrow. New types of treatment are constantly being tested in clinical trials.

The three antineoplastic compounds used in the following studies are tamoxifen, paclitaxel, and cisplatin. As mentioned above, tamoxifen is a selective estrogen receptor modulator (SERM) that binds the estrogen receptor as a competitive antagonist to estrogen signaling. Tamoxifen was first produced by AstraZeneca in the early 1970s [131] and is used for a variety of disorders, including bipolar disorder [132], gynecomastia [133], and thyroiditis [134]. Tamoxifen is currently used for the treatment of early and advanced estrogen receptor positive breast cancer in female patients, and it is the most common hormone therapy for male breast cancer [135]. It blocks the binding of estradiol to its estrogen receptor, preventing the induction and growth of hormone-dependent mammary carcinomas [136-138]. Drug resistance does occur with long-term tamoxifen treatment. Adjuvant tamoxifen treatment was evaluated for 5 year and 10 year treatment durations, where 10 years of treatment resulted in an accumulation of serious side effects and an increase in tumor recurrence [139]. Five years of tamoxifen therapy has become the standard treatment strategy since the 1990s, resulting in long-term benefits for patients [140].

Cisplatin is the first member of a group of anticancer drugs that are platinum containing compounds. Cisplatin is administered intravenously for short-term treatment of solid cancers. The compound *cis*-$[Pt(NH_3)_2(Cl)_2]$ was first described by Michele Peyrone in 1845 and was known as Peyrone's salt [141]. In 1965, it was discovered that electrolysis of platinum electrodes generated a platinum complex that inhibited cell division in *E. coli* bacteria [142]. This finding later led researchers to the observation that cisplatin was highly effective at reducing tumor

growth in rats [143]. The FDA approved cisplatin for the treatment of testicular and ovarian cancer in 1978 (9). The mechanism of action for cisplatin involves the displacement of chloride by water, and water by a nucleobase, most commonly the purine guanine, forming DNA crosslinks and interfering with mitosis [144]. The initial responsiveness to cisplatin is high, but the majority of cancer patients receiving this treatment becomes resistant and eventually relapse.

Paclitaxel (taxol) is a mitotic inhibitor isolated from the bark of the Pacific yew tree, *Taxus brevifoli.* It was first discovered in 1967 and developed commercially by Bristol-Myers Squibb. It wasn't until 1978, when researchers showed that taxol was effective in leukaemic mice [145] and xenograft studies [146]. It was FDA approved in 1992 for the treatment of patients with head and neck, lung, ovarian, and breast cancer [146]. Paclitaxel is a cytoskeletal drug that binds tubulin to stabilize the polymer and protect it from disassembly, thereby preventing mitotic spindle assembly, chromosome segregation, and cellular division [147]. Until 1993, almost all taxol production was derived from harvesting the bark from the Pacific yew, which killed the tree in the process. At this time it was discovered that taxol was synthesized by endophytic fungi living in the bark [148]. Currently taxol is produced through plant cell fermentation with the endophytic fungus *Penicillium raistrickii* followed by direct extraction and purification [149].

The nonspecific nature of chemotherapy, hormone therapy, and radiation treatment leads to a variety of unpleasant side effects for patients. Radiation damages normal tissue that is adjacent to the cancer. Side effects associated with radiation therapy of the breast, chest wall, and regional lymph nodes include myelosuppression, hyperpigmentation of the skin, lymphedema, pneumonitis, myocardial infarction, and second cancers [150]. The risk of cardiac toxicity increases when daily radiation doses are high [151], while lymphademia post radiation varies

from 5-25% in breast cancer patients [152]. The risk of developing contralateral breast cancer post radiation therapy also increases, most likely due to “scatter” radiation towards the noncancerous tissue [153-155].

Treatment with chemotherapeutic compounds damage normal, healthy cells that are rapidly dividing, such as hair follicles, nails, and bone marrow. Guidelines form the National Institutes of Health and the National Comprehensive Cancer Center Network recommend adjuvant chemotherapy, tamoxifen, for women with invasive breast cancer whether or not lymph nodes are involved [156, 157]. Common side effects with adjuvant chemotherapy include myelosuppression, nausea and vomiting, neurological toxicity, weight gain, ovarian failure, cardiac toxicity, secondary cancers, fatigue, and cognitive dysfunction [150, 158]. Approximately 10-14 days post adjuvant chemotherapy patients often experience a small to moderate reduction in white blood cell count, termed myelosuppression. Due to this there is an increase in the incidence of infection, of which 2% are life-threatening [150]. Secondary cancer formation depends on the specific chemotherapeutic drug, the cumulative dose, and duration of treatment. Patients receiving both chemotherapy and radiation have a higher risk of developing acute myeloid leukemia [159, 160]. The most common side effect experienced by breast cancer patients receiving chemotherapy is moderate to severe fatigue, affecting two out of three patients [161, 162].

Taking a closer look at tamoxifen and taxol, it is apparent that these compounds have detrimental side effects. The taxanes, such as paclitaxel and docetaxel, cause both sensory and motor peripheral neuropathy [150]. The severity of nerve damage is related to the individual dose, cumulative dose, and schedule of administration. Taxanes can also cause hypersensitivity reactions, myalgias, and arthralgias. The effects of tamoxifen depend on the target tissue.

Interestingly it has an estrogen-like effect on the endometrium, skeleton, coagulation system, and lipid metabolism [150]. Tamoxifen treatment results in a decrease in plasma concentration of antithrombin III, protein S, and fibrinogen [163-165], increasing the risk of developing coagulopathy. Hormone treatment with tamoxifen is also associated with a doubled risk of developing endometrial cancer [166-168], most likely due to an increase in estrogen-like signaling. It is clear that the optimal adjuvant therapy has not been determined. More research is needed to determine what patients would likely benefit from specific adjuvant therapies, increase the efficacy of treatment, and reduce the toxicity to minimize effects on normal tissue.

1.6 Gap junction enhancers as anticancer compounds

The restoration of GJIC in cancer cells is a novel therapeutic approach for cancer research. GJIC may be altered by a number of small molecules, shown through electrophysiology and dye transfer studies [169]. Gap junction enhancers may be utilized as anticancer compounds alone or used in combination with antineoplastic compounds to elicit the bystander effect, a mechanism involving the transportation of cytotoxic compounds/molecules from a treated cell to an adjacent cell [170]. Establishment of functional GJIC in tumor cells may aid delivery of a drug throughout the tumor. This could potentiate drug effects, similar to its improvement of radiation and chemotherapy [171, 172].

Low density structure of the gap junction was reported by Makowski *et al.* [173], providing a general shape of the gap junction channel using X-ray diffraction and electron microscopy. Unger *et al*. [174] further explored the transmembrane protein architecture by constructing a recombinant gap junction that was used in computational docking experiments with multiple small molecules that are known to restore GJIC, such as caffeic acid pehnethyl ester (CAPE) [58], liarozole [175], and lycopene [176]. Due to the high effective concentration

needed to elicit a response by CAPE and the lack of response in cancer patients to lycopene and liarozole, new gap junction enhancers are needed to study the mechanism of GJIC restoration and their anticancer effect.

Quinoline derivatives are commonly used in medicinal chemistry and biomedicine, and may be isolated from natural resources or prepared synthetically. The quinoline ring is used as the base for the design of many synthetic compounds with a variety of activities, including antimalarial [177], anti-inflammatory [178], antibacterial [179], and anticancer [180-183] functions. Using the partial crystal structure of Cx43 [173, 184] the interactions of a class of substituted quinolines (PQs) were examined using Autodock computational docking software [185-187]. The substituted quinolines (PQs) were observed to bind to the inert core of the connexon of the gap junction channel [188]. Specifically the CF_3 and OCH_3 of PQ1 bound to the H-N of Leu144 and CH_2 of Phe81 of connexin, respectively [188]. From this observation a class of substituted quinolines were synthesized from 4-acetaminoanisole to determine potential anticancer activities [188].

Gakhar *et al*. [188] showed that the substituted quinoline PQ1 was predicted to have a high binding affinity for connexin from computational docking experiments. Tissue cell culture studies further examined PQ1 as a GJIC enhancer and anticancer agent. In T47D human breast cancer cells, 200nM PQ1 treatment induced an 8.5 fold increase in GJIC, determined by scrape load dye transfer experiments [188]. Additionally PQ1 was shown to inhibit colony growth and reduce cell viability of T47D cells, while having no adverse effect on normal human mammary epithelial cells (HMEC) [188]. This suggests that PQ1 has a low toxicity to the normal tissue. At the molecular level, PQ1 significantly decreased the expression of phosphorylated Cx43, while increasing the expression of active caspase 3 [188]. This was the first study demonstrating the

ability of PQ1 to enhance GJIC in cancer cells, while showing promising results as an anticancer agent.

The effect of PQ1 on normal tissue was determined through evaluation of vital organs post administration of 25 mg/kg PQ1 via oral gavage to healthy mice [189]. This study focused on the distribution of PQ1 throughout the body after a single exposure. Histological evaluation of the tissue showed no observable alteration due to treatment with PQ1 [189], indicating low toxicity to the vital organs. Molecular analysis of the organs revealed an increase in survivin expression, and a decrease in active caspase 3 and caspase 8 [189]. Thus treatment of healthy tissue with PQ1 showed no adverse effects at the molecular level and the tissue level. Since PQ1 was previously shown to be a GJIC enhancer in neoplastic tissue, the expression level of Cx43 was determined in the normal tissue. All the organs treated had a decrease in Cx43 expression; suggesting that PQ1 has a different mechanism of action in normal tissue compared to what has been seen in cancer cells [189].

Based on previous studies showing the enhancement of GJIC and the anticancer activities of PQ1, analogs of this quinoline derivative were synthesized by varying the N1' substituent. Of the six compounds synthesized, PQ7 was determined to have the most potent anticancer activity in T47D cells with an IC_{50} value of 15.6 nM [190]. In T47D cell culture 500nM PQ7 increase GJIC determined by a 16 fold increase in dye transfer experiments [191]. Additionally PQ7 was shown to inhibit T47D colony growth, reduce viability, and decrease tumor growth in a T47D xenograft model [191]. Molecular analysis of cell lysate showed that PQ7 increased both Cx43 expression and activate caspase 9 expression [191]. These studies indicate that PQ7 may be a promising anticancer therapy for breast cancer.

1.7 Animal models of breast cancer

Mouse models for human breast cancer are categorized into three groups: xenograft models; chemically, virally, or ionizing radiation-induced models; and genetically engineered models (GEM) such as knockout or transgenic mice [192-194]. The limited understanding of the cell biology in normal breast tissue and breast cancer prevents the development of new therapeutic approaches. Animal models of mammary gland development and tumorigenesis are critical in the advancement of our knowledge of both normal and neoplastic tissue. Molecular characterization and functional analysis of isolated mouse mammary cells from both the normal gland and tumor models provide insight into the nature of different epithelial cell types and the origins of mammary carcinomas. Additionally animal models are used to predict the safety and efficacy of new drugs prior to human use. Drugs are being cited as having unpredictable toxicities and/or a lack of efficacy due to the early identification of the therapeutic properties of a compound, which are primary goals for preclinical animal models.

Preclinical animal studies allow the opportunity to measure pharmacodynamics and pharmacokinetic properties of the drug, such as absorption, distribution, metabolism, elimination, and toxicity. Rodent models are being utilized to identify chemopreventive agents, environmental and dietary risk factors, reproductive toxicity, and mechanisms of action. *In vivo* models are advantageous in that they incorporate multiple cell types, histology, and processes in a single biological system with accelerated tumorigenesis, due to the short life span of the mouse [195]. Animal models are the best option for the development of new drugs and testing therapeutic interventions. The main disadvantage of *in vivo* modeling is the difficulty in identifying molecular pathways and specific cellular processes. It is important to determine if tumorigenesis in a murine model resemble features of human breast cancer. The main paradigm that exemplifies the difference between human and murine species is the function of telomeres

and telomerase, which limit the life span of human cells [196]. Murine cells have longer telomeres and a basal telomerase activity [195]. Another important difference between rodents and humans is the mammary stroma; murine mammary stroma consists largely of adipose tissue, while human stroma has high amounts of fibroblasts. Normal and transformed cells do not just need the right hormones, but also an appropriate environment that supports epithelial cells growth.

Every animal model has specific advantages and disadvantages. Xenograft models are the most widely used [197, 198] and have a dominant role in preclinical trails. This is due to factors such as being relatively inexpensive and easy to generate, short latency for tumor formation after subcutaneous injection, and the ability to utilize ER-positive cancer cell lines. The use of breast cancer cell lines and xenograft models has been adopted by NCI for current drug screening programs [199]. One issue with the use of human xenograft models is that they do not replicate histopathology of breast cancers displayed in human patients. This is due to the xenograft tumors consisting mainly of proliferating epithelial cells with a lack of stromal infiltration. This led to the use of recombinant xenografts which use a mixture of benign epithelium, normal stromal cells, and/or neoplastic cells implanted into nude mice [200]. This has become an ideal system for examining the characteristics of heterotypic interaction in cancer initiation and progression [201]. Unfortunately numerous chemotherapeutic agents have shown promising results in preclinical models and yet had minimal activity in clinical settings. This has led to skepticism about xenograft tumor models. The lack of a competent immune system in xenograft models is a common weakness in preclinical trials. Additionally, these subcutaneous tumors are relatively predictable and are sensitive to most treatments, but metastasize infrequently. Tumorigenesis is

a multistep process and all stages of development need to be considered in the design of effective therapies.

The chemically induced rodent model is ideal for studying early events in chemical carcinogenesis and malignant progression. The 7,12-dimethyl-benz(a)anthracene (DMBA)-induced model of breast cancer requires hepatic activation, where the metabolites are present in the animal for several days. Co-administration of treatment with DMBA could cause an alteration in tumorigenicity due to pharmacokinetic properties of the different compounds; this can lead to inadequate modeling of adverse pharmacokinetic, pharmacodynamic, toxicologic, and/or molecular feedback signaling interactions.

Genetically engineered mice (GEM) are used to determine the biological function of genes during normal development and tumorigenesis. The first generation GEM uses oncogene expression or tumor suppressor inactivation to form mammary tumors. To prevent tumor formation in other tissues, transgene expression is placed under the control of promoters such as the mouse mammary tumor virus (MMTV) to target expression or loss to the mammary epithelial. The second generation GEM is comprised of conventional knockout models with targeted mutations. A main issue with the vast majority of mammary tumors developed from GEM is that they are ER-negative [201]. A variety of transgenic models have been created, but few have been characterized for their potential use in preclinical research. Specific strains are now being used in chemopreventive and chemotherapeutic trials [202, 203]. The following studies utilize a transgenic mouse model with the MMTV promoter upstream of the cDNA encoding the Polyoma Virus middle T antigen (PyVT) to initiate tumor formation in the mammary epithelium.

For studying the role of connexins in carcinogenesis, *in vitro* models such as the use of cell lines may not be optimal. Connexin expression may depend on the cellular environment, which is lost *in vitro*. A shift in connexin expression has been observed between *in vitro* and *in vivo* techniques. Specifically the hepatoma cell line 9618A expresses mainly Cx43 *in vitro*, but Cx32 *in vivo* [69]. There are similar findings with skin carcinoma cell lines that have little to no Cx26 expression *in vitro*, while as a xenograft they begin to express Cx26 [204]. Connexin data is contradictory depending on the model. This is seen for breast cancer as well. In normal breast tissue Cx26 is not detectable, in breast cancer cell lines it is known to be a tumor suppressor, but in invasive and metastatic lesions from patients it is shown to be overexpressed [62, 205]. Indeed cell lines present the advantage of minimizing the uncontrollable parameters, but the formation and progression of solid tumors is likely more complex than this experimental system, indicating a need for suitable animal models.

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Chapter 2 - Hypotheses and Objectives

2.1 Hypotheses

1. The anticancer effects of the gap junction enhancers PQs on T47D cells *in vitro* have been reported. The hypothesis is that PQs can be used as a combinational treatment with antineoplastic compounds to increase their efficacy in attenuating T47D xenograft tumor growth through the bystander effect.
2. Xenograft models are useful models for testing novel anticancer compounds, but have limitations. Here the PyVT transgenic model is hypothesized to be a useful biological system for studying tumorigenesis. The effects of common antineoplastic compounds are shown on tumor development.
3. PQ1 was shown to be an effective anticancer compound in a xenograft model. Using a spontaneous mouse model of mammary carcinoma development, we hypothesize that PQ1 can be used to attenuate tumor growth at three developmental stages of the PyVT transgenic model.
4. Minimal toxicity of PQ1 was observed *in vitro* and *in vivo*. PQ7 was observed to have minimal toxicity on human normal mammary epithelial cells (HMEC) while having an effective anticancer response in human breast cancer cells (T47D). The hypothesis is that PQ7 can be administered as anticancer drug with low toxicity to normal organs *in vivo*.
5. Male breast cancer is a rare disease with limited cohort studies to determine the anticancer effects of commonly used antineoplastic compounds. Using the male PyVT mouse as a biological system of male mammary carcinoma development, the hypothesis is that tamoxifen, cisplatin, and paclitaxel can attenuate tumor growth in a male mammary carcinoma model.

2.2 Objectives

1. Evaluate the combinational effects of PQs and cisplatin
    a. Effects of combinational treatment on tumor growth
    b. Determine the changes of protein expression due to treatment
    c. Histological evaluation of vital organs post exposure
        Appendix A: combination of PQs and paclitaxel.
2. Characterize the transgenic mouse model as a biological system to study mammary carcinoma development.
    a. Examine the differential pattern of hormonal receptors
    b. Determine connexin expression at different stages of tumor development
    c. Establish histological description for different stage of tumor development
3. Examine the effects of PQ1 in the PyVT model.
    a. Determine the effects of treatment on tumor growth and burden
    b. Examine the expression of connexins during tumor development
    c. Observe potential changes in lung metastasis
    d. Evaluate toxicity of PQ1 to normal tissue

4. Determine the potential toxic effects of PQ7 on normal tissue and the anticancer effects on tumor development.
    a. Examine the distribution of PQ7 to normal tissue
    b. Evaluate the toxicity of PQ7 to the vital organs
    c. Observe the effects on tumor growth at different stages of tumor development
    d. Pathological evaluation of isolated mammary tumors post treatment
    e. Determine connexin expression at different stages of tumor development post treatment
5. Evaluate treatment of male mammary carcinoma development with tamoxifen, cisplatin, and paclitaxel.
    a. Examine the hormone receptor profile of male mammary carcinomas
    b. Determine the efficacy of three antineoplastic drug treatment
    c. Evaluate the expression of molecular markers of male breast cancer
    d. Pathological evaluation of isolated mammary tumors post treatment

Chapter 3 - Gap junction enhancer increases efficacy of cisplatin to attenuate mammary tumor growth

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3.1 Introduction

Breast cancer is the most common cancer in women worldwide and mortality from breast cancer is consistently due to tumor metastasis [1]. Defects in neoplastic cells, such as excess proliferation, invasion, and metastasis, have a crucial role in the loss of tissue homeostasis [2, 3] [4]. Gap junctions are the only communicating junctions found in animal tissues, in all species, which are responsible for the direct trafficking of ions and molecules with molecular weights less than 1,200 Daltons [5]. Gap junctions directly connect the cytoplasms of neighboring cells, to allow the passage of intercellular signaling molecules and homeostatic regulators such as anti-growth signals and apoptotic factors. Intercellular junctions are important in the maintenance of the cellular homeostasis, cell differentiation, and cellular death. A main characteristic of cancer formation is the loss of gap junction intercellular communication (GJIC) through the decreased expression or absence of gap junctions [6].

Restoring GJIC in tumor cells is one approach that increases the spread of cytotoxic drugs and subsequently enhances antineoplastic therapies. Use of a gap junction enhancer may potentiate the bystander effect of cytotoxic compounds, such as cisplatin and paclitaxel. Recently, a new class of substituted quinolines (PQs) was synthesized and found to possess potent inhibitory activities against T47D breast cancer cells (IC_{50} value of PQ7 is 16 nM and PQ1 is 119 nM) through the enhancement of GJIC [7, 8]. PQ7 has the ability to enhance the

GJIC between neoplastic cells by increasing the expression of connexin 43 (Cx43) [9]. In addition, *in vivo*, the treatment of PQ7 on nude mice with T47D xenografts showed a 100% decrease in tumor growth after seven intraperitoneal injections [9]. This agent is capable of normalizing GJIC and has cancer-preventive properties.

Cisplatin is one of the most widely used cancer chemotherapeutic agents used clinically, but renal failure is a common problem in patients. Cisplatin nephrotoxicity is dose-related and used to be considered dose limiting [10]. The primary mechanism for cisplatin toxicity is via formation of platinum-DNA adducts that induce cell cycle arrest [11,12]. Other main mechanisms of action include DNA-protein cross linking, ROS generation leading to oxidative stress [13], and a gap junction-mediated cell-interdependent pathway [14]. The cell interdependent pathway of cisplatin toxicity requires DNA dependent protein kinase (PK) signaling and intercellular communication through gap junctions [14]. He *et al*. [15] showed that Cx32-composed gap junctions are required components of toxicity suggesting a dependence on cells being GJIC competent. Cisplatin damage in one cell triggers DNA-PK dependent signal and is transmitted by GJIC to neighboring cells. Jensen and Glazer [14] showed that by inhibiting GJIC with lindane, immortalized mouse embryonic fibroblasts (MEFs) were protected from cisplatin toxicity, while increasing GJIC by transfecting MCF-7 breast cancer cells with Cx43 enhanced drug sensitivity. Induction of apoptosis/necrosis from cisplatin in one cell may cause a "death signal" that is transmitted to neighboring cells through gap junctions.

Increasing gap junction activity or enhancing GJIC in tumor cells provides the targets to enhance antineoplastic therapies. Tanaka and Grossman [16] showed that by transfecting human bladder cancer cells with Cx26, tumor formation could be prevented. In combination with cisplatin an increase in GJIC promoted apoptosis, cell cycle arrest, and down regulated Bcl-

2 [16] . A new class of substituted quinolines (PQs) possesses inhibitory activities against breast cancer cells through the enhancement of GJIC. The objective of this study was to examine the effect of combinational treatment of PQ and antineoplastic drugs in a xenograft tumor model, showing an increase in efficacy of the antineoplastic drug, cisplatin (cis-diamminedichloroplatinum), via the enhancement of gap junctions.

3.2 Material and methods

3.2.1 Ethics statement

Husbandry of animals is conducted by the Comparative Medical Group (CMG) at the College of Veterinary Medicine at Kansas State University. The CMG animal facilities are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International (AAALAC). The compliance to aspects of animal welfare law is regularly monitored by the veterinary staff. Animal care and use protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Kansas State University (Protocol Number: 2985), Manhattan following NIH guidelines. This applies to all animal studies conducted.

3.2.2 Compounds

Compounds PQ1, 6-methoxy-8-[(3-aminopropyl)amino]-4-methyl-5-(3-trifluoromethyl-phenyloxy)quinolines, and PQ7, 6-methoxy-8-[(2-furanylmethyl)amino]-4-methyl-5-(3-trifluoromethylphenyloxy)quinoline, were graciously provided by Dr. Duy H. Hua (Kansas State University, Manhattan, KS). Cisplatin, *cis*-Diamminedichloroplatinum (II), was purchased from Sigma Aldrich (St. Louis, MO).

3.2.3 Cell line and cell culture

The T47D human breast cancer cell line was purchased from American Type Cell Culture (ATCC, Manassas, VA). Cells were grown in RPMI medium supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) at 37° C with 5% CO_2 in T-125 cm^2 flasks.

3.2.4 Xenograft tumors of T47D cells in nude mice

Nu/Nu female mice were ordered from Charles River Laboratories International, (Wilmington, MA, USA) and implanted with 17-β-estradiol (1.7 mg/pellet, Innovative Research of America, Sarasota, FL) before injection of $1x10^7$ T47D breast cancer cells subcutaneously into the inguinal region of the mammary fat pad. Cell viability of T47D cells was performed prior to the injection. Tumor size was measured in two dimensions with calipers every 2 days starting at day 7. Tumor volume was determined by the equation: Volume = ½(Length)*$(Width)^2$. Mice were observed for any change in behavior, appearance or weight. When tumors reached >50 mm^3, six animals were randomly assigned to each treatment group. Mice were administered 25 mg/kg PQ1 or PQ7 in succinic acid salt, 3.5 mg/kg cisplatin, or a combination of PQ and cisplatin via intraperitoneal injection of 100 µl. Compounds were dissolved in DMSO, which was used as a vehicle control at the same volume. Tissue was harvested from mice two days after the last injection.

3.2.5 Western blot analysis

Tissue was harvested from the mice and whole cell extractions conducted using lysis buffer (20 mM Tris pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, and 0.5 % Triton X-100) with 1:1000 dilution of protease inhibitors (Sigma-Aldrich, Saint Louis, MO, USA). Tissue was homogenized via the OMNI Bead Ruptor 24 at a speed of 5.65 m/s for 45 seconds, followed by

centrifugation at 13,000 rpm for 30 minutes at 4˚C. Twenty-five µg of whole-cell extract was resolved by 10% SDS polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membrane (Midwest Scientific, Saint Louis, MO, USA). Nitrocellulose membrane was blocked in 5% milk for an hour at room temperature and then incubated with monoclonal antibodies against anti-Cx43, -Cx32, -Cx26, -caspase 3, -caspase 8, and -caspase 9 (200 µg/ml; Santa Cruz Biotechnologies, Santa Cruz, CA, USA) and beta actin (500 µg/ml; Sigma-Aldrich) at a dilution of 1:1,000. Western blots were detected by enhanced chemiluminescence detection reagents (Pierce, Rockford, Illinois, USA) and visualized by Fluorochem E imaging system (ProteinSimple).

3.2.6 Immunohistochemistry

All tumors were removed and fixed in a solution of 10% formaldehyde and embedded into paraffin prior to sectioning them onto slides at a 5 µm thickness. Paraffin sections (5 µm) were dried at 60°C for 25 minutes. Deparaffinization was performed with 100% xylene and 100%, 90%, 75%, 50% ethanol. Antigen retrieval was performed in 1× citrate buffer solution and steam for 20 minutes. Endogenous peroxidase was blocked using 3% hydrogen peroxide. Slides were then incubated overnight at room temperature with antibody (1:50 dilution). Antibodies include: connexin 43, 32, 26; caspase 3, 8, 9; survivin; Cyclin D1; Ki-67; and Mig (Santa Cruz Biotechnologies, Santa Cruz, CA, USA). After washes in PBS, slides were successively incubated with biotinylated secondary antibodies (1:1000) for 15 minutes. Slides were washed and immunostains were amplified by incubation with Avidin Biotin Complex (ABC) for 10 minutes accordingly. Cells were visualized with 3,3-diaminobenzidine (DAB) followed by a hematoxylin counterstain. The sections were viewed and the images captured with a Nikon 80i microscope under 40X and 60X magnification.

3.2.7 Statistical Analysis

Significance was considered at a p-value ≤ 0.05 using Student's t-test analysis. All data are presented as mean ± 95% confidence interval of at least three independent experiments.

3.3 Results

3.3.1 T47D xenograft tumor growth in nude mice

Mice were implanted with 17ß-estradiol (1.7 mg/pellet) before the injection of 1 x 10^7 T47D breast cancer cells subcutaneously into the inguinal region of mammary fat pad. Seven days post cell injection, animals was randomly assigned to each treatment group. Animals were treated intraperitoneally with DMSO as a control of drug solvent, cisplatin, PQ1, PQ7, or a combining treatment of cisplatin and PQ in a total volume of 100 µl. All treatments significantly reduced tumor size (Figure 3.1) compared to control. Cisplatin alone induced a 1.8-fold decrease in mammary tumor growth while combinational treatment of cisplatin and PQ1 showed a 2.6-fold reduction after 7 treatments at every 2 days (p-value = 0.012). PQ1 alone induced a 2.8-fold reduction in tumor growth after seven injections compared to control and 1.5-fold reduction compared to cisplatin treatment alone with a p-value of 0.001. The data demonstrates that PQ7 alone and in combination with cisplatin significantly reduced T47D xenograft tumor group compared to control (p-values < 0.001). With PQ7 treatment alone, there was a 2.0-fold reduction in tumor size compared to control mice. Combinational treatments resulted in a 2.6- and 2.1-fold reduction in T47D xenograft tumor size for PQ1 (p-value = 0.028) and PQ7 combinations with cisplatin, respectively, compared to control mice. Combinational treatment of cisplatin with PQs showed greater reductions in tumor volume compared to cisplatin alone.

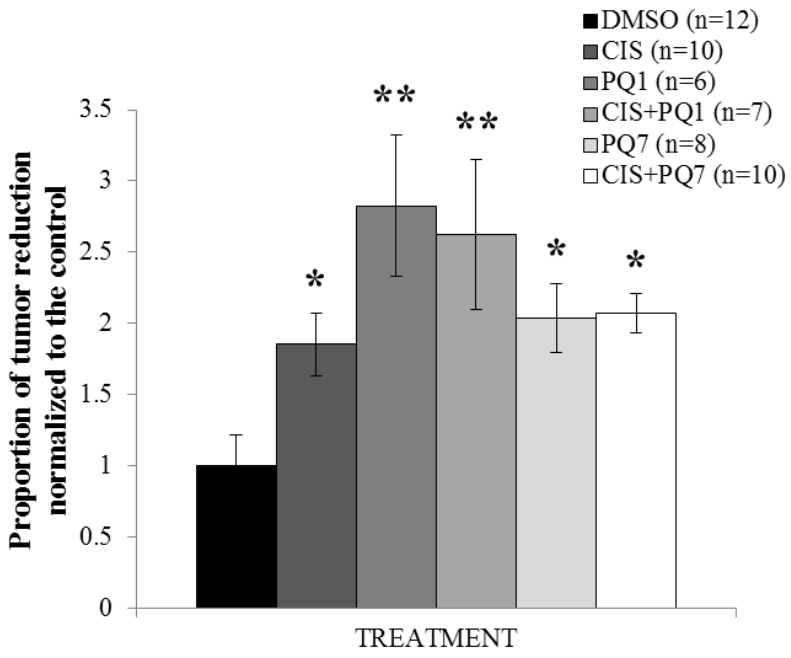


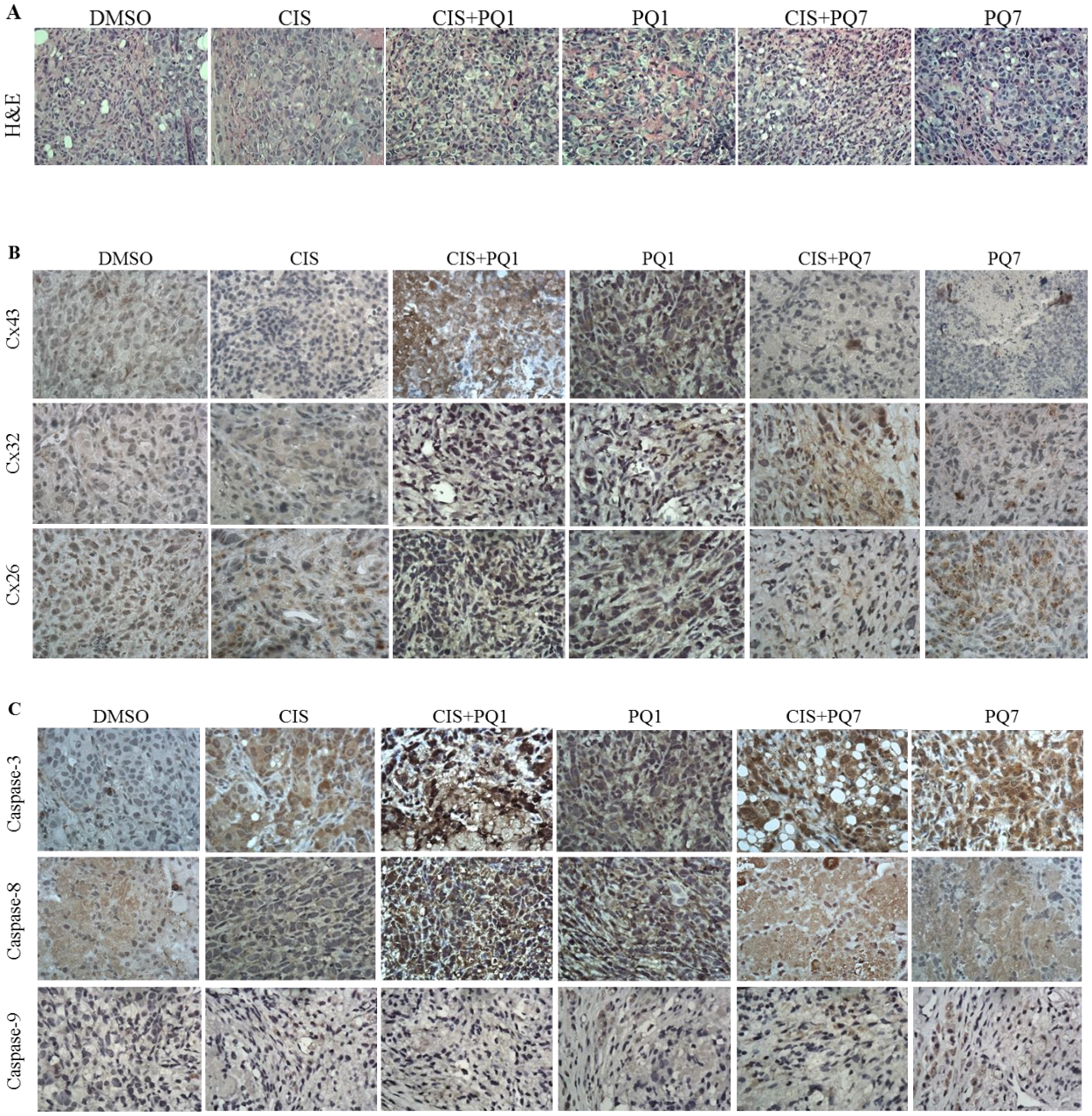
Figure 3.1 Xenograft tumor growth in nude mice. The graphical presentation shows the proportion of tumor reduction normalized to control after 7 IP injections of DMSO, cisplatin (3.5mg/kg), PQ1 (25mg/kg), PQ7 (25mg/kg), or a combination of cisplatin and PQ. * *P*-value is <0.05 compared to control. ** *P*-value is <0.05 compared to control and cisplatin treatments.

3.3.2 Protein expression of xenograft tumors

Morphological changes are the basis for contemporary cancer diagnosis. Hematoxylin and eosin (H&E) staining showed consistent morphology of all xenografts despite treatment received (Figure 3.2A). Tumor sections showed a solid nest of predominately poorly-differentiated tumor cells with large, irregular nuclei, coarse granular chromatin, prominent nucleoli, and high mitotic activity. Neoplastic cells were larger than normal epithelium with a characteristic epithelioid morphology and marked nuclear pleomorphism. Histological staining does not show any prominent features of apoptosis or necrosis for any treatment group.

Adjacent cells are able to exchange homeostatic regulators, such as anti-growth signals and apoptotic factors, through hydrophilic gap junction channels. Each gap junction is

composed of two hemichannels (connexons) that are embedded in the plasma membrane. These connexons are formed by six connexin proteins [3] , of which there are 21 different human connexin genes identified [17] . Only three connexin proteins are expressed in the human breast tissue: Cx43, Cx32, and Cx26 [18]. Immunoblot analysis and immunohistochemistry were conducted on T47D xenograft tumors harvested from mice after 7 intraperitoneal injections of DMSO, cisplatin, PQ1, PQ7, or a combining treatment of cisplatin and PQ. Tumors treated with PQ alone and in combination showed an increase in connexins (connexin 43, 32, and 26), compared to controls and cisplatin treated tumors (Figure 3.2B). Cisplatin treatment decreased the expression of Cx43 compared to control (Figure 3.2B). Western blot analysis of tumor homogenates showed that PQ1 significantly increased Cx43 expression in T47D xenografts by a 3.9 fold increase compared to control (p-value = 0.003) and 4.9 fold change compared to cisplatin (p-value = 0.007) treated mice (Figure 3.3A).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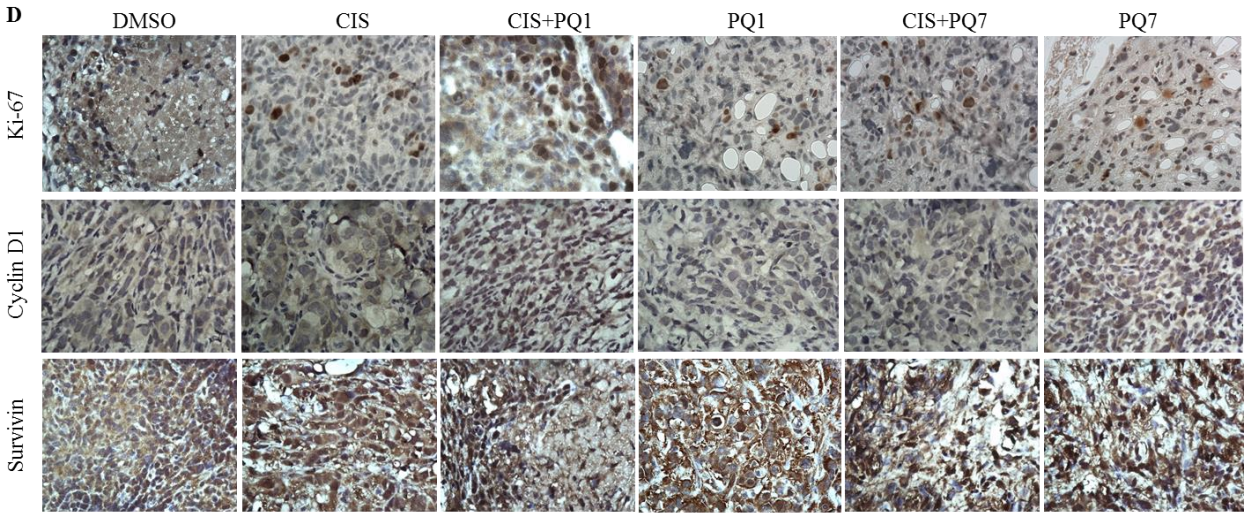

Figure 3.2 Immunohistochemistry of T47D xenograft tumors. Protein expression of A) Hemoxylin and Eosin staining under 40x magnification, B) connexins (Cx 43, 32, and 26) under 60X magnification, C) apoptotic proteins (caspase-3, -8, and -9) under 60X magnification and D) proliferative (Ki-67 and Cyclin D1) and survival proteins under 60X magnification in T47D xenograft tumors harvested after 7 IP injections treated with either DMSO (control), cisplatin, PQ1, PQ7, or a combination of cisplatin and PQs.

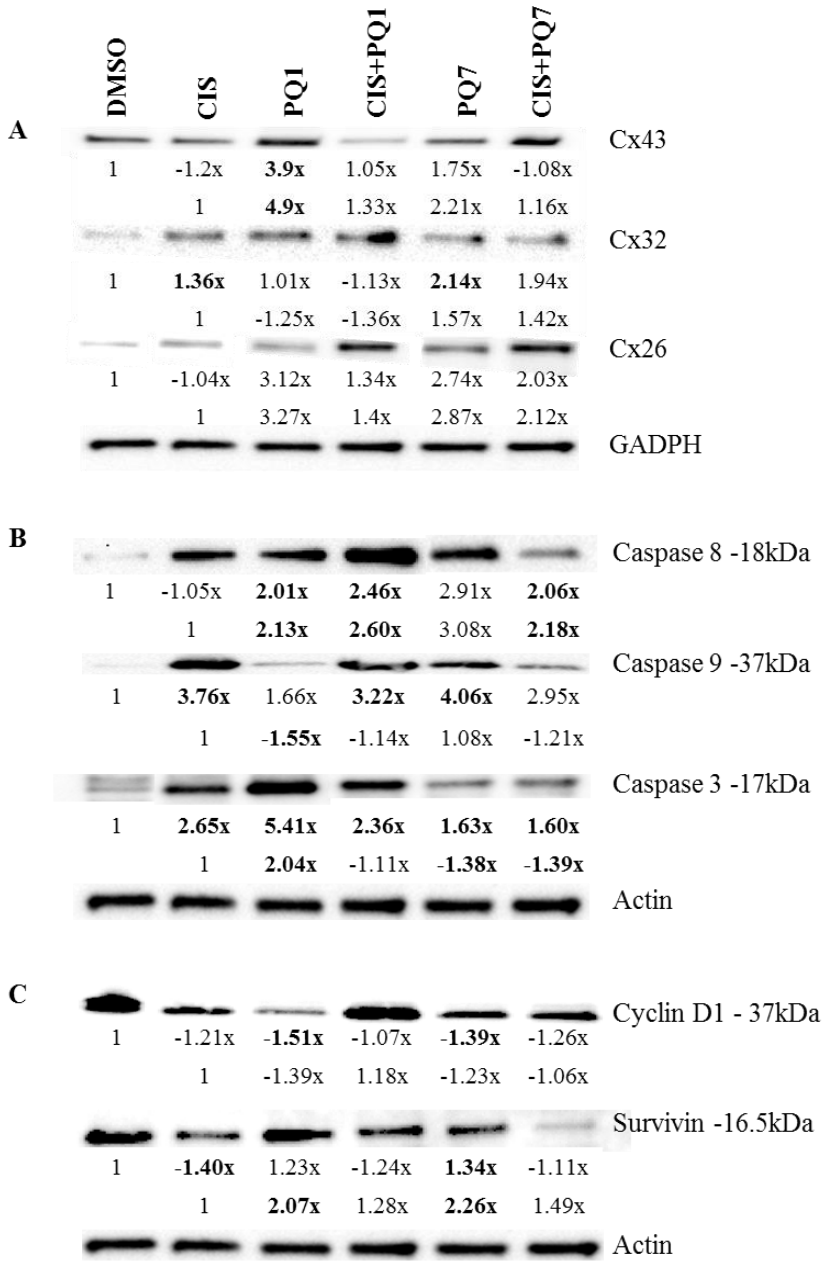

Figure 3.3 Protein expression of T47D xenograft tumors. Protein expression of A) connexins (Cx43, 32, and 26), B) apoptotic proteins (caspases), C) Cyclin D1 and survivin from T47D xenograft tumors harvested after 7 IP injections treated with either DMSO (control), cisplatin, PQ1, PQ7 or a combination of cisplatin and PQs. Actin and GADPH are loading controls. Numbers indicate the fold difference from control (top row) and cisplatin (bottom row). x = fold induction. Bold *p*-value is <0.05 compared to control or cisplatin (n=3).

The apoptotic signaling pathway induced by the treatment was determined by analysis of caspase expression. Two major signaling pathways lead to apoptosis. One is mitochondrial release of pro-apoptotic effectors such as caspase-9, which leads to caspase-dependent or independent apoptosis [19]. The other involves the interaction of death receptors with associated proteases and activation of caspase-8 [20]. Data indicate there is an increase in the density of apoptotic proteins (caspase-3, -8, and -9) staining with PQ treatment compared to control and cisplatin alone (Figure 3.2C), which is confirmed by Western blot analysis (Figure 3.3B). Cisplatin treatment increased capase-9 expression by 3.7x (p-value = 0.007) and caspase-3 expression by 2.7-fold (p-value = 0.0004) in T47D xenografts compared to control (Figure 3.3B). PQ1 treatment increased caspase-3,-8,-9 expression in tumors compared to control by a 5.4-fold (p-value < 0.0001), 2.0-fold (p-value = 0.003), and 1.6-fold change respectively. Compared to cisplatin treatment PQ1 increased caspase-3 expression by 2.0-fold (p-value = 0.0007). Additionally PQ7 also increases caspase-3, -8, and -9 expression compared to control by 1.6-fold (p-value = 0.015), 2.8-fold, and 3.8-fold (p-value = 0.001) respectively. This suggests that PQs upregulate the expression of apoptotic signaling molecules to increase cellular induced death. Combinational treatment of PQs and cisplatin did not increase caspase-3 or -9 expressions significantly from cisplatin alone. Caspase-8 expression was significantly increased with combinational treatment of PQ and cisplatin by 2.6-fold (p-value = 0.02) and 2.2-fold (p-value = 0.01) for PQ1 and PQ7 combinations, respectively. There is a significant increase in apoptosis in PQ7 treated cells compared to those treated with cisplatin alone, but the tumor sizes between groups are not significantly different.

Proteins that inhibit apoptosis provide protection for tumor cells against cytotoxic compounds. Survivin is a member of the inhibitors of apoptosis protein family that is expressed

during embryogenesis and in tumor cells as an anti-apoptotic protein that is capable of regulating mitosis [21-23] . Survivin is highly expressed in a range of tumors and its expression correlates with both accelerated relapse and chemotherapy resistance [24]. T47D xenograft tumors were isolated after treatment to determine the expression of survivin. All tumors that received treatment showed an intense immunohistological staining for survivin, but expression was not evenly distributed through the tissue similar to the control (Figure 3.2D). Western blot analysis of the tumors indicates cisplatin treatment significantly reduced survivin expression compared to control by 1.4-fold (*p*-value = 0.02; Figure 3.3C). PQ1 treatment increased survivin expression by 2.1-fold (*p*-value = 0.04) compared to cisplatin. PQ7 showed a 0.3-fold increase in survivin expression compared to control (*p*-value = 0.006) and a 2.25-fold increase compared to cisplatin treatment (*p*-value = 0.0045).

Ki-67 and Cyclin D1 were used as biomarkers for cell proliferation. Ki-67 is found in rapidly dividing cells and is used to determine the rate of cellular proliferation. Cyclin D1 is a key cell cycle regulator in which over expression results in rapid progression from G1 to S phase in mitosis [25]. From immunohistochemistry all isolated xenograft tumors that were treated showed a decreased expression in Ki-67 (Figure 3.2D). The treated tumors have fewer cells expressing Ki-67, but those cells that are expressing this protein show strong positive staining compared to control. Cyclin D1 expression in T47D xenografts were significantly lower with PQ treatment compared to control by 1.5-fold (*p*-value = 0.0007) and 0.4-fold (*p*-value = 0.008) for PQ1 and PQ7 respectively (Figure 3.2D and 3.3C). This indicates that PQ treatment downregulates the expression of proliferative proteins Ki-67 and Cyclin D1 in xenograft tumors.

3.3.3 Histological study of metabolic organs

Histological examination of the kidney and liver from xenografted mice showed no significant difference in morphology due to the treatment received. To determine if there was any change in protein expression of the metabolic organs, immunoblot analysis and immunohistochemistry was conducted. In the kidney there was an increase in Cx43 expression and a decrease in survivin expression due to PQ treatment (Figure 3.4A). There was an increase in caspase 3 expression after cisplatin treatment, which was not surprising since nephrotoxicity is common with cisplatin treatment. The kidney showed a decrease in caspase 3 expression with PQ1 treatment. The liver showed an increase in caspase-3 expression with cisplatin treatment (Figure 3.4B), suggesting possible cisplatin-induced hepatotoxicity. PQ treatment in combination with cisplatin appeared to decrease caspase-3 expression compared to cisplatin alone. There was an additional increase in survivin expression due to PQ treatment. There was no significant difference in Cx43 expression of the liver between treatment groups.

The monokine induced by interferon-gamma (MIG) was used as a biomarker for inflammatory signaling, indicating cytotoxicity due to treatment. The enhanced release of this CXC chemokine targets activated T cells, causing an increase in intracellular calcium ion concentrations and chemotaxis [26]. The kidney isolated from cisplatin treated animals had an increase in MIG expression (Figure 3.4A). The combinations of cisplatin and PQ show less staining than cisplatin alone, while PQs alone show low levels of MIG expression. The liver showed a similar pattern of staining for MIG expression with each treatment (Figure 3.4B). The decrease in MIG expression suggests that PQs may provide protection from the inflammatory response of cisplatin treatment.

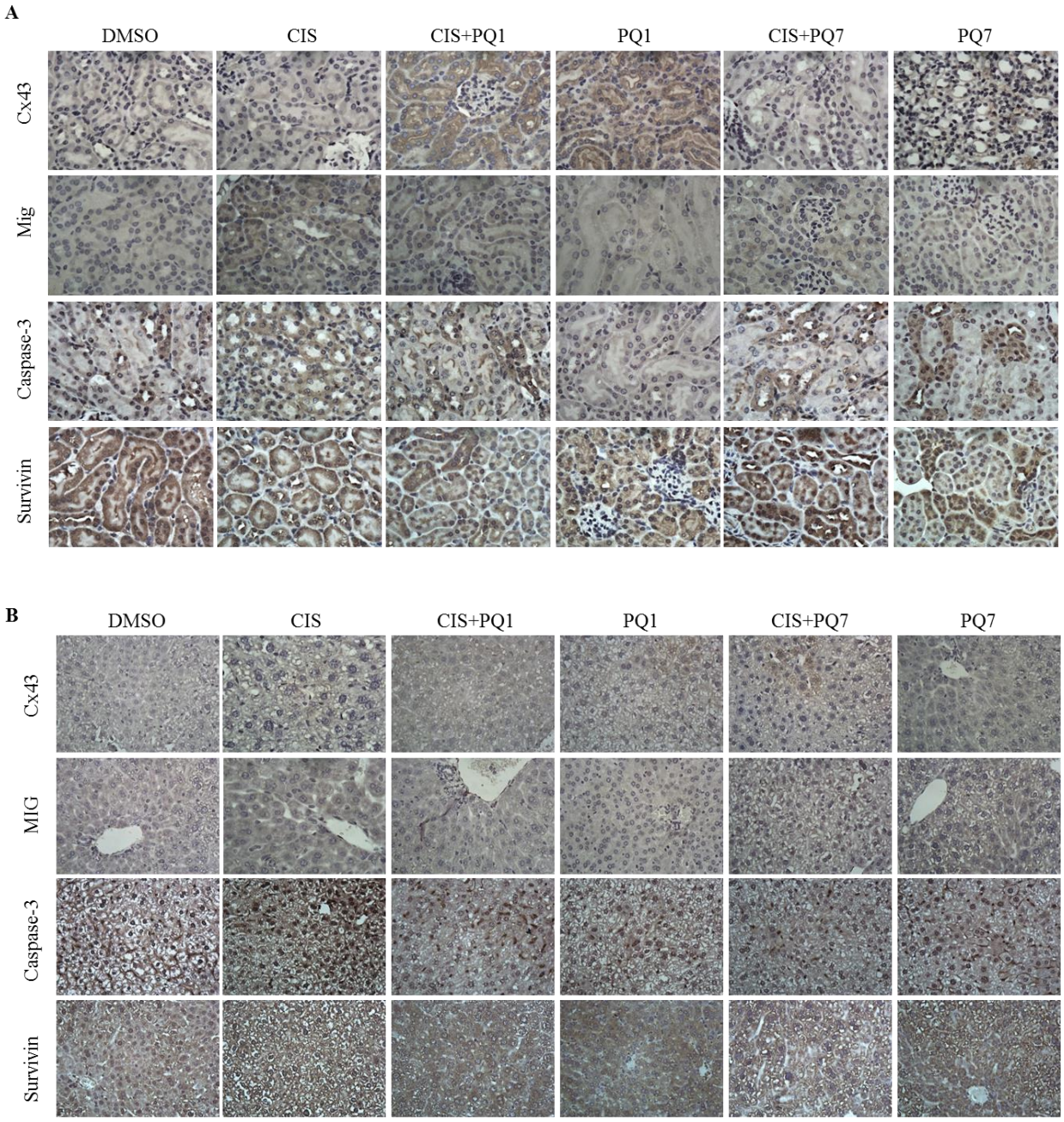

Figure 3.4 Immunohistochemistry of metabolic organs from nude mice. Protein expression of Cx43, the monokine induced by IFN-gamma (MIG), apoptotic protein (caspase 3) and survivin in A) kidney and B) liver from mice with T47D xenograft tumors harvested after 7 IP injections treated with either DMSO (control), cisplatin, PQ1, PQ7 or a combination of cisplatin and PQs (view image under 60X magnifcation).

To determine if the normal GJIC of various organs was potentially affected by treatment, the expression of Cx43 was observed. The uterus, heart, and brain showed no change in Cx43 expression with any treatment (Figure 3.5). There is no observable deleterious effect due to an increase in connexin expression of cells in the liver and kidney. Further studies must be made to determine the full effects of this response.

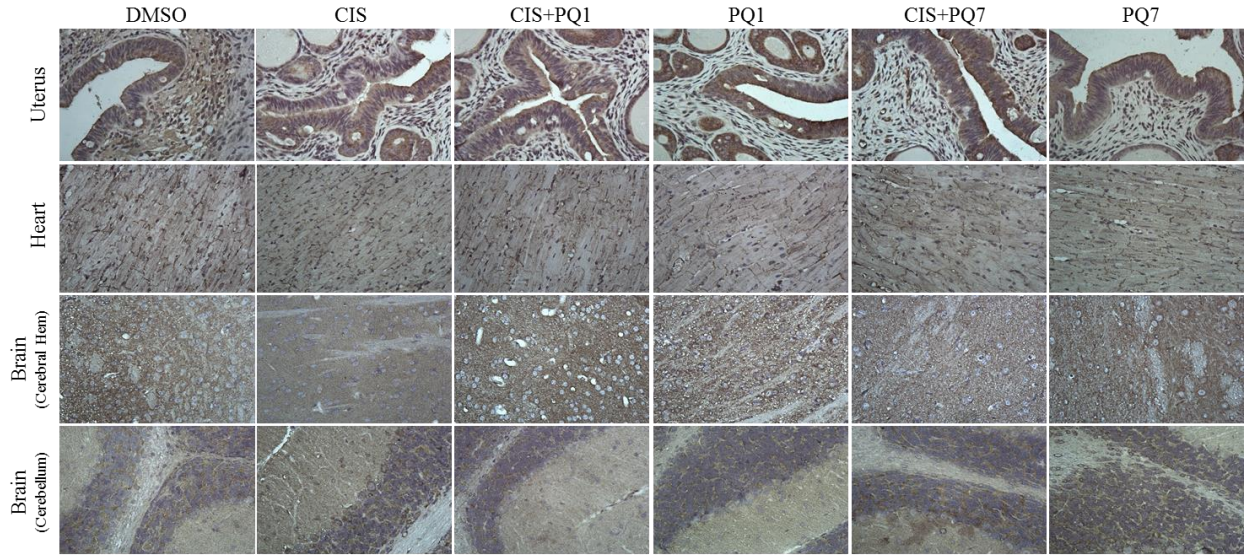

Figure 3.5 Immunohistochemistry of organs harvested from nude mice. Protein expression of Cx43 in the uterus, heart, and brain (cerebral hemisphere and cerebellum) from mice with T47D xenograft tumors harvested after 7 IP injections treated with either DMSO (control), cisplatin, PQ1, PQ7 or a combination of cisplatin and PQs. Image viewed under 40X magnification.

3.4 Discussion

This study used T47D xenografts to determine the effects of the combinational treatment of cisplatin and gap junction enhancers, PQs, in tumor-bearing mice. The results showed a decrease in tumor growth with PQ treatment, both alone and in combination with cisplatin, compared to control after seven injections (Figure 3.1). The combination of PQ1 and cisplatin

significantly reduced tumor size compared to cisplatin alone, providing evidence that PQ1 can increase the efficacy of antineoplastic drugs in this animal model. Previously PQ7 demonstrated the ability to enhance GJIC activity through an increase in connexins 43 expression [9]. Here we show an increased expression of connexins 43, 32, and 26, suggesting a corresponding increase in cell to cell communication which would allow more efficient trafficking of cisplatin. Protein expression in the tissue sections indicates that connexin 43 is being regulated by PQ treatment. Results support previous data [27] that cisplatin cytotoxicity is dependent on GJIC. There is an increase in cisplatin-mediated response with GJIC enhancement.

The decrease in tumor size seen with PQ treatment may be attributed to an increase in apoptosis as the result of an up-regulation of caspase-3, caspase-8, and caspase-9 (Figure 3.3B). These findings suggest that PQ1 and PQ7 are anticancer agents. Cisplatin treatment did not upregulate caspase-8 expression since cisplatin induced apoptosis is regulated by caspase-9 [28]. Additionally caspase expression was not downregulated with cisplatin treatment, indicating that there was not cisplatin resistance observed with the T47D xenograft tumors. There was an increase in caspase-8 expression in T47D xenograft tumors of PQ treated mice compared to cisplatin and control tumors, showing that PQ induced apoptosis through induction of both caspase-8 and capase-9 signaling.

Apoptosis is recognized as a major mode of cisplatin induced cell death. From histological results, there is a significant increase of apoptosis in PQ-treated cells compared to those treated with cisplatin alone. The tumor sizes between cisplatin alone, PQ7 alone, and cisplatin and PQ7 in combination treated groups are not significantly different despite the differences in caspase expression. The process of apoptosis produces multiple distinct populations of cells at varying stages, from early stage to secondary necrosis [29,30]; therefore,

the discrepancy in tumor size may be due to insufficient clearance from the body within the 14 day time period. Gap junctions have previously been shown to induce the synchronous cell death behavior of coupled cells [31], suggesting that PQs affect cell death by increasing connexin expression and indirectly inducing both pathways of apoptosis through an increase in caspase expression.

Western blot analysis resulted in highly variable protein expression with whole organ homogenates, most likely due to the presence of multiple tissue types in each organ. Kadle *et al*. [32] showed that different forms of Cx43 have a tissue-specific distribution, suggesting tissue wide differences in protein expression. More data on the tissue-specific distribution of proteins, specifically connexins, is needed to accurately determine the effects of utilizing a gap junction enhancer systemically.

Combinational treatment of cisplatin and PQ was not significantly different from PQs alone. Wang *et al.* [27] demonstrated that high cisplatin concentrations strongly inhibit GJIC through direct interactions with connexins and indirect reduction of connexin expression. Cisplatin thus may act as a competitor for gap junction enhancers. PQ7 may not have significantly increased the efficacy of cisplatin in a combinational treatment due to the direct competition between the compounds have when targeting gap junctions. Cisplatin was shown to inhibit Cx32/Cx26 heteromeric hemichannel in a concentration dependent manner [27] , which is supported by the data presented in immunohistochemistry and immunoblot of Cx32 and Cx26 (decrease in expression).

Cisplatin induced “death signal” is transmitted to neighboring cells via GJIC [14]. Enhancement of GJIC may allow transmission of this “death signal” more efficiently between cells. Peterson-Roth *et al*. [33] showed that the level of expressed Cx43 in a cancer cell

modulates cell-to-cell cisplatin-mediate response. Overexpression and activation of src is seen clinically in many cancer types treated with cisplatin [34-36]. Oncoproteins, such as src, promote cell growth and survival when exposed to cytotoxic agents [37-39] , therefore protecting the cancer cells from chemotherapeutics. Src is specifically induced by cisplatin to produce tyrosine phosphorylation of Cx43, decease GJIC, and increases cell survival; thus, elevates survival of the neighboring cells by disrupting GJIC of the "death signal" [33]. Enhancement of GJIC with PQ may counter the effects of src in the cancer cell to increase the efficacy of cisplatin through an increase in transmission of the "death signal". The fact that cancer cells are widely accepted to be deficient in GJIC and/or connexins and that cisplatin inhibits GJIC may contribute to the development of cisplatin resistant tumors. Development of drugs and methods that can increase or recover GJIC may be a new potent way to enhance chemotherapeutic methods and radiotherapy, which has also been shown to be GJIC-dependent [31] .

Renal failure in cancer patients is a common problem. Cisplatin nephrotoxicity is clearly dose-related and increases with frequency of administration and cumulative dose [40] . The increased cell-to-cell communications displayed in the tumor cells is seen in the kidney and liver, but not in any other vital organ. There are no observable morphological or molecular abnormalities due to the increase in connexin expression. Cisplatin treatment alone induced an increase in both caspase and MIG expression, while PQ treatment did not (Figure 3.5). The combinational treatment conducted in this study shows that PQs can be utilized to decrease the cytotoxicity of cisplatin. This provides evidence for a new combinational treatment for breast cancer using cisplatin at a reduced dose to prevent renal toxicity. Present data leads to the idea that PQ, at lower concentrations than needed for anticancer effects, may improve the efficacy of

chemotherapeutic agents. This would allow the use of lower drug concentrations, thus decreasing the extent of detrimental side effects due to the cytotoxicity of the compounds.

Gap junction enhancers prove to accelerate apoptotic cell death in breast cancer tumor cells while increasing the connexin expression. This is promising for use of PQs in gap junction-mediated intercellular transfer of toxic effects in multiple systems and the bystander effect. The decrease in survivin distribution within the neoplastic tissue after PQ treatment also indicates good prognosis for patients post treatment since survivin is highly expressed in a range of human tumors and its expression directly correlates with both accelerated relapse and chemotherapy resistance [24]. Future studies will focus on potentiating other antineoplastic drugs through the enhancement of gap junctional activity, as well as expanding the treatment period to 21 or 28 days and look at the reoccurrence of tumors post treatment remission.

The growth suppressive effect of PQs has been previously established in multiple breast cancer cell lines [8,9]. The antitumor effects of PQs on breast cancer xenografts in combination with antineoplastic agents in nude mice show did not show an additive or synergistic effect. The combinational treatment was not significantly different than treatment with PQs alone, suggesting that these compounds may function along the same pathway but that the effects in tumor growth may be due primarily to PQ exposure. This indicates that PQs can attenuate tumor growth independent from cisplatin treatment. Interestingly cisplatin decreased cell communication to antagonize connexin expression, and potentially PQ function. The combinational treatment of PQs and antineoplastic drugs show promising treatment for breast cancer. The efficacy of antineoplastic compounds can be increased via enhancement of gap junctions. The outcome of our findings has introduced a new class of anticancer drugs, enhancing current treatment for breast cancer.

3.5 References

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Data for the combinational treatment of PQs and paclitaxel is summarized in Appendix A.

Chapter 4 – The PyVT transgenic mouse as a multistage model for mammary carcinoma and the efficacy of antineoplastic treatment.

A research article of the following findings has been published in Journal of Cancer Therapy (2013) 4(7) pp. 1187-1197. doi: 10.4236/jct.2013.47138.

4.1 Introduction

In vivo models are important for translational research and may be used to explore the mechanism of tumorigenesis, or the pharmacodynamics and the development of therapeutics for cancer. Animal models to study cancer formation should include features such as molecular targets, drug metabolism, pharmacokinetics/dynamics, drug distribution, anatomic similarities, microenvironment, angiogenesis, and metastasis. Change in tumor size is the most widely used end point for drug efficacy, but tumor frequency, survival, and tumor burden are other important factors. Thus, specific model systems have advantages for measuring tumor burden, drug sensitivity, and metastatic potential. The antitumor activity of chemotherapeutics is commonly determined in immune deficient mice transplanted with human tumors, but these xenograft tumors have the potential loss of tumorigenicity, limited metastatic potential of many cell lines, and loss of linearity with increasing tumor volume [1].

Animal models have evolved from the transplantable syngeneic mouse tumor models to chemically induced, transgenic, and spontaneous animal tumor models. Numerous chemotherapeutic agents have shown promising results in preclinical models and yet had minimal activity in clinical settings. This has led to skepticism about xenograft and syngeneic tumor models. Newer techniques, including transgenic mouse models, have the potential to be more predictive. Tumorigenesis is a multistep process and all stages of development need to be

considered in the design of more effective therapies. Since several transgenic mammary models of human breast cancer progress through well-defined cancer stages, they are useful pre-clinical systems to test the efficacy of chemopreventive and chemotherapeutic agents. The use of transgenic mammary carcinoma models allows for detailed study of stage-specific responses to antineoplastic agents, defining the appropriate timing for intervention with specific compounds.

The transgenic strain FVB/N-Tg(MMTV-PyVT)634Mul/J (known as PyVT) is a novel *in vivo* model for the study of mammary carcinoma formation and metastasis with important clinical utility. The PyVT mouse model carries the Polyoma Virus middle T antigen with the mouse mammary tumor virus (MMTV) promoter that drives mammary tissue-specific expression [2]. The PyVT oncogene activates multiple oncogenic pathways, such as src and phosphatidylinositol-3-kinase [3], leading to an aggressive tumor phenotype. Previous studies indicate that virgin females carrying the transgene develop multi-focal, poorly differentiated, highly invasive ductal carcinoma by 10–12 weeks of age, with a high incidence of lung metastases stemming from the primary mammary tumor [4]. At 5 weeks of age females develop noninvasive focal lesions which are classified into four groups: simple, solid, cystic, and mixed (solid and cystic) [5]. Solid lesions consist of large foci with a dense mass of atypical cells in nodular sheets. Cystic lesions vary in size and complexity, and are lined by multilayered epithelium with significant amounts of clear fluid [5]. The premalignant tumors are morphologically heterogeneous and highly proliferative neoplastic cells with atypical nuclei that contain abnormal microvasculature, and stay within the basement membrane [5]. The MMTV-PyVT transgene expression is variable in tumors [5], which suggests that the transgene is not necessary for the maintenance of the malignancy. The PyVT model represents a multistep process due to lesions progressing from hyperplasia to an adenoma/mammary intraepithelial

neoplasia mixed phenotype, followed by early and late carcinoma with pulmonary metastasis [4, 5]. Metastasis of the primary tumor to distant sites remains a significant cause of death in many cancer types, highlighting the importance for a metastatic model. This model of spontaneous mammary carcinogenesis is a powerful tool for studying the mechanism associated with tumor progression and development of novel chemotherapeutics.

This model has not been used to test the efficacy of many antineoplastic compounds despite its clinical relevance to human breast cancer. The compounds utilized here are cisplatin, paclitaxel, and tamoxifen. The mechanism of action is briefly explained. Cisplatin induces damage to tumors via formation of DNA adducts followed by cell growth inhibition and induction of apoptosis. Paclitaxel prevents cellular proliferation by binding to tubulin and inhibiting disassembly of the microtubules in the cell. Tamoxifen is a selective estrogen receptor modulator (SERM) that competitively inhibits the binding of estradiol to the estrogen receptors. The effects of these compounds on the MMTV-PyVT transgene-induced mouse have not yet been reported.

The present study reviews the characteristics of tumor development and determines the effects of antineoplastic treatment on tumorigenesis and metastasis using the MMTV-PyVT transgene-induced mammary tumor model. Interestingly with the progression of malignancy there is an increase in expression levels of Ki-67 and survivin, with a decrease in estrogen receptor (ER) and progesterone receptor (PR) expression. This supports previous work done on this model. We showed for the first time the anticancer activity of tamoxifen, not cisplatin or paclitaxel, against a multistage mammary tumor model.

4.2 Materials and methods

4.2.1 Animals

A colony of PyVT transgenic mice (The Jackson Laboratory; Bar Harbor, ME) was established. To identify transgenic progeny, genomic DNA was extracted from a 1.5-cm tail clipping. All mice carrying the PyVT transgene developed mammary tumors. Tumor development of positive female mice was closely monitored every 2–3 days. Tumor onset was recorded as the age of the animal at which palpable abnormal masses were detected. Tumor size was measured in two dimensions with calipers every 2 days. Tumor volume was determined by the equation: Volume = ½(Length)*(Width)^2. Female mice at each stage of tumor development were randomly divided into four experimental groups: (1) control animals given the vehicle solvent (DMSO); (2) animals treated with 3.5 mg/kg cisplatin; (3) animals treated with 10 mg/kg paclitaxel; and (4) animals treated with 20 mg/kg tamoxifen. All treatments were administered as an intraperitoneal (IP) injection every other day for 14 days.

4.2.2 Compounds

cis-Diammineplatinum(II) dichloride (P4394), paclitaxel (T1912), and tamoxifen (T5648) were purchased from Sigma Aldrich (St. Louis, MO, USA).

4.2.3 Antibodies

Primary antibodies: Anti-ERα (sc-8002, mouse monoclonal), anti-ERβ (sc-8974, rabbit polyclonal), anti-PR (sc-166170, mouse monoclonal), anti-survivin (sc-374616, mouse monoclonal), and anti-Ki67 (sc-23900, mouse monoclonal), from Santa Cruz Biotechnology (200 µg/ml; Santa Cruz, CA); anti-E-cadherin (3195, rabbit monoclonal; 43 µg/ml) and anti-GAPDH (2118, rabbit monoclonal; 24 µg/ml) from Cell Signaling (Boston, MA); anti-pan epithelial (MAB1631) and anti-pigment epithelium (MAB1059) from Chemicon (1.0 mg/ml;

Temecula, CA); and anti-Her2 (AP7629e, rabbit polyclonal) and anti-p53 (AP6266b, rabbit polyclonal) from ABGENT (250 µg/ml; San Diego, CA) were used for both western blot and immunohistochemistry (IHC).

4.2.4 Western blot analysis

Mammary gland tumor tissue was homogenized in 500 mL of lysis buffer (20 mM Tris pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 0.5% Triton X-100) with protease inhibitors at 1:1000 dilution (Sigma-Aldrich, Saint Louis, MO). Tissue was homogenized via the OMNI Bead Ruptor 24 at a speed of 5.65 m/s for 45 seconds, followed by centrifugation at 13,000 rpm for 30 minutes at 4˚C. Twenty-five µg of whole-cell extract was resolved by 10% SDS polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membrane (Midwest Scientific, Saint Louis, MO). Nitrocellulose membrane was blocked in 5% milk for an hour at room temperature and then incubated with monoclonal antibodies (1:1,000). Western blots were detected by enhanced chemiluminescence detection reagents (Pierce, Rockford, IL) and visualized by Fluorchem E imaging system (ProteinSimple, Santa Clara, CA).

4.2.5 Immunohistochemistry

Mammary carcinomas and organs were removed and fixed in a solution of 10% formaldehyde and embedded into paraffin prior to sectioning them onto slides at a 5 µm thickness. Paraffin sections (5 µm) were dried at 60°C for 25 minutes. Deparaffinization was performed with 100% xylene and 100%, 90%, 75%, 50% ethanol. Antigen retrieval was performed in 1× citrate buffer solution and steam for 20 minutes. Endogenous peroxidase was blocked using 3% hydrogen peroxide. Slides were then incubated overnight at room temperature with primary antibody (1:50 dilution). After washes in PBS, slides were successively incubated with biotinylated secondary antibodies (1:1,000) for 15 minutes. Slides were washed and

immunostains were amplified by incubation with Avidin Biotin Complex (ABC) for 10 minutes accordingly. Cells were visualized with 3,3-diaminobenzidine (DAB) followed by a hematoxylin counterstain. The sections were viewed and the images captured with a Nikon 80i microscope under 40X and 60X magnification.

4.2.6 Statistical analysis

Significance was considered at a p-value ≤ 0.05 using Student's t-test analysis. All data are presented as mean ± 95% confidence interval of at least three independent experiments.

4.3 Results

Female PyVT transgenic mice developed tumors as early as 4 weeks of age. All 10 mammary pads developed tumors with the maximum tumor burden achieved around 12 weeks of age. Tumor development was divided into 3 stages based on the extent of tumor size and the frequency of tumor formation. The Pre stage of tumor development began at 4-5 weeks of age, consisting of a pre-cancerous condition where no tumors were palpated and the mammary tissue appeared normal on gross observation. The Early stage of development was confined to 6-8 weeks of age, represented by the gross observation of 1-2 solid tumors within the mammary tissue. The Late stage was based on the presence of all 10 primary mammary tumors with secondary lung metastasis, which appeared after 10 weeks of age. Representative sections of the lung tissue were stained with hematoxylin and eosin (H&E) for histopathological review to determine the presence of metastases.

To confirm the PyVT tumors were of epithelial origin, immunohistochemistry was conducted at each stage of development with pan epithelium, pigment epithelium-derived factor, and E-cadherin (Figure 4.1). There was a strong positive staining for pan epithelium in the Pre and Early stages, while the Late stage showed weak staining. This was consistent for the

pigment epithelium-derived factor (PEDF) and E-cadherin. This is indicative of a transition in cellular phenotype, demonstrating the process of epithelial-mesenchymal transition (EMT) in this model.

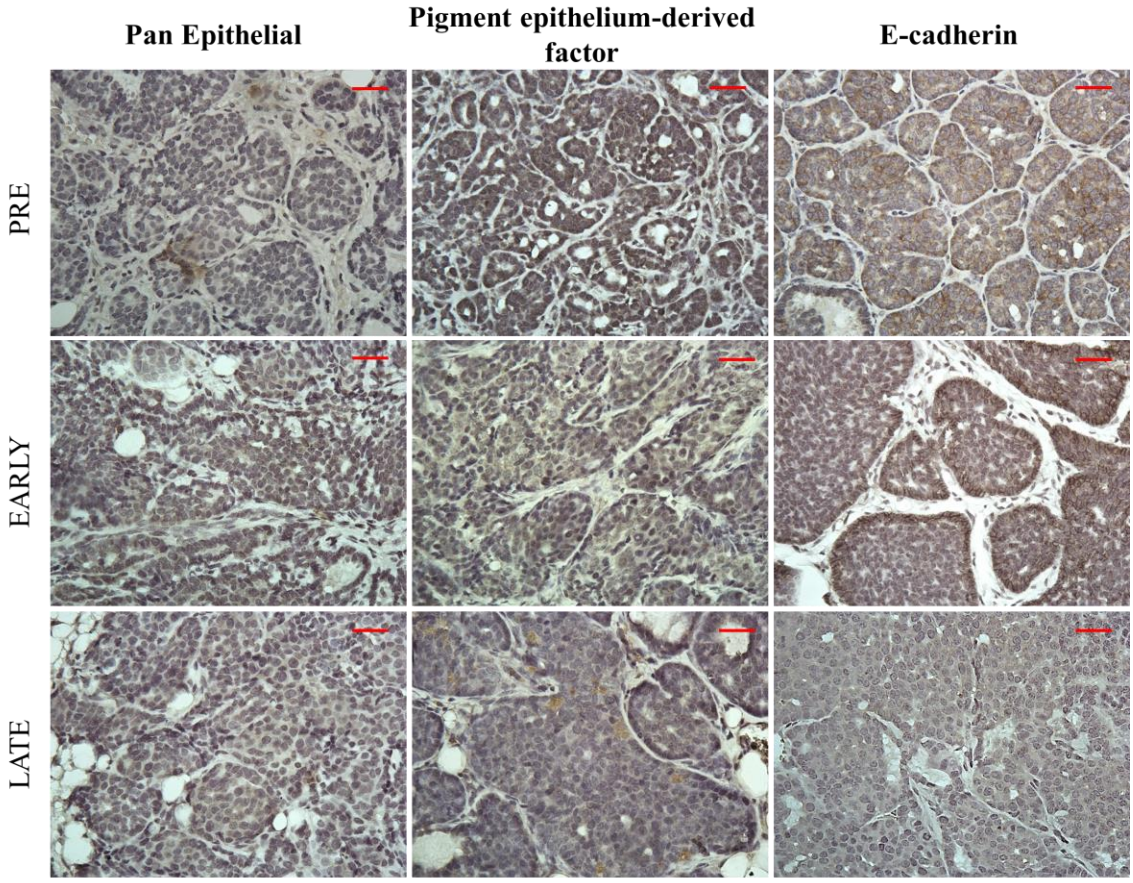

Figure 4.6 Immunohistochemisty of tumor epithelial phenotype from PyVT females during tumor development. Paraffin-embedded sections stained with antibodies against pan epithelial, pigment epithelium-derived factor, and E-cadherin at Pre, Early, and Late stages. Proteins staining: brown, counterstaining: blue (hematoxylin). Images represent only 1 of n = 3 per group at a 40X magnification. Scale bar = 50 μm.

Breast cancers are routinely assessed for hormone receptor status [estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2)] due to their relation to different subtypes and impact on prognosis, treatment, and overall survival. PyVT

tumors isolated from all stages of development were shown to be ER- and HER2-positive (Figure 4.2). Tumors from all three stages of development expressed detectable levels of PR. This was confirmed with Western blot analysis (data not shown). Estrogen receptor (ER), progesterone receptor (PR), and expression of human epidermal growth factor receptor 2 (HER-2) are recognized prognostic and predictive factors. ER found in 50-80% of breast cancers [6]. PR is a surrogate marker of a functional ER and is expressed in 60-70% invasive breast carcinomas with a higher positivity in older age and postmenopausal women [7]. HER-2/neu also known as C-erb B2 (HER-2), is a proto-oncogene located on chromosome 17 and the protein (HER-2) is overexpressed in 15-25% of invasive breast carcinoma with associated poor prognosis [8].

Tumors were shown to express p53 (Figure 4.2). P53 is an additional prognostic marker for inflammatory breast cancer, a more aggressive form than locally advanced breast cancer [9]. It is a sensor of cellular stress and master regulator of apoptotic programming [10]. The role of p53 protein is to maintain genomic stability as a multifunctional transcriptional regulator participating in the cell cycle [11]. In cells with active p53, it functions as a survival gene, and its loss sensitizes the cell to genotoxic stress [12]. Elevated levels of p53 expression have been associated with poor prognoses [13].

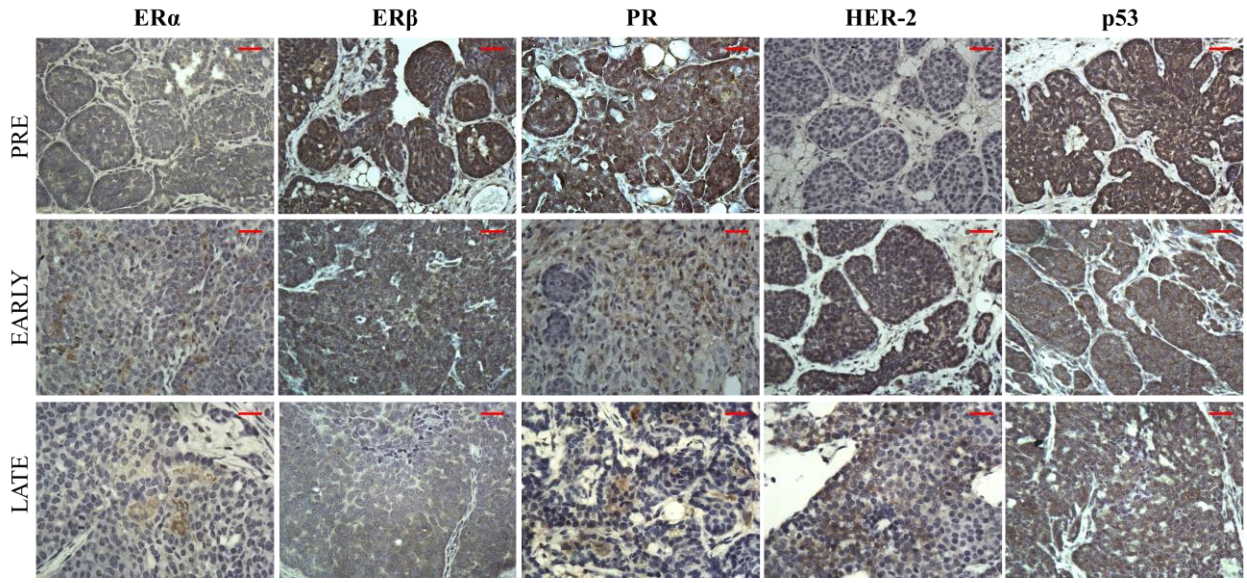

Figure 4.7 Immunohistochemisty of tumor phenotype from PyVT females during tumor development. Paraffin-embedded sections stained with antibodies against estrogen receptor (ERα and ERβ), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER-2), and tumor protein 53 (p53) at Pre, Early, and Late stages. Proteins staining: brown, counterstaining: blue (hematoxylin). Images represent only 1 of n = 6 per group at a 40X magnification. Scale bar = 50 µm.

Neoplastic cells have multiple survival techniques against death signals, such as the use of inhibitors of apoptosis. The anti-apoptotic protein survivin is a member of the inhibitors of apoptosis protein family expressed in a range of tumor types that regulates mitosis [14-16]. Survivin expression correlates with chemotherapeutic resistance and accelerated relapse [17]. Immunoblot analysis of tumor homogenate indicated that the expression of survivin increased as the tumor developed (Figure 4.3A). This was confirmed by immunohistochemistry (Figure 4.3C).

Cellular proliferation was determined by the expression of Ki-67, which is detectable during all activate phases of the cell cycle (G1, S, G2, and mitosis), but absent in the resting cell

(G0) [18]. Elevated Ki-67 expression is associated with increased breast cancer recurrence and poor patient survival [19]. Ki-67 is one of only five genes for proliferation, out of 16 cancer-associated genes, that contribute significantly to the Oncotype score [20]. Immunoblot and immunohistochemistry of Ki-67 on PyVT tumors showed an increase in expression levels as the tumor progressed to a malignant phenotype (Figure 4.3B). This was confirmed by immunohistochemistry (Figure 4.3C).

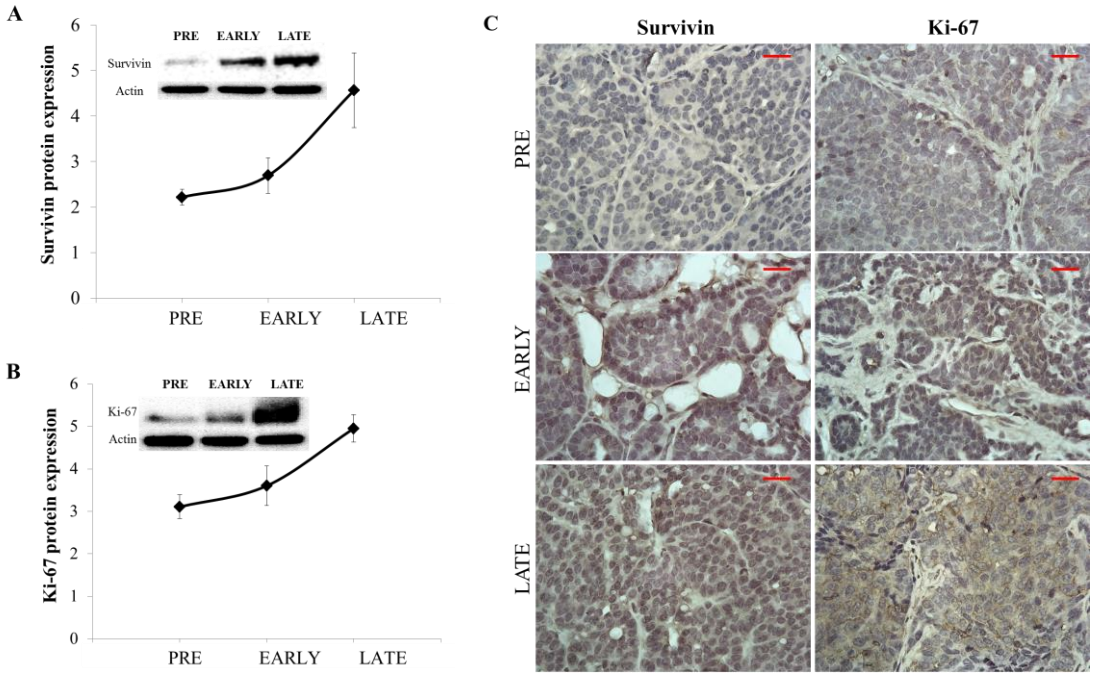

Figure 4.8 Expression of molecular markers in tumors from each stage of development. A) Raw and graphical representation of protein expression in tumors from Western blot analysis. Data presented as fold-pixel intensity of survivin in PyVT female tumors in each of the three stages of tumor development. n = 4. B) Raw and graphical representation of protein expression in tumors from Western blot analysis. Data presented as fold-pixel intensity of Ki-67 in PyVT female tumors in each of the three stages of tumor development. n = 4. C) Immunohistochemisty of tumors from PyVT females. Paraffin-embedded sections stained with antibodies against survivin and Ki-67 from PyVT females at Pre, Early, or Late stage of tumor

development. Proteins staining: brown, counterstaining: blue (hematoxylin). Images represent only 1 of n = 6 per group at a 60X magnification. Scale bar = 20 µm.

Histopathological examination of the mammary tumors was conducted for each treatment group in the three stages of tumor development. When present, tumors were categorized as adenoma/mammary intraepithelial neoplasia (MIN), early carcinoma, or late carcinoma (Figure 4.4). Adenoma/MIN involved expansion of acini and ducts by a proliferation of polygonal neoplastic epithelial cells with multifocal coalescence of the affected ducts and acini. Neoplastic cells exhibited minimal cellular atypia and a low mitotic index (averaged 1/400X field). The neoplastic proliferation was confined by the basement membrane and there was a lack of fibrous connective tissue within the neoplasm. Early carcinomas were unencapsulated and moderately well-demarcated, with closely packed nests and acini of neoplastic cells with mild to moderate cellular atypia and an average of 2 mitotic figures per high powered field. Neoplastic cells breached the basement membrane and were multifocally separated by a small to moderate amount of fibrovascular stroma. Late carcinomas were unencapsulated, poorly demarcated and invasive, composed of sheets of tightly packed nest and acini of neoplastic cells separated by moderate amounts of fibrovascular stroma. Anisocytosis and anisokaryosis were moderate and mitoses averaged 2/400X field. The Pre tumors were either adenoma/MIN or early carcinomas, while the Early tumors were all early carcinomas. The Late tumors were both late carcinomas.

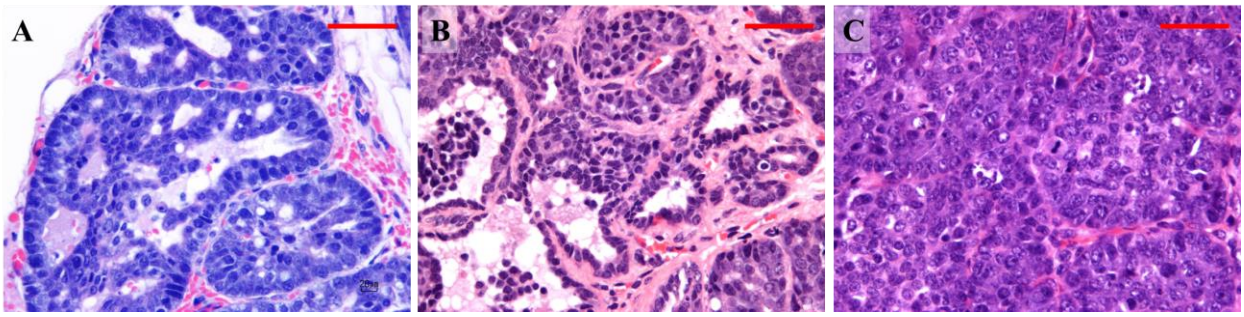

Figure 4.9 Pathological evaluation of hematoxylin and eosin (H&E) stained female PyVT mammary tumors. A) Adenoma/MIN: expansion of acini and ducts by a proliferation of polygonal neoplastic epithelial cells which exhibited minimal cellular atypia and a low mitotic index (averaged 1/400X field). The neoplastic proliferation was confined by the basement membrane and there was a lack of fibrous connective tissue within the neoplasm. B) Early Carcinoma: unencapsulated and moderately well-demarcated, with closely packed nests and acini of neoplastic cells with mild to moderate cellular atypia and 1-3 mitotic figures per high powered field. Neoplastic cells breached the basement membrane and were multifocally separated by a small to moderate amount of fibrovascular stroma. C) Late Carcinoma: unencapsulated, poorly demarcated and invasive, composed of sheets of tightly packed nest and acini of neoplastic cells separated by moderate amounts of fibrovascular stroma. Anisocytosis and anisokaryosis were moderate and mitoses averaged 2/400X field. Images represent only 1 of n = 3 per group at 40X magnification. Scale bar = 50 µm.

The mammary carcinoma developed by the PyVT transgenic model is characterized by a metastatic pattern involving the lung tissue. No lung metastasis was observed for the Pre stage of tumor development. Metastatic foci were not commonly found in the Early stage mice, but a few mice did develop small lesions by approximately 8 weeks of age (Figure 4.5A). Mice in the Late stage of tumor development formed secondary tumors in the lung epithelium, as determined by gross observation and histopathology (Figure 4.5B, 4.5C, and 4.5D). Lung tumors exhibited similar histopathologic features as those in the mammary tissue.

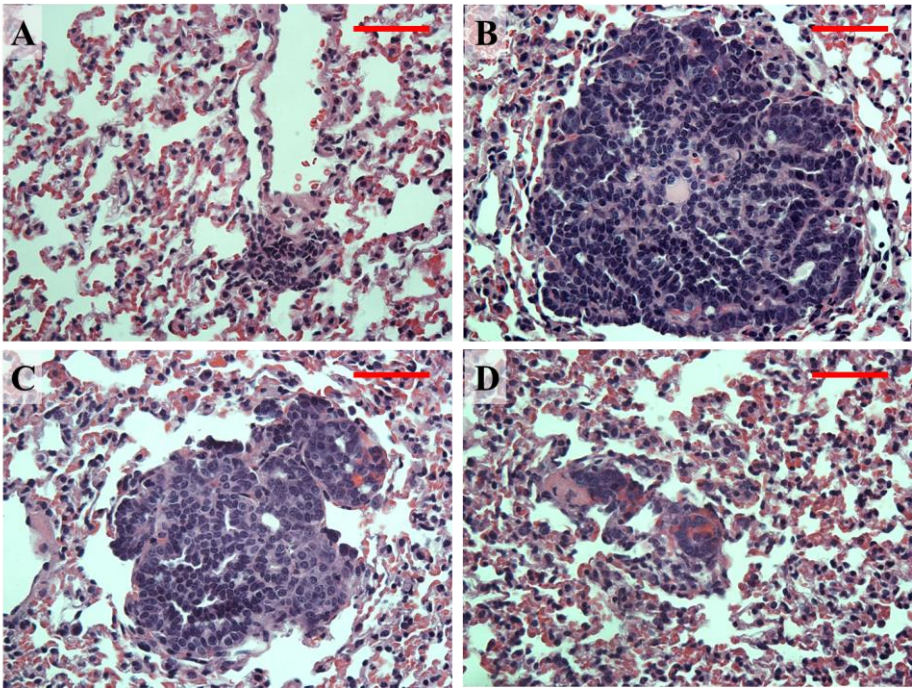

Figure 4.10 Representative images of hematoxylin and eosin (H&E) stained mammary tumors identified in the lung epithelium. Lung tissue collected from female PyVT mice at the A) Early and BCD) Late stage of tumor development. The lung contains metastatic foci of neoplastic cells. Additional morphologically similar foci were noted throughout the lungs. Images are representative of n = 3 at a magnification of 40X. Scale bar = 50 µm.

Tumor growth was monitored for two weeks during each stage of development. The initial tumor volume for Pre stage mice was 7.57 ± 10.77 mm^3 at 5 weeks of age. Two weeks after the initial measurement of the Pre stage mice, the tumors grew to an averaged total of 309.04 ± 16.63 mm^3 (Figure 4.6A). During the Early stage of development the initial tumor volume was measured at 6 weeks of age to be 133.33 ± 76.59 mm^3. By 8 weeks of age, 14 days post initial volume measurement, the Early stage mice developed an average total tumor volume of 450.71 ± 39.56 mm^3 (Figure 4.6B). The Late stage of tumor development began at 10 weeks of age, when mice had an initial tumor volume of 588.3 ± 78.87 mm^3. After 14 days post initial volume measurement, total tumor volume was 1727.21 ± 50.82 mm^3 (Figure 4.6C).

The effect of three antineoplastic compounds was tested on the development of tumors in the PyVT mouse model. Treatment with cisplatin or paclitaxel did not significantly attenuate tumor growth during the Pre, Early, or Late stage of development (Data not shown). However treatment with tamoxifen did significantly attenuated tumor growth during the Pre and Early stages of tumor development (Figure 4.6A and B). There was a significant difference in tumor volumes between tamoxifen and DMSO treated mice during the Pre stage of tumor development from day 10 to day 14 (Figure 4.6A). The change in tumor volume over this 14 day period showed a significant reduction of 124 mm^3 with tamoxifen treatment compared to control (*p*-values = 0.018). During the Early stage of development there was a significant difference in tumor volumes between tamoxifen and control groups at day 14 (Figure 4.6B). Tamoxifen significantly reduced tumor growth, leading to a final volume of 306 mm^3 after 14 days of treatment (*p*-value = 0.013). The average size of tumors after 14 days of tamoxifen treatment was 144 mm^3 smaller than the final control volume, which is a reduction of 31%. Tamoxifen treatment did not significantly reduced tumor growth during the Late stage of tumor development (Figure 4.6C). There was no observable change in the formation of metastatic lung lesions.

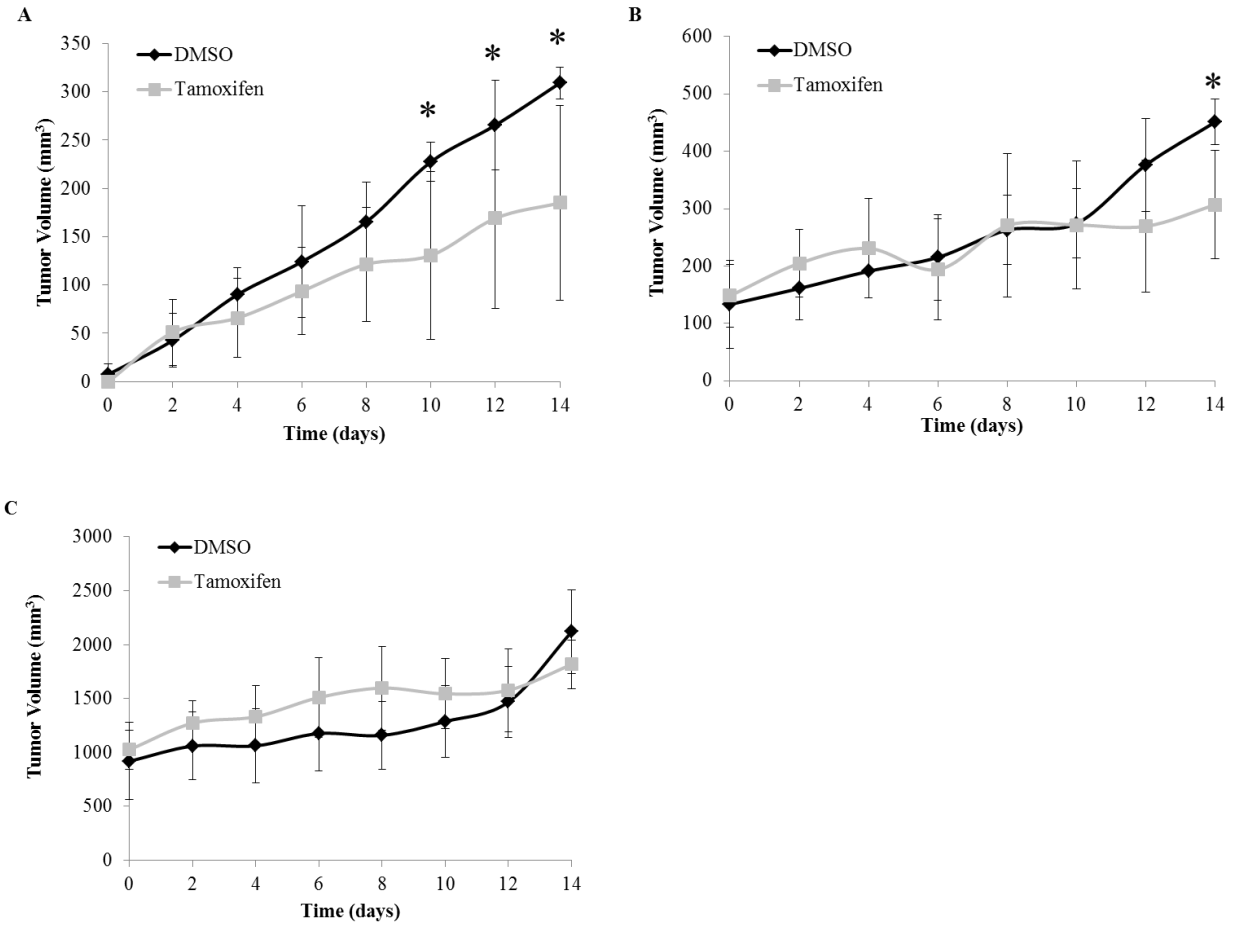

Figure 4.11 Tumor growth (mm^3) in PyVT female mice. Tumors measured in two dimensions with calipers every 2 days for Pre (A), Early (B), and Late (C) stages of tumor development. The tumor size is expressed over a 14 day period for treatment with tamoxifen (20mg/kg) of the vehicle control (DMSO) via 7 IPs. Days 0-12 represent the days of the 7 IP injections, day 14 represents the end of the study with measurements prior to tissue harvest. * *P*-value < 0.05 compared to controls.

4.4 Discussion

Throughout history, scientists have utilized a multitude of animal models to solve medical problems, develop new techniques and treatments, and cure disease. Genetically engineered mice are pursued in the cancer field, allowing researchers to investigate multiple

aspects of cancer. Many types of transgenic animal have been developed, most of which have been used in small numbers as tools for investigating gene function *in vivo*. Several characteristics of the PyVT model make it ideal for research, such as its colony stability, predictable tumor growth behavior, metastatic phenotype, and its clinical similarity to human neoplasms.

The process of EMT involves epithelial carcinoma cells acquisitioning mesenchymal markers, such as vimentin, for increased metastatic potential [21], as well as loss of epithelial cell adhesion molecules [21, 22]. Alteration in E-cadherin expression is the typical epithelial cell marker of EMT [23]; additionally loss of E-cadherin functionality promotes EMT [24]. The observed changes in epithelial marker expression in the isolated PyVT tumors are consistent with the EMT process. In addition the decrease in PEDF, a multifunctional secreted protein with anti-angiogenic and anti-tumorigenic functions, is associated with progression towards malignancy and poor patient outcome [25].

The mammary tumors isolated from PyVT mice were shown to be ER+, PR+, P53+, and HER-2+ via immunohistochemistry. This contradicts the results from Maglione *et al*. [5], which indicated that tumors identified as mammary intraepithelial neoplasia (MIN) had no detectable levels of PR antigen. Maglione *et al*. used 9 week old virgin females with the PyVT transgene; this is in the age range between the Early and Late stages of development defined above, in which a reduction in PR was observed as the tumors progressed towards malignancy. Here we used the PR (F-2) mouse monoclonal antibody raised against amino acids 375-564 of PR. It is recommended for detection of progesterone receptors A and B by immunohistochemistry with a starting dilution of 1:50 and provided at a concentration of 200 µg/ml PBS. Strong signal was observed in tissue samples using this antibody. Additionally, the expression of hormone

receptors tends to show a cyclic change during the estrous cycle in mice [26, 27]. This variation implies that the phase of the cycle, and thus the time of tissue harvest, may also affect the expression of PR in the mammary carcinomas collected.

Other studies on the PyVT mouse tumor phenotype indicate a loss of ERα and PR during tumor progression towards malignancy [4]. The results presented here support these conclusions. In addition ERβ is shown to also decrease with increased malignancy (Figure 4.2). The ER genes share a large proportion of homology, but differ in their distribution and functions in many tissue types. While ERα is a known prognostic marker in breast cancer, the role of ERβ is less clear. A decrease in ERβ protein expression has been shown in the transition of the normal mammary gland to a pre-invasive tumor [28]. The loss of ERβ protein in invasive carcinomas correlates with reported mRNA levels in breast tumors [29]. Overall, these results demonstrate for the first time a downregulation of ERβ during carcinogenesis in the PyVT model.

Interestingly the expression of ER and Ki-67 are mutually exclusive in normal premenopausal breast tissue, but co-expression occurs in ER positive breast cancer. With age the ER+ cell population increases, while the number of Ki-67+ cells decrease in the healthy mammary tissue [30]. The transformation of normal tissue to a neoplasia may reverse this phenotype. The results shown here indicate that ER+ cell decrease and Ki-67+ cells increase with tumor progression towards a malignant phenotype. The observed increase in Ki-67 expression corresponds to previous finding that cyclin D1, a regulator of the cell cycle also used as a molecular marker for proliferation, increases during tumor progression in the PyVT mouse model [4].

Strong expression of survivin is observed in the majority of cancer types, and is associated with tumor progression and chemoresistance [31]. Elevated survivin expression is associated with aggressive disease and has a strong correlation with poor patient outcome. The PyVT model has an increased expression of survivin with progression of the tumor phenotype towards malignancy. Survivin can regulate the cell cycle, cytokinesis, and apoptosis through multiple interactions, such as with heat shock protein 90 [32], Smac/Diablo [33], Cdk4 [34], and p53 [35]. The observable increase in survivin expression suggests it plays a role in tumor formation and development in the PyVT model.

The p53 protein is a tumor suppressor that prevents progression of the cell cycle and may induce apoptosis [36, 37]. The PyVT mouse mammary tumors were shown to be p53 positive at each stage of development. Interestingly, the survivin promoter sequence has two p53-binding sites [38]. Overexpression of the survivin protein saves neoplastic cells from p53-induced apoptosis [35]. The increase in survivin expression with tumor progression combined with a strong expression of p53 suggests that the cell is avoiding p53-dependent apoptosis through the upregulation of survivin.

Previous investigations on the PyVT transgenic model have been limited to studies focusing on gene function or histological characterization to prove clinical similarity to human breast cancer. This model has not been utilized to test the efficacy of antineoplastic compounds despite its multistage tumor development and similarities to human breast cancer. The compounds cisplatin, paclitaxel, and tamoxifen were chosen due their popularity in cancer treatment. Interestingly cisplatin and paclitaxel were not effective anticancer treatments, while tamoxifen successfully attenuated tumor formation in the PyVT model. The lack of response to cisplatin and paclitaxel suggest potential mechanisms of resistance, such as a reduction in the

accumulation of compound insides the neoplastic cells due to membrane barriers or increased efflux, faster repair mechanism, or modulation of the apoptotic pathways.

There is a pressing need for effective and low-toxicity chemotherapeutics and chemopreventive agents against mammary carcinomas. Here the inhibitory effect of tamoxifen is demonstrated on neoplasm development in the PyVT model. Tamoxifen has been extensively studied, but this is the first time it is shown to be effective in a multistage mammary carcinoma model with lung metastasis. Exposure to tamoxifen prior to appearance of palpable mammary tumors significantly reduced tumor burden and attenuated tumor growth. However when tamoxifen exposure began after the gross appearance of mammary tumors, it was less effective at attenuating tumor growth and metastasis, without changing primary tumor burden. The means by which tamoxifen suppressed tumor development may be associated with the MMTV promoter sequence in this transgene-induced model. The MMTV promoter sequence contains a hormone-responsive element (HRE) that can bind progestin, glucocorticoid and androgen receptors. Hormone that has bound to its receptor can enter the nucleus where the complex can bind the HRE and stimulate the MMTV sequence. MMTV is activated by high levels of estrogen and progestin, upregulating expression of the target genes and inducing cancer formation [39]. Exogenous estradiol and progesterone treatment were shown to increase MMTV mRNA expression [40]. Therefore by treating with tamoxifen, hormone is unable to bind the ER, translocate to the nucleus, and bind the HRE in the MMTV promoter sequence. This prevents promoter activation and reduces the expression of the PyVT antigen.

Tamoxifen was shown to be effective only during the Pre and Early stages of tumor development. Previous research showed that the tumors had variable expression of the PyVT antigen, indicating an importance of the transgene expression in initiation of tumor formation but

not in the perpetuation of a malignant phenotype [5]. This suggests that during the Pre and Early stages of tumor development, when the transgene is more highly expressed, tamoxifen may be preventing MMTV activation and subsequent PyVT antigen expression. Additionally the finding that tamoxifen efficiently suppressed the spontaneous mammary tumor development at these stages provides evidence that this compound may be beneficially utilized as a chemopreventive and chemotherapeutic in early intervention to reduce morbidity and mortality associated with this neoplastic disease.

The long terminal repeat (LTR) region of the MMTV promoter has previously been shown not to be down-modulated by the antiestrogenic effects of tamoxifen [41], but this study was conducted in the T47D human breast cancer cell line stably transfected with the LTR of the MMTV. There is not data indicating systemic estrogen concentrations or tamoxifen treatment in the transgenic mouse model does not alter promoter activation. Multiple studies conducted on transgenic mice using the MMTV promoter sequence do show anticancer effects with the use of tamoxifen [42-45], though they do not indicate if the promoter sequence plays a role in the compound's efficacy.

This study looks at the transgenic PyVT mouse as a model for breast cancer research and drug development. Tumor growth was monitored from a precancerous stage to a metastatic stage. The tumor phenotype was determined for three distinct periods of development emphasizing a change in cellular phenotype (i.e. EMT) and hormone receptor expression, proliferative shift, survival techniques, and a detailed histopathological analysis of mammary carcinoma lesions. For the first time this model was used to determine the efficacy of three common antineoplastic compounds in the development of spontaneous mammary carcinomas. Tamoxifen can attenuate the formation of breast carcinoma if given early enough to inhibit

transformation of the normal cell. The mechanism of tamoxifen chemoprevention may involve a reduction of the number of total cells transformed by the MMTV-PyVT transgene. These data suggest that tamoxifen, not cisplatin or paclitaxel, may improve the clinical outcome in patients prior to diagnosis of metastatic disease, and could potentially reduce morbidity and mortality associated with breast cancer.

4.5 References

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Chapter 5 - The anticancer effect of PQ1 in the PyVT mouse model

A research article of the following findings has been published in the International Journal of Cancer (2013) doi: 10.1002/ijc.28461.

5.1 Introduction

Cancer treatments using chemotherapies that target proliferating cells for destruction lead to severe side effects due to the lack of specificity for cancer cells. One characteristic of cancer cells is the loss of gap junction intercellular communication (GJIC). GJIC maintains tissue homeostasis through the sharing of small metabolites via channels in the cellular membrane of neighboring cells. Loewenstein and Kanno first reported a lack of electrical coupling in rat hepatomas in 1966 [1]. This was observed in chemically-induced and transplanted hepatomas [1, 2], which differed significantly from the normally well-coupled liver cells. The lack of electrical coupling soon became a common characteristic found in solid tumors, whether chemically-induced, transplanted, or spontaneously formed. Over 40 years, research has confirmed that a deficiency in gap junctions and thus GJIC is associated with the cancer phenotype [3, 4]. Many tumor promoting agents have been shown to inhibit GJIC [5], such as phenobarbital [6]. This reinforced the hypothesis that a deficiency of GJIC leads to tumorigenesis.

Treatments that target gap junctions provide specificity for the neoplastic cells, while the restoration of GJIC could effectively attenuate tumor growth with less detrimental effects to the host. PQ1 was found to possess potent inhibitory activities against T47D breast cancer cells through the enhancement of GJIC [7, 8]. This compound is capable of increasing GJIC in cancer cells and has anti-cancer properties. Previous studies indicate that administration of PQ1 via oral gavage has a low toxicity to normal tissue with no observable adverse effects [9], while significantly attenuating tumor growth [10].

This study focuses on utilizing PQ1 as a treatment for mammary carcinoma in the PyVT spontaneous mouse model. We have taken advantage of the *in situ* generation of mammary tumors in transgenic mice to determine the biological and histological effects of PQ1 on spontaneous tumorigenesis and metastasis. Development of tumors was divided into three time periods: Pre-tumor (4 weeks old), Early tumor stage (6 to 8 weeks old) and Late tumor stage (more than 10 weeks old). For each stage, treated and control groups were evaluated. The results demonstrated a significant reduction of tumor growth in the PQ1 treated mice compared to control with DMSO treatment or without treatment. Furthermore, immunoblot and immunohistochemical studies showed an increase in the expression of connexin in the treated tumors. For the first time, the change of connexin expression has been shown to correlate with tumor growth in a spontaneous mammary carcinoma animal model.

5.2 Materials and methods

5.2.1 Compounds

PQ1, 6-methoxy-8-[(3-aminopropyl)amino]-4-methyl-5-(3-trifluoromethyl-phenyloxy) quinolone, was synthesized by Dr. Duy H. Hua's laboratory [7].

5.2.2 Animals

A colony of PyVT transgenic mice (The Jackson Laboratory; Bar Harbor, ME) was maintained. Genomic DNA was extracted from a 1.5-cm tail clipping to identify transgenic progeny. Tumor development of positive female mice was closely monitored. Tumor onset was recorded as the age of the animal at which palpable abnormal masses were detected. Tumor volume was measured in two dimensions with calipers every 2 days starting at 4 weeks of age. Tumor volume was determined by the equation: Volume = ½(Length)*(Width)^2. Mice were observed for any change in behavior, appearance or weight. When animals reached the specific

age range, six female mice were randomly assigned to each treatment group and administered either DMSO (vehicle control) or 25 mg/kg PQ1 via intraperitoneal (IP) injection.

5.2.3 Antibodies

Primary antibodies: Anti-Cx46 (sc-20859, goat polyclonal), anti-PKCα (sc-8393, mouse monoclonal), anti-Ki-67 (sc-23900, mouse monoclonal), anti-survivin (sc-374616, mouse monoclonal), and anti-Cx43 (sc-13558, mouse monoclonal), from Santa Cruz Biotechnology (200 µg/ml; Santa Cruz, CA); anti-GAPDH (2118, rabbit monoclonal; 24 µg/ml) from Cell Signaling (Boston, MA); anti-p53 (AP6266b, rabbit polyclonal) from ABGENT (250 µg/ml; San Diego, CA) were used for both western blot and immunohistochemistry (IHC).

5.2.4 Western blot analysis

Mammary gland tumor tissue and selected organs (brain, heart, liver and kidney) were homogenized in 500 mL of lysis buffer (20 mM Tris pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 0.5% Triton X-100) at 1:1,000 dilution of protease inhibitors (Sigma-Aldrich, Saint Louis, MO). Tissue was homogenized via the OMNI Bead Ruptor 24 at a speed of 5.65 m/s for 45 seconds, followed by centrifugation at 13,000 rpm for 30 minutes at 4˚C. Twenty-five µg of whole-cell extract was resolved by 10% SDS polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membrane (Midwest Scientific, Saint Louis, MO). Nitrocellulose membrane was blocked in 5% milk for an hour at room temperature and then incubated with monoclonal antibodies (1:1,000). Western blots were detected by enhanced chemiluminescence detection reagents (Pierce, Rockford, IL) and visualized by Fluorchem E imaging system (ProteinSimple, Santa Clara, CA).

5.2.5 Immunohistochemistry

Mammary carcinomas and organs were removed and fixed in a solution of 10% formaldehyde and embedded into paraffin prior to sectioning them onto slides at a 5 µm thickness. Paraffin sections (5 µm) were dried at 60°C for 25 minutes. Deparaffinization was performed with 100% xylene and 100%, 90%, 75%, 50% ethanol. Antigen retrieval was performed in 1× citrate buffer solution and steam for 20 minutes. Endogenous peroxidase was blocked using 3% hydrogen peroxide. Slides were then incubated overnight at room temperature with antibody (1:50 dilution). After washes in PBS, slides were successively incubated with biotinylated secondary antibodies (1:1,000) for 15 minutes. Slides were washed and immunostains were amplified by incubation with Avidin Biotin Complex (ABC) for 10 minutes accordingly. Cells were visualized with 3,3-diaminobenzidine (DAB) followed by a hematoxylin counterstain. The sections were viewed and the images captured with a Nikon 80i microscope under 40X and 60X magnification.

5.2.6 Statistical analysis

Significance was considered at a p-value ≤ 0.05 using Student's t-test analysis. All data are presented as mean ± 95% confidence interval of at least three independent experiments.

5.3 Results

Tumor development was divided into the 3 stages based on the tumor size and the frequency of tumor formation: Pre, Early, and Late. Tumor growth over a 14 day period with 7 IP injections of either control DMSO or PQ1 indicated a significant effect of PQ1 treatment on neoplastic development at all three stages of tumor development in female PyVT mice (Figure 5.1). There was no significant difference between the controls (no treatment group and DMSO treatment group). The initial tumor volume for all Pre stage mice was 14.74 ± 11 mm^3. There

was a significant difference in tumor volumes between PQ1 and DMSO treated mice during the Pre stage of tumor development from day 4 to day 14 (Figure 5.1A). PQ1 significantly decreased the initial tumor size (day 0) with PQ1 treatment to 6.4 mm^3 over the 14 day treatment period (*p*-value = 0.046). The final tumor growth of the control DMSO treated mice was 377 mm^3. The change in tumor volume over the 14 day period showed a significant reduction of 40 mm^3 with PQ treatment compared to both controls (*p*-values < 0.0001; Figure 5.1B). There was a 56% reduction from initial tumor size in the Pre stage mice after treatment with PQ1.

The initial tumor volume for all Early stage mice was 129 ± 49 mm^3. During this stage of development there was a significant difference in tumor volumes between treatment groups from day 8 to day 14 (Figure 5.1C). PQ1 significantly decreased the initial tumor size to a final volume of 72 mm^3 after 14 days of treatment (*p*-value = 0.0002), while the final volume for the control DMSO treated group was 410 mm^3. The average size of tumors after 14 days of PQ1 treatment was 57 mm^3 smaller than the initial volume, which is a significant reduction of 44% from initial tumor size compared to the controls (*p*-value < 0.0001; Figure 5.1D). PQ1 treatment during the Early stage of development resulted in a 53% and 56% reduction from initial tumor volume compared to the no treatment and DMSO controls, respectively.

During the Late stage of tumor development, mice began treatment with the initial tumor volume of 610 ± 104 mm^3. PQ1 attenuated tumor growth compared to control, indicated by a final volume of 1727 mm^3 for DMSO control and 968 mm^3 after PQ1 treatment over 14 days (*p*-value = 0.0001; Figure 5.1E). PQ1 treatment lead to a significant reduction in tumor growth compared to controls ($p\text{-value}_{\text{No Trt}}$ = 0.001, $p\text{-value}_{\text{DMSO}}$ = 0.016); the controls increased by 1463 and 1008 mm^3 for no treatment and DMSO, respectively, while PQ1 treated tumors grew

only 382 mm^3 (Figure 5.1F). This was a 26% and 38% reduction in tumor growth with PQ1 treatment compared to control no treatment and DMSO, respectively.

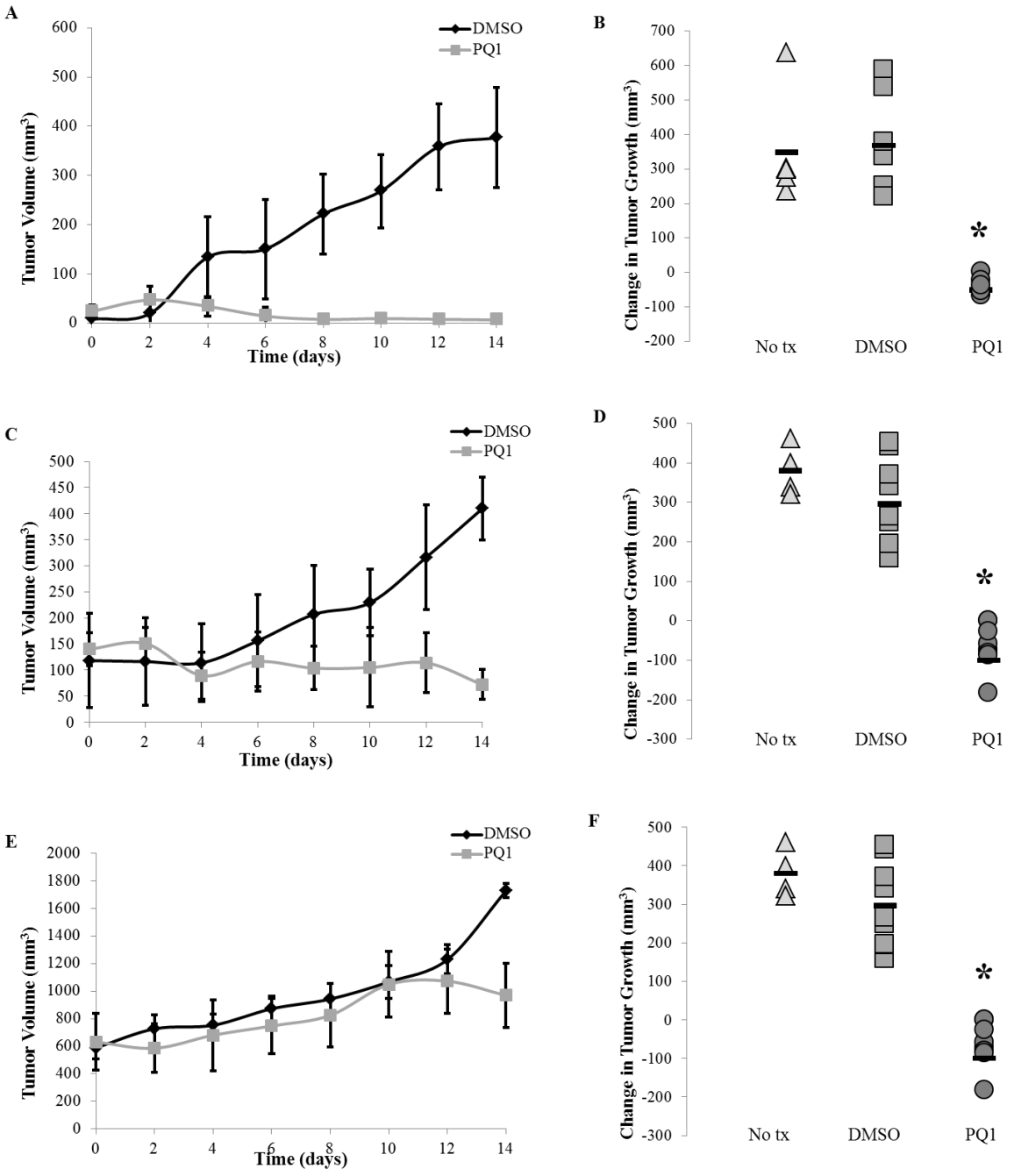

Figure 5.1 Tumor growth (mm^3) in PyVT female mice. Tumors measured in two dimensions with calipers every 2 days prior to administration of treatment for AB) Pre, CD) Early, and EF)

Late stages of tumor development. A, C, E) The tumor size is expressed over the 14 day treatment period for the DMSO (control) and PQ1 (25 mg/kg) treated PyVT mice. Days 0-12 represent the days of the 7 IP injections, day 14 represents the end of the study with measurements prior to tissue harvest. Data points represent the mean ± the 95% confidence interval. B, D, F) The overall change in tumor size after no treatment, or treatment with DMSO (control) or PQ1 (25 mg/kg) via 7 IPs. Data points represent the individual sample per treatment group, while the mean is represented as a horizontal bar. n = 6. * *P*-value < 0.05 compared to control no treatment and DMSO.

PyVT mice have a total of 10 mammary fat pads that may develop tumors during their lifetime. The tumor burden was monitored during the course of treatment, and the final tumor number for each treatment in each stage of development is represented by Figure 5.2. During all stages there was no significant difference between the tumor burdens of the control groups. Treatment with PQ1 during the Pre stage significantly reduced the number of tumors developed after treatment (*p*-value = 0.0002; Figure 5.2A). During the Early stage of tumor formation, PQ1 treatment significantly reduced tumor burden compared to the control DMSO group (*p*-value = 0.02; Figure 5.2B). The no treatment control group for this stage had a greater variation in the number of tumors developed than the control DMSO treated group, though it was not significantly different. There was no difference in the tumor burden between experimental groups of the Late stage of tumor development (Figure 5.2C).

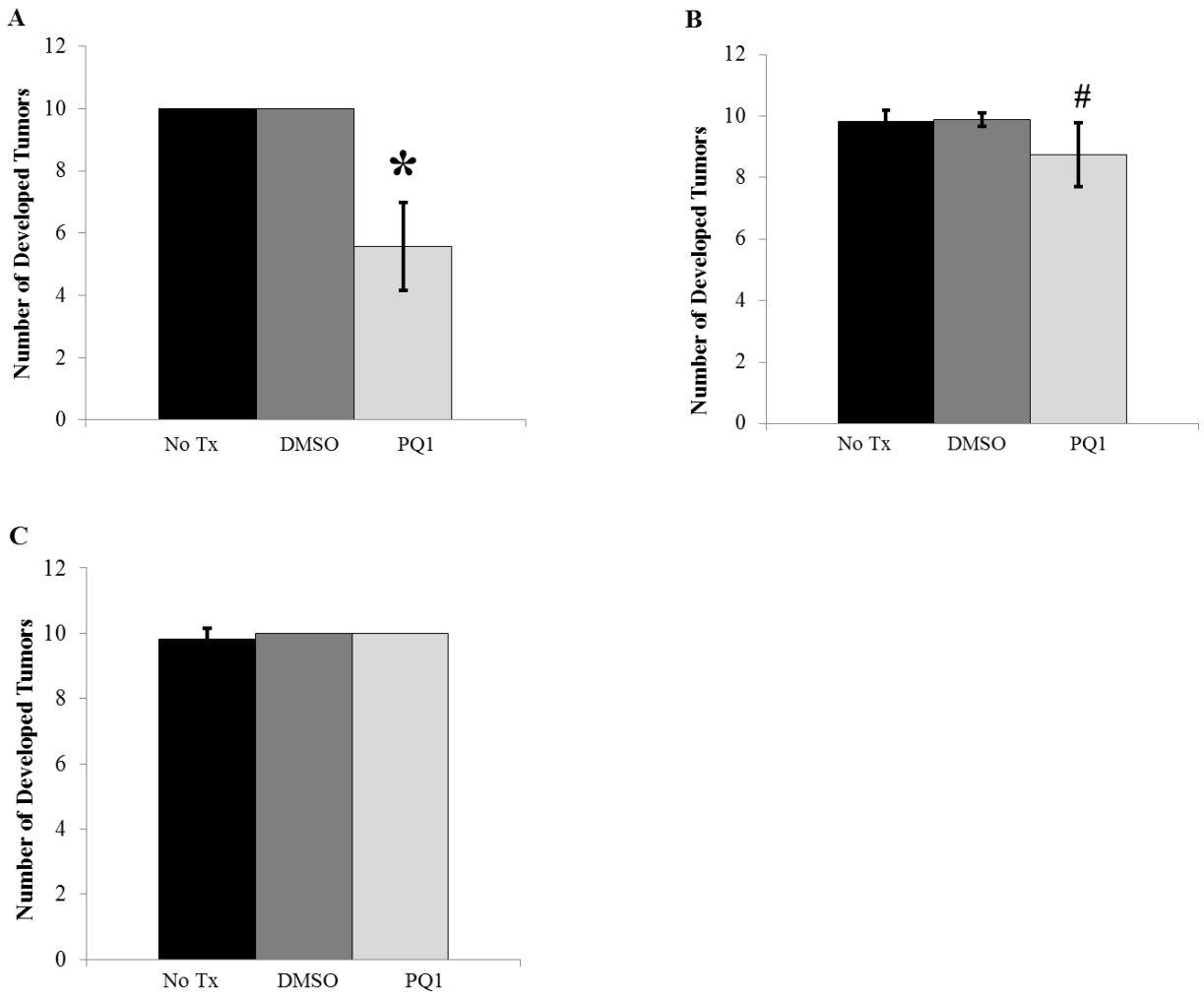

Figure 5.2 Number of developed tumors in PyVT female mice during development. Tumors identified grossly during the A) Pre, B) Early, and C) Late stages of tumor development after a 14 day period with either no treatment, or treatment with DMSO (control) or PQ1 (25 mg/kg) via 7 IPs. n = 6. * *P*-value < 0.05 compared to control no treatment. Columns represent the mean ± the 95% confidence interval. # *P*-value < 0.05 compared to control (DMSO).

Histopathological examination was conducted of the mammary tumors for each treatment group in the three stages of tumor development. Tumors were categorized as adenoma/mammary intraepithelial neoplasia (MIN), early carcinoma, or late carcinoma. The Pre control tumors were either adenoma/MIN or early carcinomas, while the Pre PQ1 treated tumors appeared to be early

or late carcinoma. The Early control tumors were all early carcinomas. The Early PQ1 treated tumors varied from adenoma/MIN, early carcinoma, and late carcinoma. No lung metastasis was observed for the Pre or Early stages of development for either treatment group. The Late control and PQ1 tumors were both late carcinomas. The Late control mice had several metastatic foci in the lung, while Late PQ1 treatment resulting in less mice with metastatic lesions and fewer metastatic foci per mouse.

Immunoblot analysis of connexin expression indicates that PQ1 treatment decreased Cx46 expression (Figure 5.3A) and increased Cx43 expression (Figure 5.3B) during carcinogenesis. During the control PyVT mouse tumor development there was an increase in Cx43 and Cx46 expression from Pre to Late stage. Data suggests that connexin 43 is expressed at higher levels in PQ1 treated animals compared to controls and the contrary for connexin 46. Cx46 expression in PyVT mouse tumors treated with PQ1 from the Pre and Late stages of development had significantly lower levels than that of the controls (p-value $_{Pre}$= 0.019, p-value $_{Early}$= 0.007). Pre and Early stage tumors treated with PQ1 had a significantly greater level of Cx43 expression compared to controls (p-value $_{Pre}$= 0.0003, p-value $_{Early}$= 0.03). During the Late stage of tumor formation, connexin 46 was expressed less in treated mice than controls, while there was no significant change in connexin 43 expression. This is explained by the overall increase in both connexin 43 and connexin 46 during tumor development and metastasis.

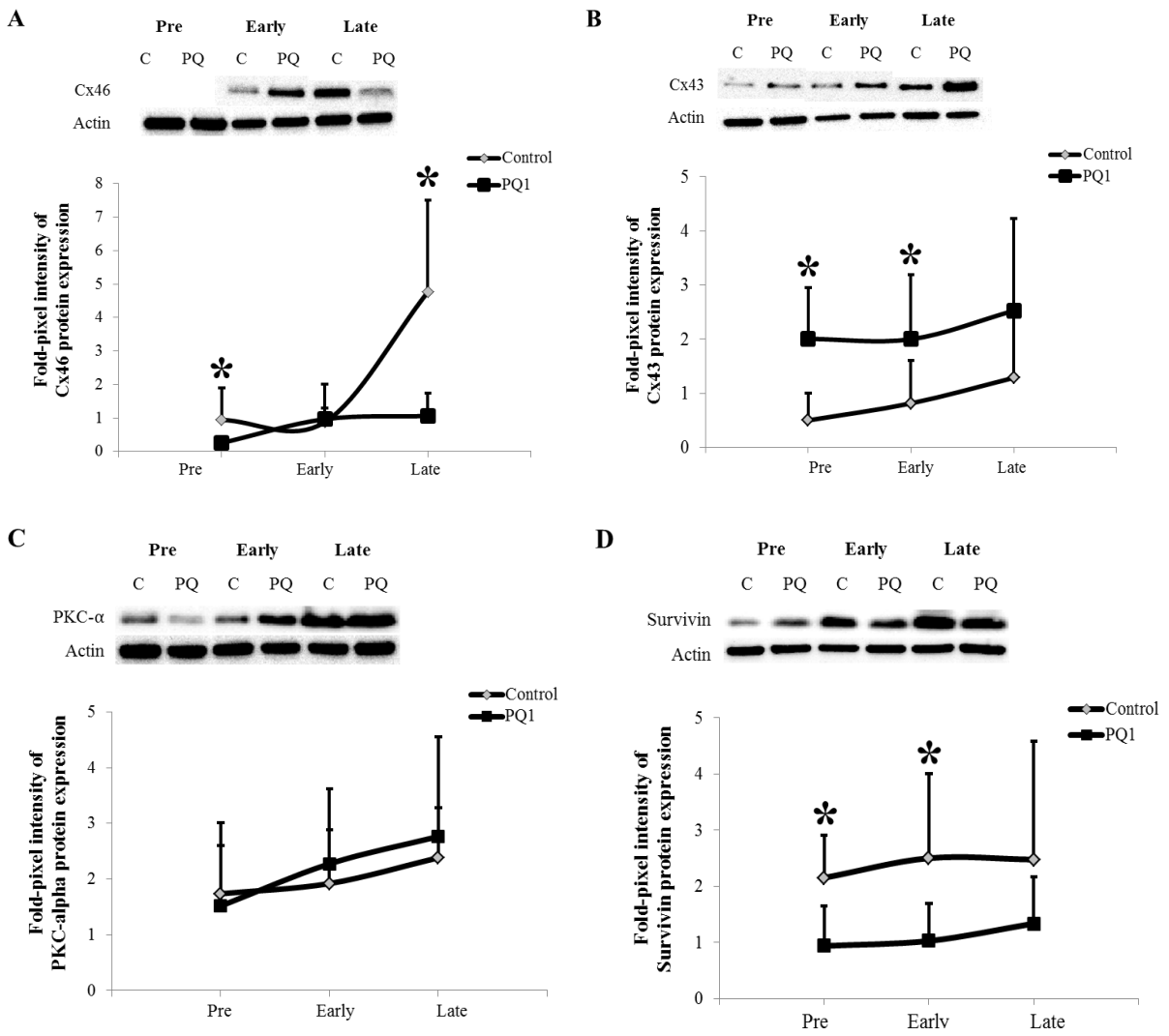

Figure 5.3 Raw data and graphical representation of protein expression in tumors from Western blot analysis. Fold-pixel intensity of A) Cx46, B) Cx43, C) PKC-alpha, and D) survivin in PyVT female tumors treated with DMSO (control) or PQ1 (25 mg/kg) via 7 IPs in each of the three stages of tumor development. Data points represent the mean ± the 95% confidence interval. n = 4. * *P*-value < 0.05 compared to control.

Connexin expression is regulated by a number of factors, such as transmembrane and transjunctional voltage, cytosolic ions (Ca^{2+} and H^{+}), and post-translational modifications, predominantly the phosphorylation status [11-13]. All connexins, except Cx26, are phosphoproteins that are targeted by several kinases such as sarcoma (Src) kinases, protein

kinase C (PKC), protein kinase A (PKA) and Mitogen-Activated Protein Kinase (MAPK), which are required for efficient trafficking, assembly and disassembly, degradation, and gating of hemichannels and/or gap junctions [12, 14, 15]. GJIC regulation by phosphorylation is both connexin and kinase specific [12, 16]. The role of PKCα in the PQ1 induced altered connexin expression was determined in tumors isolated from the PyVT mouse at each stage of development by western blot analysis (Figure 5.3C) and immunohistochemistry (Figure 5.4). There was no significant change in PKCα.

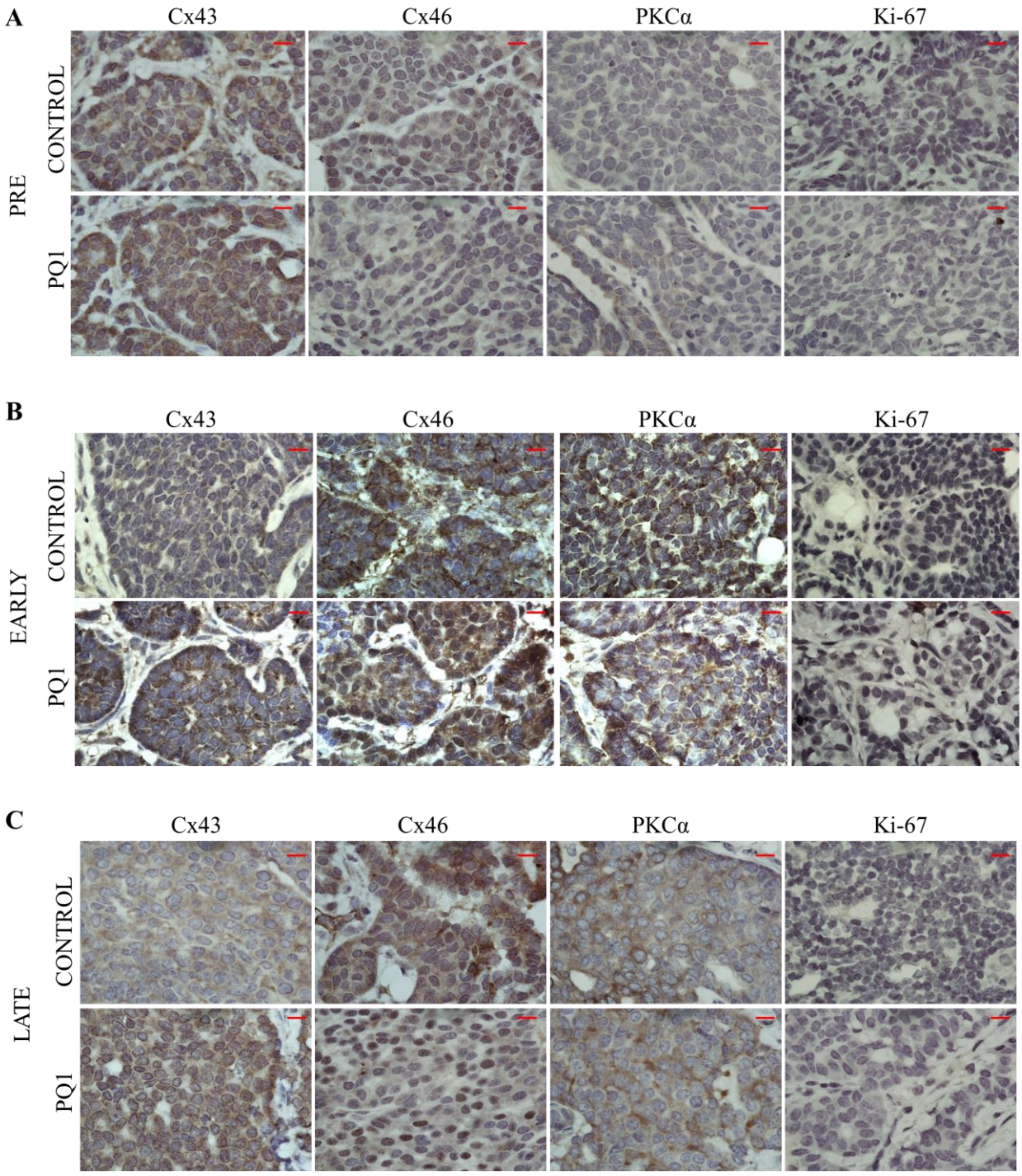

Figure 5.4 Immunohistochemisty of tumors from PyVT females. Paraffin-embedded sections stained with antibodies against Cx43, Cx46, PKCα, and Ki-67 from PyVT females treated with DMSO (control) or PQ1 (25 mg/kg) via 7 IPs at either A) Pre, B) Early, or C) Late stage of tumor development. Proteins staining: brown, counterstaining: blue (hematoxylin). Images represent only 1 of n = 6 per group at a 100X magnification. Scale bar = 10μm.

Neoplastic cells use inhibitors of apoptosis as protection against cytotoxic compounds. The anti-apoptotic protein survivin is a member of the inhibitors of apoptosis protein family expressed in a range of tumor types that regulates mitosis [17-19]. Survivin expression correlates with chemotherapeutic resistance and accelerated relapse [20]. PyVT tumors treated with DMSO or PQ1 at each stage of development were analyzed for the expression of the anti-apoptotic protein survivin. PQ1 treatment significantly decreased the expression of survivin in mammary tumors during the Pre (p-value = 0.014) and Early (p-value = 0.028) stages of development (Figure 5.3D).

Histopathology of the harvested PyVT mouse tumors showed no significant difference in morphology. Immunohistochemistry of PQ1 treatment at all stages of tumor formation showed stronger positive cytoplasmic staining in Cx43 and a weaker positive cytoplasmic staining of Cx46 compared to control tumors (Figure 5.4). There was no significant change in Ki-67 expression.

The mammary carcinoma developed by the PyVT transgenic model is characterized by a metastatic pattern involving the lung tissue. Mice in the Late stage of tumor development had many large secondary tumors in the lung epithelium, as determined by gross observation and histopathology. With PQ1 treatment, there was a decrease in the size and frequency of these secondary lung tumors (Figure 5.5). Lung tumors exhibited similar histopathologic features as those in the mammary tissue.

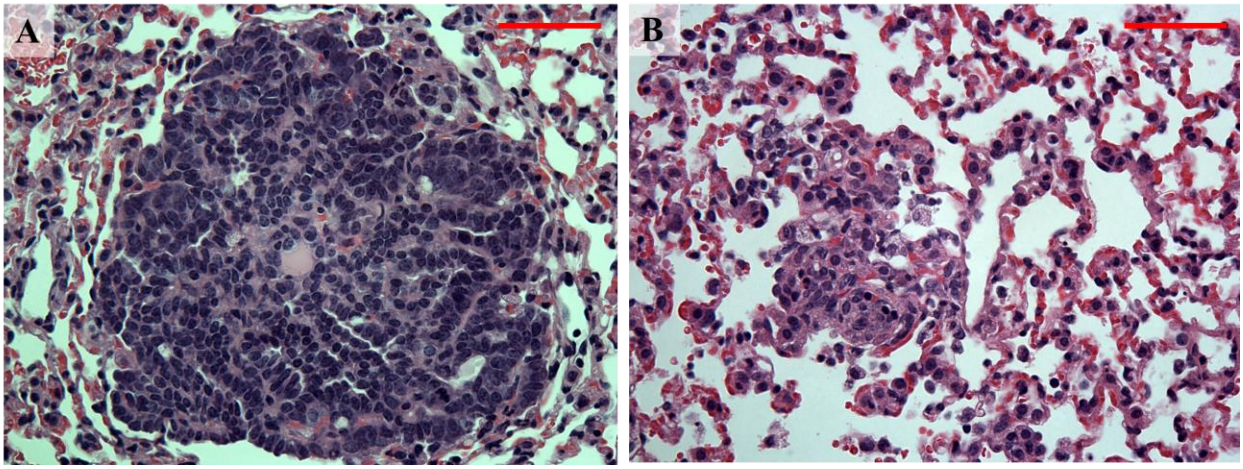

Figure 5.5 Representative images of hematoxylin and eosin (H&E) stained mammary tumors identified in the lung epithelium. A) Female PyVT mouse treated with DMSO (control) at Late stage of tumor development. The lung contains a large metastatic focus of neoplastic cells. Additional morphologically similar foci were noted throughout the lungs. B) Female PyVT mouse treated with PQ1 at Late stage of tumor development. The lung contains small metastatic foci of neoplastic cells. However, the foci are of decreased size and frequency, as compared to the control animals. Images are representative of n = 3 at a magnification of 40X. Scale bar = 50μm.

Multiple vital organs (brain, heart, liver and kidney) were also examined using histopathology to determine any potentially detrimental effects of PQ1 administration. There was no morphological change in the tissues compared to control, indicating that PQ1 had specificity for targeting neoplastic cells rather than the untreated tissue (Figure 5.6A). Further examination of Cx43, Cx46, PKCα, and Ki-67 in the liver showed a strong positive cytoplasmic staining of Cx43, Cx46, and PKCα in PQ1 treated tissue (Figure 5.6B) from immunohistochemistry. There was no significant change in Ki-67. Systemic exposure to PQ1 over the 14 day period had no observed adverse effects on the health or behavior of the animals.

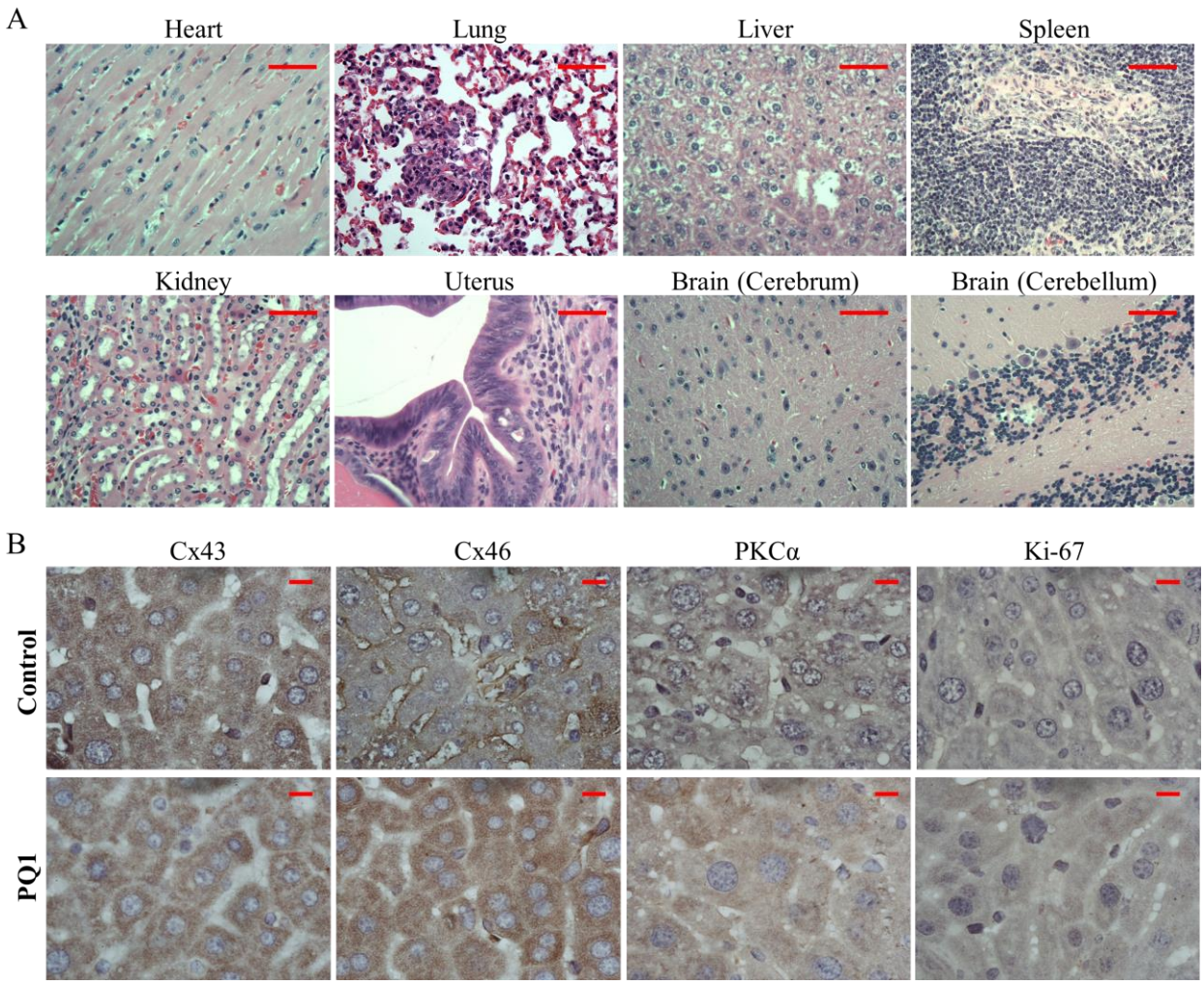

Figure 5.6 Evaluation of normal tissue isolated from PyVT mice. A) Representative images of hematoxylin and eosin (H&E) stained tissues from untreated PyVT mice in the Late stage of tumor development. Images are representative of n = 3 at a magnification of 40X. Scale bar = 50µm. B) Immunohistochemistry of Liver isolated from PyVT mice. Paraffin-embedded sections stained with antibodies against connexins (Cx43 and 46), PKCα, and Ki-67 in female PyVT mouse liver harvested after 7 IP injections treated with either DMSO (control) or PQ1 (25 mg/kg) via 7 IPs during the Late stage tumor development. Proteins staining: brown, counterstaining: blue (hematoxylin). Images represent only 1 of n = 6 per group at a 100X magnification. Scale bar = 10 µm.

5.4 Discussion

PQ1 is the first compound targeting gap junctions as an anticancer drug in a spontaneous mammary tumor model. It has previously been shown to be a specific enhancer of GJIC *in vitro* [8] and resulted in an increase in connexin expression leading to a significant decrease in tumor growth on a T47D human breast cancer cell xenograft model in nude mice [21]. This study translates the *in vitro* results of PQ1 previously obtained to an *in vivo* context using a spontaneous mammary carcinoma model. Genetically engineered mice are pursued in the cancer field, allowing researchers to investigate multiple aspects of cancer. Several characteristics of the PyVT model make it ideal for studies, such as its colony stability, predictable tumor growth behavior, and its similarity to human neoplasms. The efficacy of PQ1 in reducing tumor cell growth is demonstrated in the PyVT mouse model over a two week period. The results showed a 43% and 53% reduction in tumor volume from the initial size in pre and early stages, respectively, while there was a 26% reduction from final tumor volume in the Late stage of development with PQ1 treatment compared to the DMSO control after seven injections of treatment.

Molecular analysis of the protein expression demonstrated a general increase of expression of Cx43 and a decrease for Cx46 in PQ1-treated PyVT mice. In the Pre stage of tumor development, there was an increase in Cx43 expression and a decrease in Cx46 expression with PQ1 treatment. PQ1 alters the overall connexin profile by upregulating Cx43 and downregulating Cx46. The general loss of GJIC correlates with tumorigenic phenotypes, but the different connexins play distinct roles in specific stages of cancer progression. It has been observed in former studies that the proportion of the connexins constitutive of gap junctions differed in tumor stages. Cx46 protein is expressed highly in both breast cancer cell lines and human breast tumors [22]. Knockdown of Cx46 in human breast tumor xenografts is shown to

inhibition tumor growth in nude mice [22]. This indicates that Cx46 is upregulated to promote tumor growth and development in early breast cancer tumors. Other gap junction connexins (Cx43, Cx32, and Cx26) are considered tumor suppressors since loss of these correlates with breast tumor progression [23].

Cx46 is a novel gap junction protein in mammary tissue that is hypoxia-specific. Burr *et al*. [24] hypothesized that Cx46 has pro-tumor effects due to its ability to prevent hypoxic death. Solid tumors may have an upregulation of Cx46 to survive the hypoxic conditions prevalent during late stage development while also inducing a loss of Cx43, which promotes tumor growth. Treatment with PQ1 alters the expression of both Cx43 and Cx46 in such a way that it normalizes the tumor tissue and prevents tumor growth. The increase in Cx43 could induce a downregulation of Cx46, sensitizing the tumor cells to hypoxic conditions and attenuate further tumor development. The advanced stages of solid tumor development typically consist of tumors with more hypoxic centers, making them difficult to treat due to the slower rate of cell proliferation. PQ1 could be utilized to restore the hypoxic cells to normal homeostasis and induce cellular death of unregulated cells.

The decrease in survivin protein expression in the neoplastic tissue due to PQ1 treatment found during the Pre and Early stages of tumor formation supports the hypothesis above. Survivin expression in normal tissue is developmentally regulated and is low in most terminally differentiated tissues. In cancer cells, elevated survivin is commonly associated with enhanced proliferative index [25], reduced levels of apoptosis [26], resistance to chemotherapy [27], and increased rate of tumor recurrence [28]. PQ1 treatment decreased survivin, suggesting that the tumor cells are sensitized to stressful conditions, such as hypoxia. Our data suggest that PQ1

may act as a survivin disruptor, independent from its gap junction function, to sensitize cancer cells.

The proliferation rates of the tumors have been evaluated in an attempt to correlated expression with prognosis. Ki-67 is one of several cell-cycle regulators that can be determined by immunohistochemistry. The Ki-67 antibody reacts with a nuclear non-histone protein that is highly expressed in all active phases of mitosis, except G_0 [29]. A high Ki-67 labeling index for immunohistochemistry is associated with poor differentiation of tumors, large tumor size, and an increased risk in recurrence [30]. There was no observable change in Ki-67 expression due to PQ1 treatment, indicating that PQ1 is not involved in the proliferation of neoplastic cells in the PyVT model.

As the PyVT tumor develops, there is an increase in Cx43 and Cx46 expression, which may be characteristic of an increase in the metastatic potential of the lesion. The metastatic tumor, represented by the Late stage, had a significantly greater expression of Cx46 compared to the other stages of development (Figure 4A), suggesting that this upregulation may contribute to tumorigenesis. With PQ1 treatment there is a decrease in the elevation of Cx46 during development, suggesting that the treatment is attenuating tumor formation and delaying the process of metastasis. This is supported by reduced incidence of secondary tumor formation in the lung with PQ1 treatment. Primary tumors that are initially GJIC impaired become GJIC competent during the metastatic stage[31]. Increased expression of connexins and GJIC correlate with invasiveness and metastasis in a variety of cancer cell types, including breast cancer. Connexin expression profiles change from a metastatic cell to that more similar to a normal breast epithelial cell with expression of metastasis-suppressor gene *BRMS1* [32]. This suggests that the connexin composition of gap junctions contributes to the lesions metastatic

potential. Though functionality of the gap junctions was not conducted, the data presented shows a high expression level of Cx43 and Cx46 during late tumor development and metastasis.

PKCα expression did not vary between treatment groups. PKCs are the principal kinases that regulates GJIC by phosphorylation of the C-terminal domain [12], which has been shown to be necessary for the Cx43 disassembly in lens epithelial cells [33]. Morley *et al*. [34] showed that PKC inhibition (PKCα and/or PKCβ1) increases GJIC to reduce the proliferation of tumor cells, which was due to altered trafficking of connexins. The data presented here show an alteration in connexin expression without a change in PKCα expression. This suggests that PQ1 affects connexin expression through a mechanism that either does not affect PKCα, but rather a different isoform of PKC; or does not involve phosphorylation of the C-terminal domain of connexins.

The MMTV promoter is a glucocorticoid hormone inducible promoter [35] that has been used as an ideal system for the generation of transgenic mice modeling tumor development in the breast and urogenital tissues due to its inducible and high activity in these tissues [36]. Pathological evaluation of the tumors post treatment lead to the observation that Pre stage PQ1 treated tumor were histologically described as early or late carcinomas, while control tumors were adenoma/MIN or early carcinoma, implying that treatment lead to an increase in malignancy. To clarify the results, only one of the six tumors evaluated in this treatment group was characterized as late carcinoma, which may be due to biological variation within the transgenic model. This increase in malignancy may be explained by different levels of transgene expression due to copy number variation, or different spatial or temporal expression of the transgenes in the tissue. An increase in incidence of tumors is common with higher expression of the transgene in the mammary tissue with hormonal effects on the MMTV [37]. Different

quantitative levels of expression are possible due to differences in transgene copy number, leading to a variation in tumor incidence and phenotype. Therefore the single tumor characterized as more aggressive by pathological evaluation may simply have higher levels of transgene copy number. Additionally the Pre stage of tumor development occurs in concurrence with reproductive development, in which relatively mature levels of circulating hormone are present. Murine sexual maturity normally coincides with rising circulating gonadotropin some point after 4 weeks of age. Precisely when reproductive maturity occurs is highly variable between mice. High levels of circulating hormone may affect the activation of the promoter sequence, leading to variation in tumorigenesis. Transgene expression levels were not determined for the tumor samples.

The effectiveness of many anticancer drugs is limited by their toxicity to normal rapidly growing cells and vital tissues. Administration through oral gavage of PQ1 indicated that exposure of the vital organs has low toxicity [9]. For this study the liver and kidney were examined histologically to determine potentially detrimental effects of systemic exposure to a gap junction enhancer. PQ1 treatment showed no observable detrimental effects to any of the organs. It is uncertain if the observed increase in Cx43 and Cx46 expression in the immunohistochemistry of the liver may induce damage. No acute toxicity was seen in this study. Long term survival studies after PQ1 treatment are needed to determine if the normal tissue is truly affected.

Cancer has been characterized as a disease of differentiation or stem cell disease [38]. Despite the heterogeneity within a single tumor, they have all been derived from clonal expansion of a single cell. Stem cells are naturally immortal and become mortal when they differentiate [39]. The initiation process of carcinogenesis irreversibly inhibits the differentiation

of stem cells. Adult stem cells do not express connexins or have functional GJIC. Normal human breast epithelial stem cells [40], limbral progenitor cells [41], and mouse embryonic stem cells [42] have been shown to lack expression of connexin genes and functional GJIC. Gap junctions are needed to differentiate stem cells. Stem cell growth is controlled without functional GJIC through secreted negative growth regulators from either their terminally differentiated daughter cells [43] or from some stroma-derived secreted factor [44]. If stem cells are deficient in functional GJIC but are targeted by the initiation phase, then they would remain in a primitive, less differentiated phenotype leading to cancer formation. Chemoprevention of the promotion/ progression of the initiated stem cell would require either interference with those secreted factors that stimulate the growth of stem cells or induction of the connexin genes and functional GJIC. PQ1 is shown in previous studies [7, 8] and the above results to satisfy the later.

PQ1 is an enhancer of GJIC, which could lead to stem cell differentiation and prevention of cancer initiation. Here we show the attenuation in tumor formation with PQ1 treatment at the Pre stage. This may be explained by PQ1 enhancing GJIC in tumor stem cells, pluripotent and/or early progenitor cells. Introduction of GJIC by PQ1 could lead to differentiation of the pluripotent stem cell that has a normally low expression of connexins and deficient in GJIC, eliminating the potential for initiation. The early progenitor cell, which has stem-like potential and functional GJIC, if initiated is unable to terminally differentiate, but can still control its growth via GJIC. The initiated progenitor cell may then lose growth control during the promotion stage through the downregulation of GJIC, leading to proliferation, accumulation, promoter independence, invasion, and finally metastasis. PQ1 may have a role in preventing the loss of GJIC, maintaining growth control, and leading to apoptosis thereby preventing tumor formation. In vitro studies of adult mammary stem cells with PQ1 treatment are needed to

determine the mechanism of action and any adverse effects on the normal tissue. This is a new, promising approach to chemoprevention and chemotherapy.

Normal cells can signal adjacent malignant cells to reverse their phenotype or induce apoptosis [45], but the deficiency in connexin expression intervenes in the regulation of cell proliferation, differentiation, cell death, homeostatic maintenance and reduced mitosis in the late G1, S and M phases [46], suggesting an involvement in carcinogenesis [1]. A new class of gap junction enhancers alters tumor growth in a spontaneous mammary carcinoma animal model with no apparent side effects. This study shows additional evidence that connexin expression and cell growth are inversely correlated in malignant lesions, confirming the results of Saez *et al*. [47]. The previous studies on PQ1 conducted on T47D cells in nude mice, and these results obtained using a spontaneous mammary carcinoma model, are promising for the development of a new chemotherapy for breast cancer treatment, and potentially other cancer types, in humans. PQ1 may also be combined with other antineoplastic drugs to reduce their toxicity and increase efficacy.

5.5 References

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Chapter 4 -

Chapter 6 – Bioavailability and efficacy of a gap junction enhancer (PQ7) in a mouse mammary tumor model

A research article of the following findings has been published in PLoS ONE (2013) 8(6): e67174. doi:10.1371/journal.pone.0067174.

6.1 Introduction

Gap junction intercellular communication (GJIC) has an important function in maintaining tissue homeostasis. GJIC is the process in which small metabolites are shared directly by contiguous cells that have their cytoplasms connected by aqueous channels called gap junctions. The loss of GJIC is related to the pathogenesis of multiple diseases, such as deafness and hearing loss, cataracts, skin disorders, oculodentodigital dysplasia, and cancer (see review [1]). The enhancement or restoration of functional gap junctions could be therapeutic treatment option for these diseases. Here we focus on the pathogenesis of cancer in relation to the loss of connexin expression. A class of substituted quinolines was described in Shi *et al*. and the effects of the first generation compound (PQ1) as a gap junction enhancer in breast cancer cell lines has been explored [2, 3]. PQ7 was shown to enhancer GJIC activity in cancer cells, with a more powerful effect on GJIC than the first generation PQ1 [4].

Many cancer treatment methods utilize chemotherapies that target mitotic cells for destruction, but this is not specific to the cancer cells and leads to severe side effects. The loss of GJIC by cancer cells is specific, suggesting that restoration of GJIC may provide a treatment with less detrimental effects to the host. Previous studies indicate that administration of PQ1 via oral gavage has a low toxicity to normal tissue of healthy C57BL/6J mice with no observable adverse effects [5], while significantly attenuating xenograft tumor growth of nude mice [6]. Here the distribution and anti-tumor effects of PQ7 are explored.

This study first determined the systemic distribution of PQ7 after intraperitoneal injection in healthy C57BL/6J mice. The drug distribution to the vital organs was determined at various periods of time after injection. Analysis using histological observation of PQ7 treated tissue showed no significant alterations in tissue organization or structure, suggesting a low toxicity. Next PQ7 was utilized as a treatment for mammary carcinoma in a spontaneous mammary tumor mouse model. The *in situ* generation of mammary tumors in the PyVT mouse was used to determine the biological and histological effects of PQ7 on spontaneous tumorigenesis and metastasis. For each stage of tumor development previously described, the effect of PQ7 administration was evaluated and the expression of connexins was determined in treated tissue.

6.2 Materials and methods

6.2.1 Compounds

PQ7, 6-methoxy-8-[(2-furanylmethyl)amino]-4-methyl-5-(3-trifluoromethylphenyloxy) quinoline, was prepared as previously reported [2].

6.2.2 Animals

Female C57BL/6J (The Jackson Laboratories, Bar Harbor, Maine 04609 USA) mice approximately 5 weeks of age were used in the distribution experiments. All mice were housed together in a temperature controlled environment (72°F) with a 12 hour light-dark cycle and unlimited access to standard mouse chow and water. Six animals were randomly assigned to each treatment group and administered 25 mg/kg PQ7 via intraperitoneal (IP) injection. At 6, 12, 24, and 36 hours post injection, all organs were harvested from animals euthanized by carbon dioxide inhalation.

A colony of PyVT mice (The Jackson Laboratory; Bar Harbor, ME) was established for mammary carcinoma studies. To identify transgenic progeny, genomic DNA was extracted from

a 1.5-cm tail clipping. All mice carrying the PyVT transgene developed mammary tumors. Tumor development of positive female mice was closely monitored every 2–3 days. Tumor onset was recorded as the age of the animal at which palpable abnormal masses were detected. Tumor size was measured in two dimensions with calipers every 2 days as early as 5 weeks of age. Tumor volume was determined by the equation: Volume = ½(Length)*(Width)^2. Mice were observed for any change in behavior, appearance, and weight. When animals reached a specific age range, six female mice were randomly assigned to each treatment group and administered 25 mg/kg PQ7 via IP injection.

6.2.3 PQ7 distribution studies in mice (HPLC and mass spectrometry)

6.2.3.1 Extraction of PQ7 from organs and plasma

Organs were cut into small pieces followed by the addition of 4 mL of deionized water and 10 mL of a solution of 9:1 ratio of ethyl acetate and 1-propanol. Plasma samples were directly mixed with 4 mL of water and 10 mL of a 9:1 solution of ethyl acetate and 1-propanol. Tissue and plasma solutions were separately sonicated for 40 minutes and 10 minutes, respectively, and the organic layer was separated from a separatory funnel. The aqueous layer was extracted twice with 10 mL of a 9:1 solution of ethyl acetate and 1-propanol. The organic layers were combined, washed with 5 mL of brine, dried over anhydrous $MgSO_4$, and concentrated to dryness on a rotary evaporator. The residue was diluted with 1 mL of 1-propanol and filtered through a 0.2 µm filter disc (PTFE 0.2 µm, Fisherbrand) and analyzed using HPLC and mass spectrometry as described below.

6.2.3.2 Quantification of PQ7 using HPLC

HPLC analysis was carried out on a Varian Prostar 210 with a UV-vis detector and a reverse phase column (250 x 21.20 mm, 10 micron, Phenomenex, S. No: 552581-1). A flow rate

of 5 ml/min and detection wavelength of 254 nm were used. A gradient elution of solvent A, containing deionized water and 0.01% of trifluoroacetic acid, and solvent B, containing acetonitrile and 0.01% of trifluoroacetic acid, was applied for the analysis.

1,2,4,5-Benzenetetracarboxylic acid (BTA) was used as an internal standard to quantify the amount of PQ7 in the tissue extracts. Solutions of 100 µl of various mixtures of authentic PQ7 and BTA were injected into a HPLC instrument, the peak areas corresponding to PQ7 and BTA were integrated from the HPLC chromatogram, and the ratios of the peaks were obtained. Results of the ratios of HPLC peak areas and ratios from PQ7 and BTA concentrations were plotted, and a linear correlation line was obtained from the graph. Hence using this correlation diagram, the ratio of HPLC peak areas of PQ7 and BTA from tissue extract, and the added known amount of BTA to the tissue extract, the amount of PQ7 in the tissue extract was determined.

Hence, 100 µl of 1:1 mixture by volume of the tissue or plasma extract and BTA of known concentration was injected into the HPLC, the peaks corresponding to PQ7 and BTA were integrated from the HPLC chromatogram, and the ratio of their masses was determined. Comparing the ratio of the masses of the peaks of PQ7 in the extract and standard BTA to the ratio of the masses of the peaks of authentic PQ7 and standard BTA, the mass of PQ7 in the organs and plasma was quantified.

6.2.3.3 Mass spectroscopy

An Applied Biosystem API 2000 LS/MS/MS mass spectrometer was used in the analysis. The eluent corresponding to PQ7 peak from the HPLC was collected and injected into the mass spectrometer. A mass of 406 corresponding to M+1 of PQ7 was found in the mass spectrum,

and the fragmentation pattern of this M+1 mass is similar to that of the authentic PQ7 verifying the identity of PQ7.

6.2.4 Antibodies

Primary antibodies: Anti-Cx46 (sc-20859, goat polyclonal), anti-PKCα (sc-8393, mouse monoclonal), and anti-Cx43 (sc-13558, mouse monoclonal), from Santa Cruz Biotechnology (200µg/ml; Santa Cruz, CA); anti-GAPDH (2118, rabbit monoclonal; 24 µg/ml) from Cell Signaling (Boston, MA) were used for both western blot and immunohistochemistry (IHC).

6.2.5 Western blot analysis

Mammary gland tumor tissue and selected organs (heart, lung, liver, spleen, kidney, uterus, brain) were homogenized in 500 ml of lysis buffer (20 mM Tris pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 0.5% Triton X-100) with 1:1,000 dilution of protease inhibitors (Sigma-Aldrich, Saint Louis, MO). Tissue was homogenized via the OMNI Bead Ruptor 24 (Omni International, Kennesaw, GA) at a speed of 5.65 m/s for 45 seconds, followed by centrifugation at 13,000 rpm for 30 minutes at 4˚C. Twenty-five µg of whole-cell extract was resolved by 10% SDS polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membrane (Midwest Scientific, Saint Louis, MO). Nitrocellulose membrane was blocked in 5% milk for an hour at room temperature and then incubated with monoclonal antibodies (1:1,000). Western blots were detected by enhanced chemiluminescence detection reagents (Pierce, Rockford, IL) and visualized by Fluorochem E imaging system (ProteinSimple, Santa Clara, CA).

6.2.6 Immunohistochemistry

Mammary carcinomas and organs were removed and fixed in a solution of 10% formaldehyde and embedded into paraffin prior to sectioning onto slides at a 5 µm thickness. Paraffin sections (5 µm) were dried at 60°C for 25 minutes. Deparaffinization was performed in

solutions of 100% xylene and 100%, 90%, 75%, 50% ethanol. Antigen retrieval was performed in a steam chamber with 1× citrate buffer solution for 20 minutes. Endogenous peroxidase was blocked using 3% hydrogen peroxide. Slides were then incubated overnight at room temperature with primary antibody (1:50 dilution). After washing in PBS, slides were successively incubated with biotinylated secondary antibodies (1:1,000) for 15 minutes. Slides were washed and immunostains were amplified by incubation with Avidin Biotin Complex (ABC) for 10 minutes. Cells were visualized with 3,3-diaminobenzidine (DAB) followed by a hematoxylin counterstain. The sections were viewed and the images captured with a Nikon 80i microscope under 40X and 60X magnification.

6.2.7 Statistical analysis

Significance was considered at a p-value ≤ 0.05 using Student's t-test analysis. All data are presented as mean ± 95% confidence interval of at least three independent experiments.

6.3 Results

6.3.1 Distribution of PQ7

Knowledge about the distribution of PQ7 in a biological system is important for the potential usage of this compound as an anticancer agent. PQ7 at 25 mg/kg was administered to 5-week-old female mice systemically by intraperitoneal injection. The total amount of PQ7 administered to each animal was defined as 100%. Six hours after the injection of PQ7, only 8.14% of the compound was detectable in the tissue collected. At 12, 24, and 36 hours post administration 4.65, 1.53, and 0.29% of the original compound was measurable by HPLC, respectively. Six hours after treatment the majority of PQ7 was detected in the heart, liver, lung, and uterus at levels of 1.4% (107 μM), 1.3% (98.74 μM), 1.2% (90.90 μM), and 1.1% (82.02 μM) of the total amount administered, respectively (Figure 6.1). A lower detectable level was

measured in the kidney (0.85%; 65.94 µM) and brain (0.92%; 71.34 µM). At 12 hours post exposure, the concentration of PQ7 changed in the liver from 1.28% of that administered at 6 hours post injection to 0.47% (34.73 µM). At this time point PQ7 was no longer detectable in the spleen. At 24 hours post injection the compound was no longer detectable in the heart or uterus, while the lung and intestine had the highest concentration, at 0.41% (31.83 µM) and 0.48% (38.05 µM) respectively. After 24 hours of treatment, no PQ7 was found in the majority of the organs tested or the plasma. At 36 hours post exposure, the compound was detectable in limited amounts in the intestine (0.21%; 15.01 µM) and liver (0.07%; 5.21 µM). The trend in distribution of PQ7 remained fairly consistent in all tissues tested including plasma.

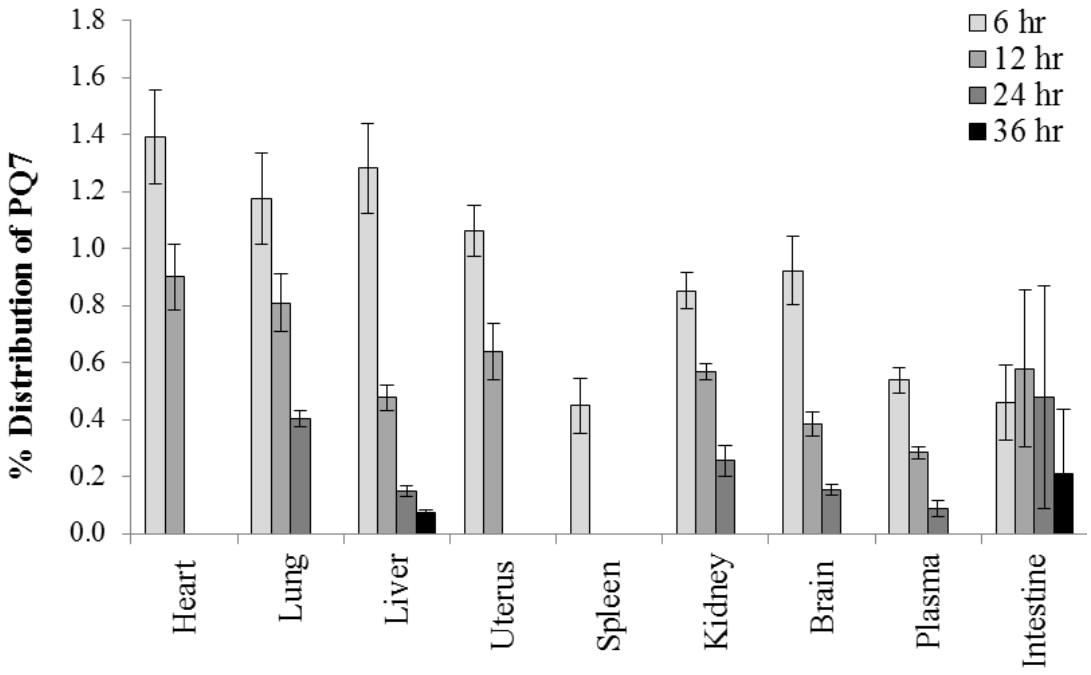


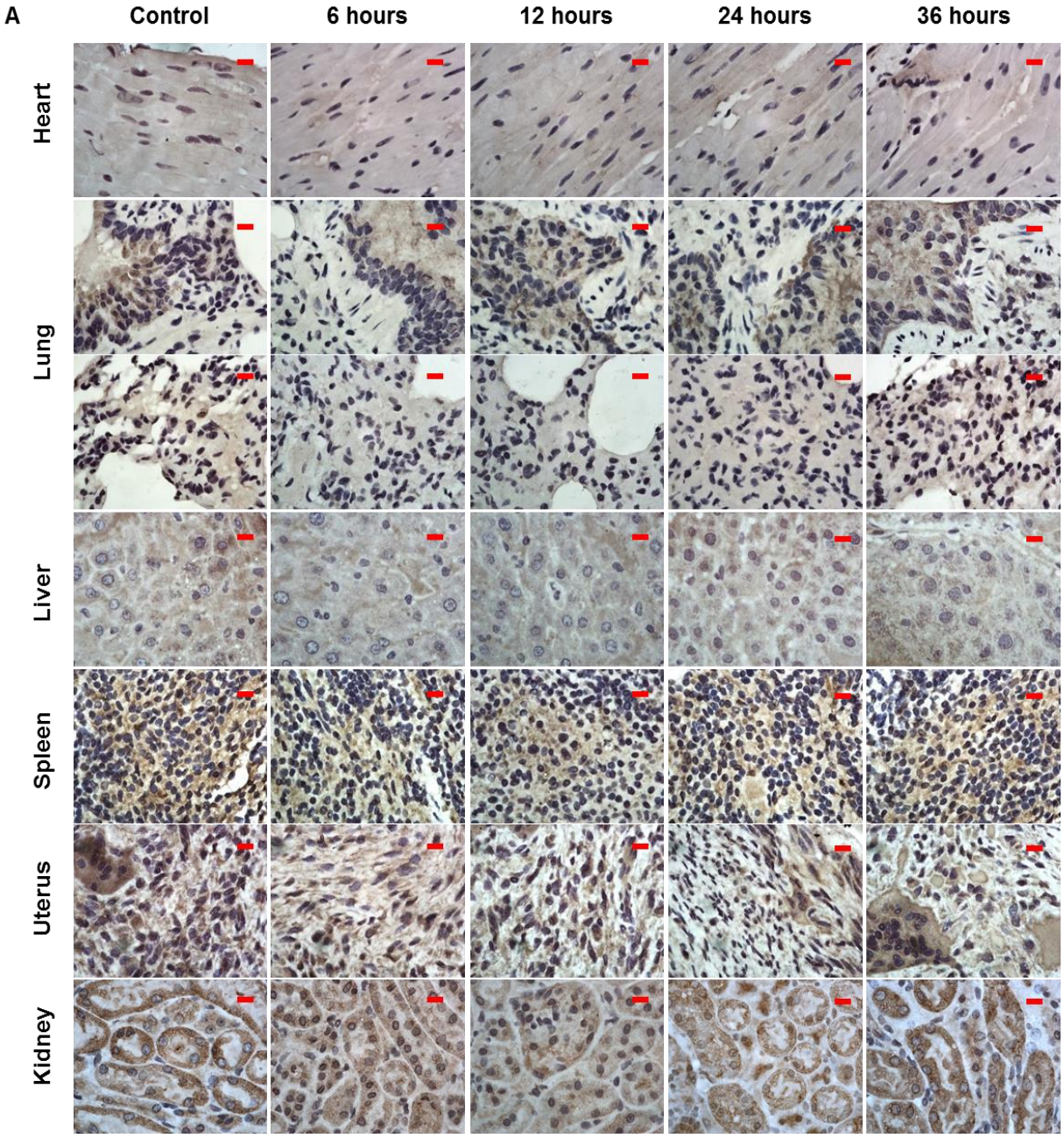
Figure 6.1 Distribution of PQ7 in mice. Mice treated with 25 mg/kg of PQ7 were euthanized at 6, 12, 24, and 36 hours. The total amount of PQ7 administered to each animal was defined as

100%. Bar graph represents the mean distribution of PQ7 with a 95% confidence interval. Data obtained from sample size of n = 6 mice.

6.3.2 Analysis of vital organs post PQ7 exposure

Multiple vital organs (brain, heart, liver and kidney) were examined using histopathology to determine any potentially detrimental effects of PQ7 administration in a single dose or in 7 doses spread over a period of 14 days. There were no morphological changes, evidence of hemorrhage, or inflammation in the tissues compared to control. This indicates that PQ7 had no toxicity to the normal tissue of healthy C57BL/6J mice. All mice exposed to PQ7 had no observed adverse effects on their health or behavior.

PQ7 has been shown to enhance GJIC and increase the expression of connexins in neoplastic cells [4,6]. The expression of Cx43 in PQ7 treated and untreated organs were compared. Cx43 was detected in all tissues tested (Figure 6.2A). PQ7 treatment initially decreased Cx43 expression in the heart, lung, liver, uterus, and brain at 6 hours post injection (Figure 6.2B). The spleen had a significant decrease in Cx43 expression at 12 hours post injection. The heart and liver recovered normal expression levels after 24 hours. Cx43 expression in the lung, uterus, and brain remained significantly lower than normal over the 36 hours observed. There was no observable side effect due to the decreased expression levels. The kidney did not have a change in Cx43 expression.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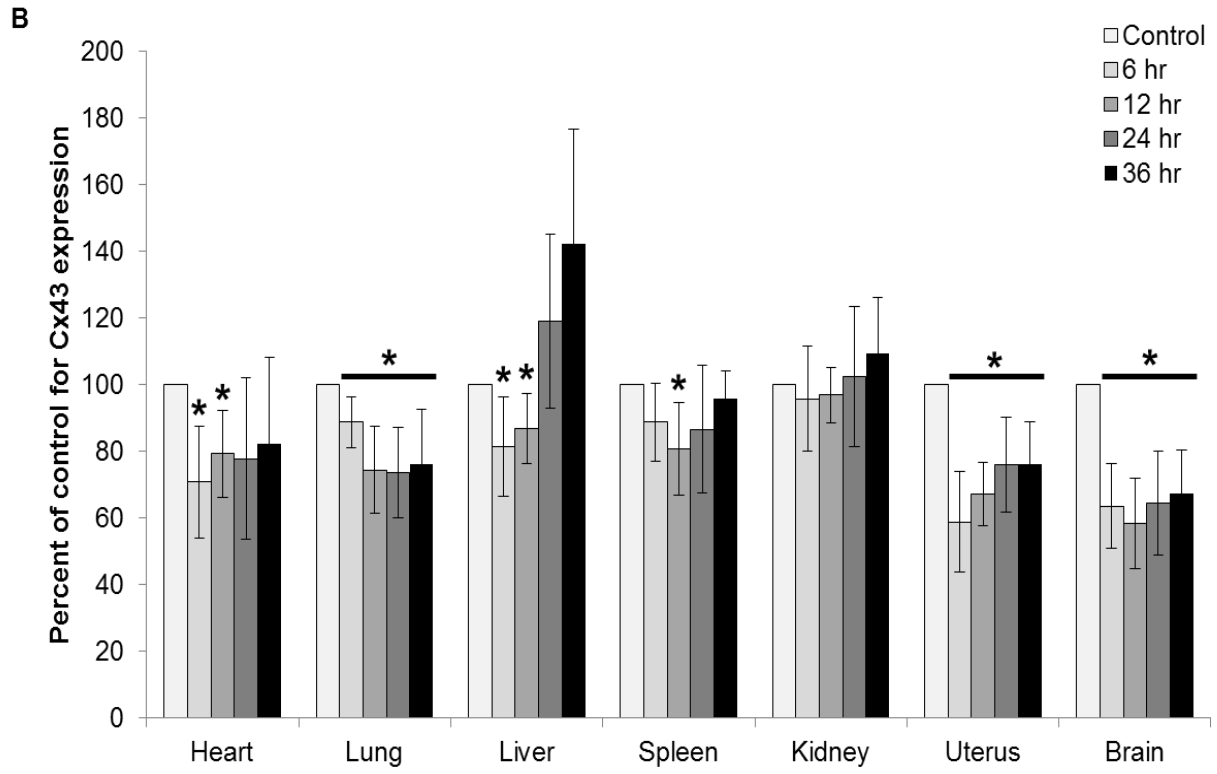


Figure 6.2 Effect of PQ7 on connexin 43 expression in normal tissue. A) Immunohistochemisty of tissue sections. Paraffin-embedded sections stained with antibodies against Cx43 in female C57BL/6J organs harvested after a single IP injection of PQ7 (25 mg/kg) at 6, 12, 24, and 36 hours. Proteins staining: brown, counterstaining: blue (hematoxylin). Images represent only 1 of n = 6 per group at a 100X magnification. Scale bar = 10 µm. B) Graphical representation of western blot analysis examining the effect of 6, 12, 24, and 36 hours of PQ7 treatment on the level of Cx43 expression. Mice without PQ treatment were used as a control. Bar graph shows the pixel intensities of protein bands normalized to the pixel intensities of loading control protein (actin) as a percentage of the control tissue. * *P*-value < 0.05 compared to control.

6.3.3 Effect of PQ7 on tumor growth in a spontaneous mammary tumor model

Tumor development was divided into 3 stages: Pre, Early, and Late. Tumor growth over a 14-day period with 7 IP injections of PQ7 or DMSO indicated a significant effect of PQ7 treatment on the Pre stage of neoplastic development in female PyVT mice. The initial tumor

volume for all pre stage mice was 14.27 ± 13 mm^3. There was a significant difference in tumor volumes between PQ7 and DMSO treated mice during the Pre stage of development from day 8 to day 14 (Figure 6.3A). PQ7 significantly attenuated tumor growth with a final volume of 27.8 mm^3 over the 14-day treatment period (*p*-value = 0.0008). The final tumor growth of the control DMSO treated mice was 377 mm^3. The change in tumor volume over the 14-day period shows a significant attenuation of tumor size with PQ7 treatment compared to both controls (p-value $_{NO\ TX}$= 0.005, p-value $_{DMSO}$ = 0.0005; Figure 6.3B). There was a 98% difference between the overall changes in tumor growth after treatment with PQ7.

The initial tumor volume for all Early stage mice was 104 ± 53 mm^3. During this stage of development there was not a significant difference in tumor growth between treatment groups (Figures 6.3C and 6.3D). During the Late stage of tumor development, mice began treatment with the initial tumor volume of 676 ± 134 mm^3. PQ7 did not attenuate tumor growth compared to control during the Late stage of development (Figures 6.3E and 6.3F).

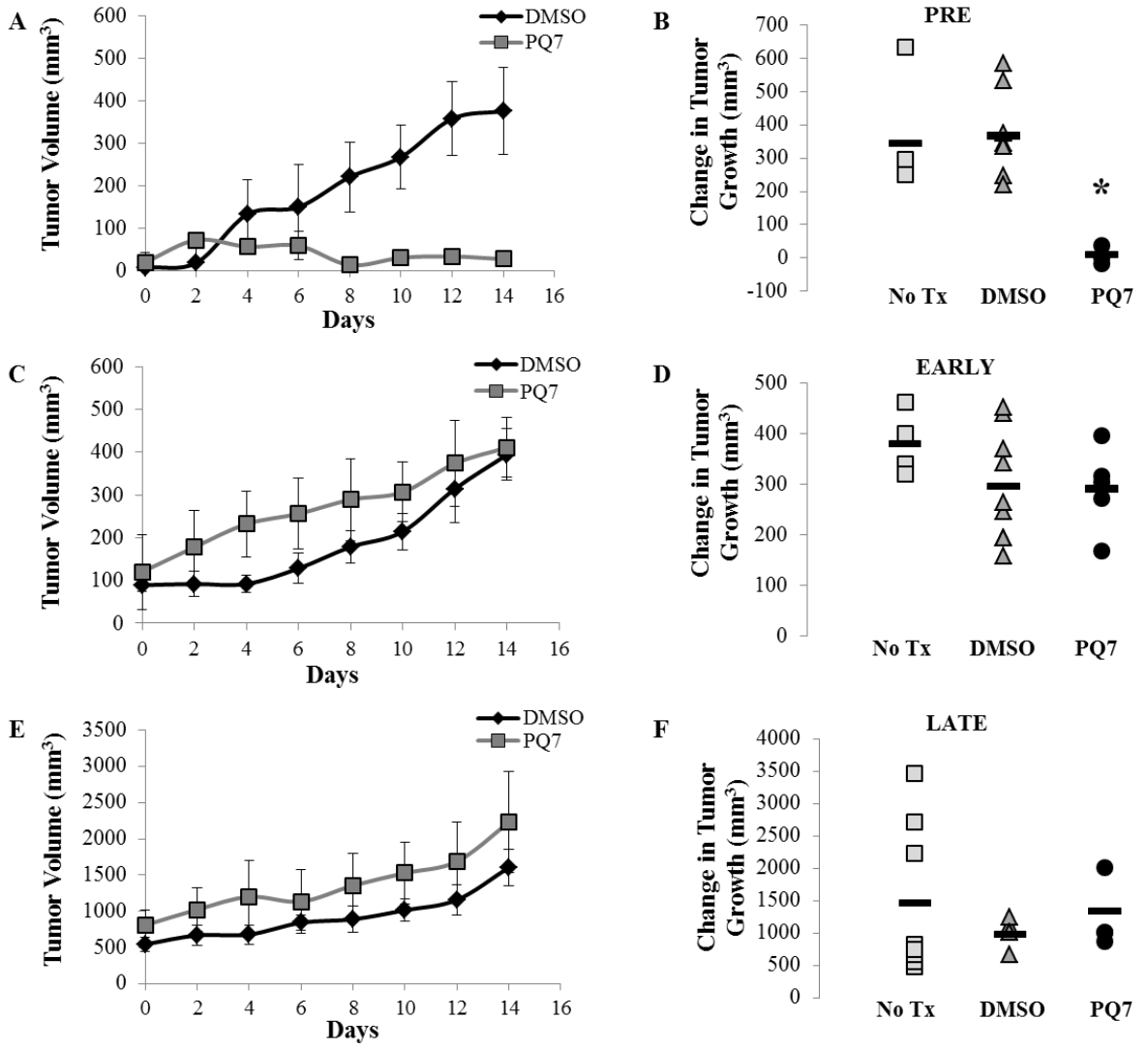


Figure 6.3 Tumor growth (mm^3) in PyVT female mice. Tumors measured in two dimensions with calipers every 2 days prior to administration of treatment for panels A and B) Pre, C and D) Early, and E and F) Late stages of tumor development. Panels A, C, and E) The tumor size is expressed over the 14 day treatment period for the DMSO (control) and PQ7 (25 mg/kg) treated PyVT mice. Days 0-12 represent the days of the 7 IP injections, day 14 represents the end of the study with measurements prior to tissue harvest. Panels B, D, and F) The overall change in tumor size after no treatment, or treatment with DMSO (control) or PQ7 (25 mg/kg) via 7 IPs. * *P*-value < 0.05 compared to controls.

PyVT mice have a total of 10 mammary fat pads that may develop tumors during their lifetime. The total number of palpable tumors, defined as the tumor burden, was monitored during the course of treatment, and the final tumor number for each treatment group in each stage of development is presented (Figures 6.4A-C). During all three stages there was no significant difference between the tumor burdens of the two control groups. Treatment with PQ7 during the Pre stage significantly reduced the number of tumors developed after treatment (*p*-value < 0.00001; Figure 6.4A). There was no difference in the tumor burden between experimental groups of the Early or Late stages of tumor development (Figure 6.4B and 6.4C).

Tumors were analyzed to determine the quantity of PQ7 detectable after approximately 48 hours after the last IP injection. At each stage of development, the parent compound was measureable in the neoplastic tissue harvested from treated animals. The Pre and Early stages of tumors were determined to have a concentration of 37 pM PQ7, while the Late stage tumors had 1.1 nM PQ7 (Figure 6.5A). This indicates that the parent compound remained in the tumor for at least 48 hours after a 14 day treatment period with 7 IP injections.

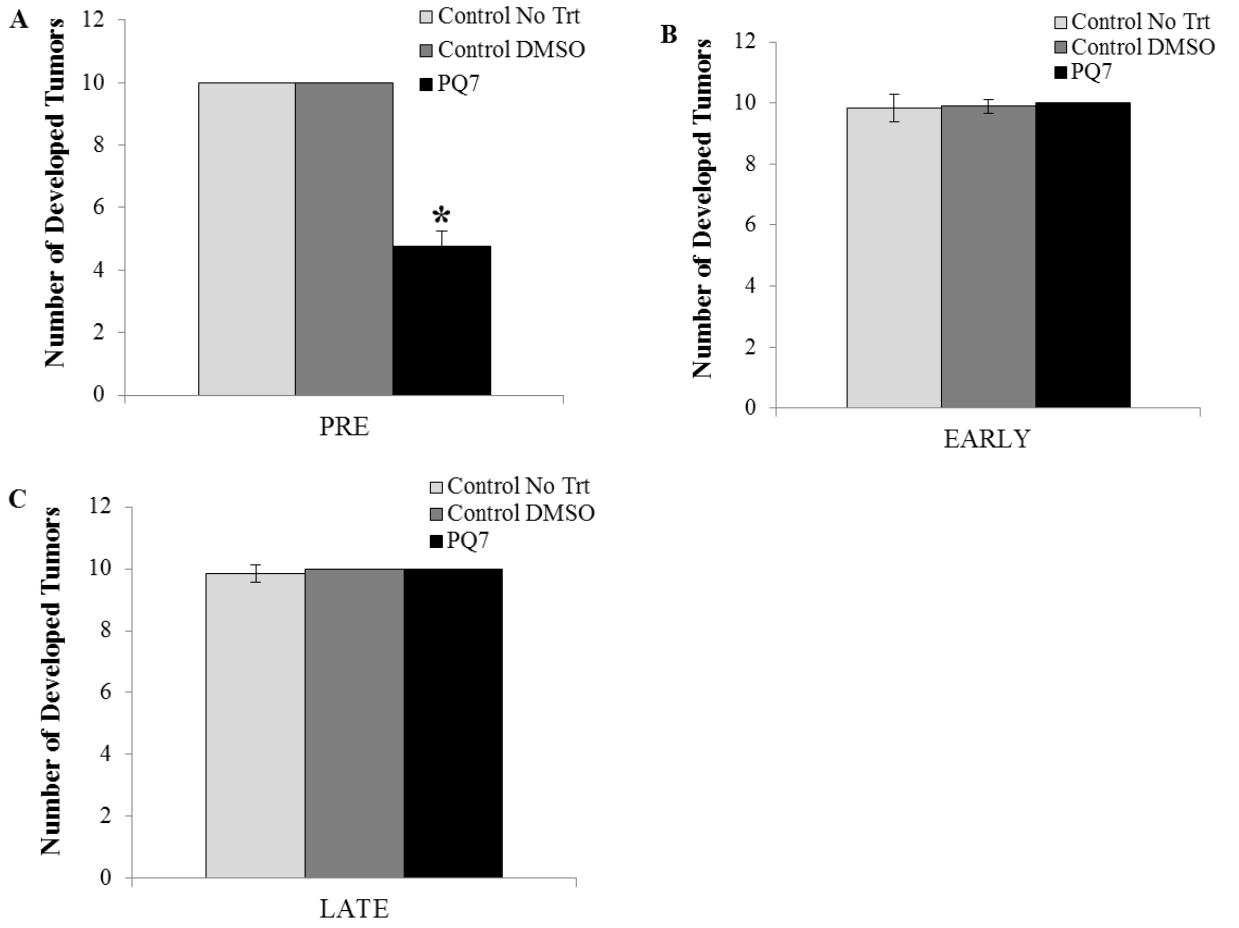


Figure 6.4 Number of developed tumors in PyVT female mice during development. Tumors identified grossly during the A) Pre, B) Early, and C) Late stages of tumor development after a 14 day period with either no treatment, or treatment with DMSO (control) or PQ7 (25 mg/kg) via 7 IPs. * *P*-value < 0.05 compared to controls.

6.3.4 Pathological analysis of PyVT tumors post PQ7 treatment

Histopathological examination of the mammary tumors of PyVT mice was conducted for each treatment group in the three stages of tumor development. When present, tumors were categorized as adenoma/mammary intraepithelial neoplasia (MIN), early carcinoma, or late carcinoma. The Pre control tumors were either adenoma/MIN or early carcinomas; while the Pre PQ7-treated tumors appeared to be focal hyperplasias or adenoma/MIN and early carcinoma.

The Early control tumors were all early carcinomas. The Early PQ7-treated tumors varied from adenoma/MIN, early carcinoma, and late carcinoma. The Late control and PQ7 tumors were both late carcinomas. In addition a few Late PQ7 tumors were identified as adenosquamous carcinomas. Histological examination of the lung tissue from all Late stage mice showed no significant difference in the presence of metastatic foci between treatment groups.

6.3.5 Effect of PQ7 on connexin expression in neoplastic tissue

PQ7 has been shown to enhance GJIC and increase Cx43 expression in breast cancer cells [4], therefore the differential pattern of connexin proteins in PQ7-treated tumors was determined. Though most connexins are tumor suppressors, Cx46 has been shown to be upregulated in breast cancer cell lines and tumors to provide protection in hypoxic conditions [10]. Immunoblot analysis of connexin expression indicates that PQ7 treatment increased Cx43 expression (Figure 6.5B) and decreased Cx46 expression (Figure 6.5C) during the early stages of carcinogenesis. During the control PyVT tumor development there was an increase in Cx43 and Cx46 expression from Pre to Late stage. Data suggest that Cx43 was expressed at higher levels in PQ7-treated animals compared to controls and the contrary for connexin 46 early in tumor formation. Cx46 expression in PyVT tumors treated with PQ7 from the Pre and Late stages of development had significantly lower levels than that of the controls (p-value $_{Pre}$= 0.016, p-value $_{Late}$= 0.0007). The Pre stage tumors treated with PQ7 had a significantly greater level of Cx43 expression compared to controls (p-value $_{Pre}$= 0.040), while during the Late stage they had significantly less Cx43 compared to controls (p-value $_{Late}$= 0.034). This may be explained by the overall increase in both connexin 43 and connexin 46 during tumor development and metastasis of the PyVT mice.

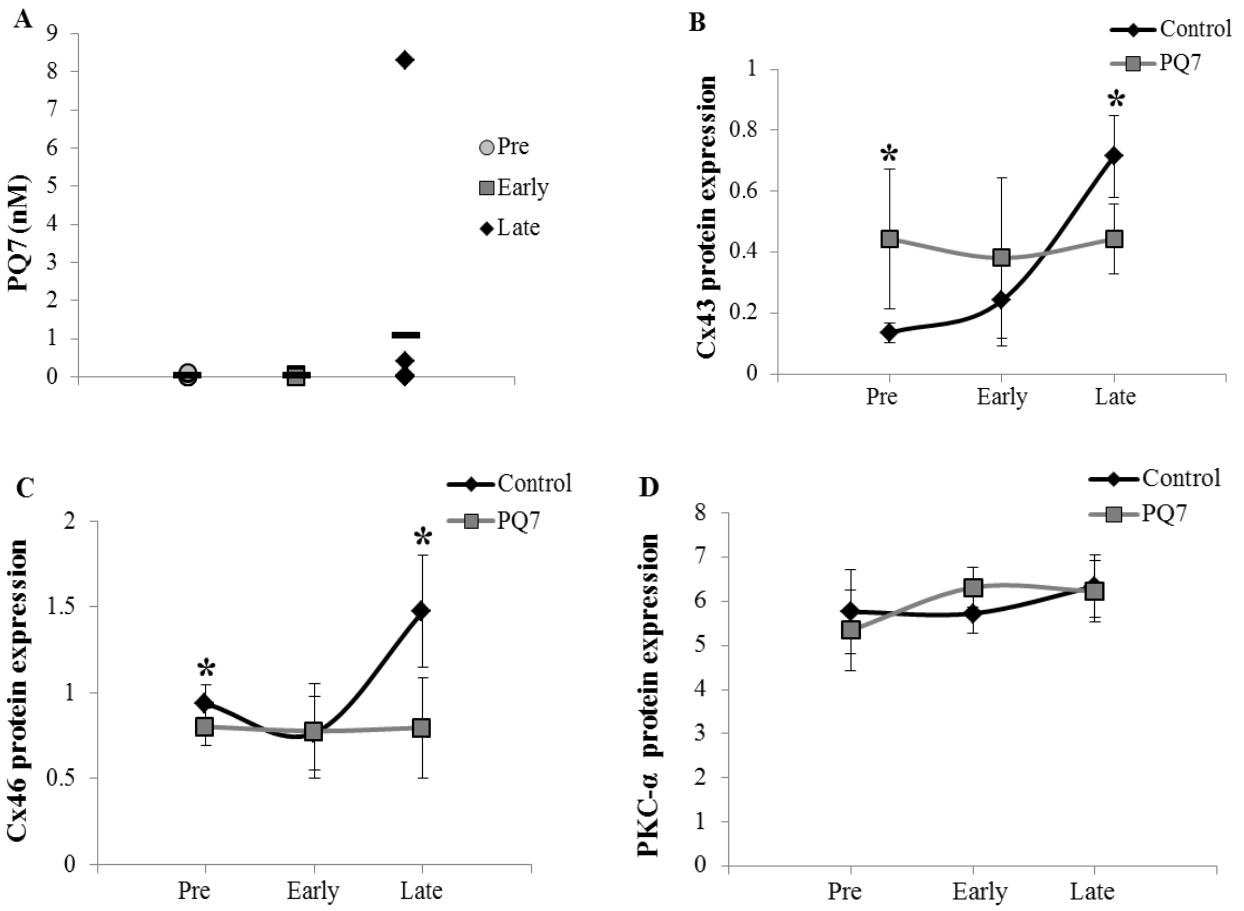
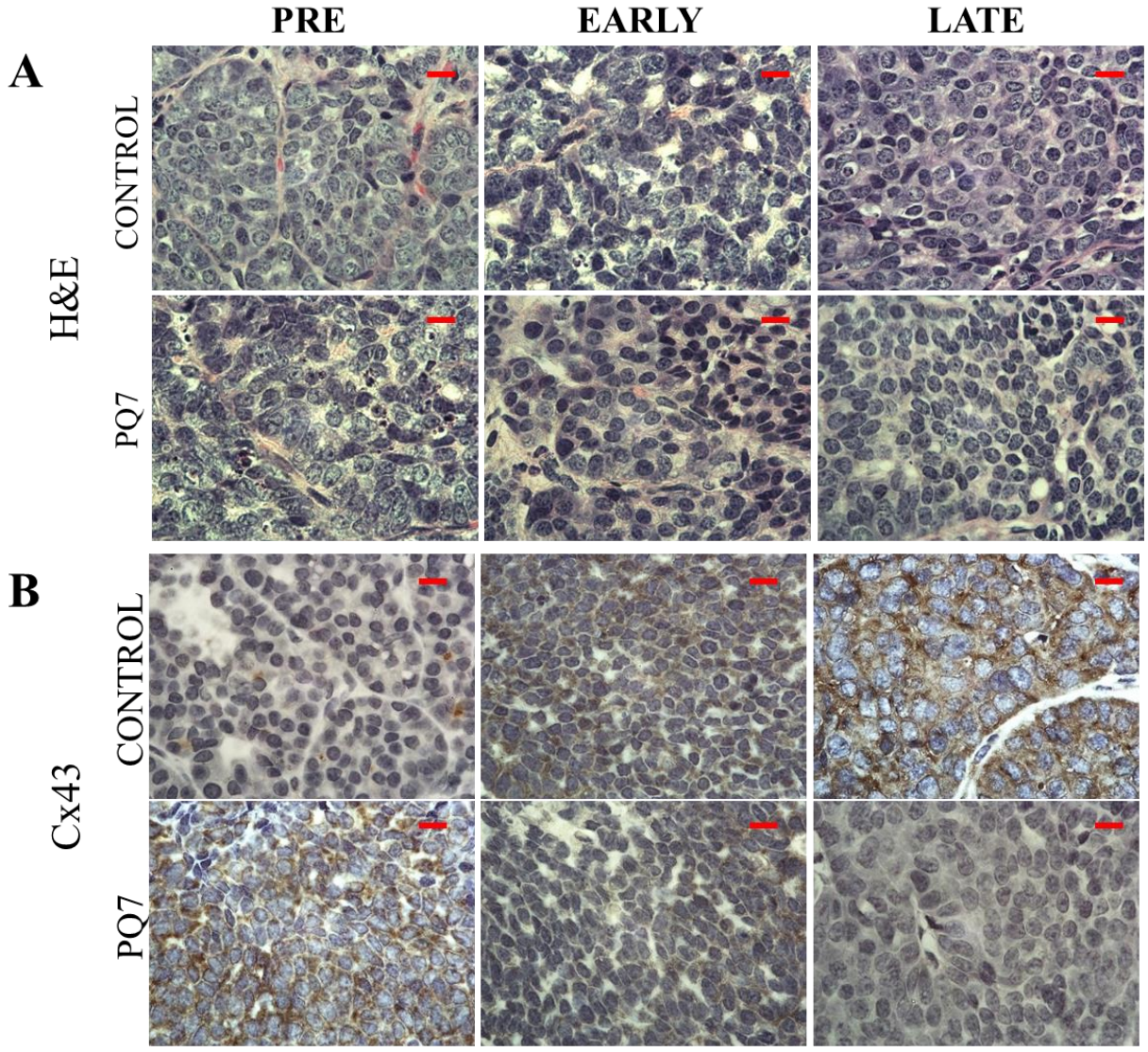


Figure 6.5 Analysis of tumors isolated from PyVT females 48 hours after the last IP injection. A) Quantitative analysis of PQ7 in the tumor homogenate. Data obtained from a minimum of three samples per developmental period. Data points represent the nanomolar concentration of PQ7 in each tumor isolated from treated mice, while the dashed lines represent the mean concentration of the PQ7 in all the tumors analyzed. B, C, and D) Graphical representation of protein expression in tumors from Western blot analysis. Fold-pixel intensity of B) Cx43, C) Cx46, and D) PKCα normalized to loading control in PyVT female tumors treated with DMSO (control) or PQ7 (25 mg/kg) via 7 IPs in each of the three stages of tumor development. n = 4. * *P*-value < 0.05 compared to control.

Histopathology of the tumors harvested from PyVT mice showed no significant difference in morphology. Immunohistochemistry of PQ7 treatment at Pre and Early stages of tumor formation showed stronger positive cytoplasmic staining in Cx43, while during the Late

stage there was stronger positive staining in the control tissue versus the PQ7 treated tissue (Figure 6.6A). The Cx46 immunohistochemistry indicated a weaker positive staining compared to controls. This supports the molecular analysis previously mentioned.

Connexins are phosphoproteins that are targeted by kinases for efficient trafficking, assembly and disassembly, degradation, and gating of hemichannels [11,12,13]. Phosphorylation regulates GJIC in both a kinase and connexin specific manner [11,14]. Since PQ7 altered connexin expression, we explored the role of PKCα in the PQ7 treated PyVT mouse tumors at each stage of development by western blot analysis (Figure 6.5D) and immunohistochemistry (Figure 6.6C). No significant change in PKCα expression was determined due to PQ7 treatment. Interestingly in the control tumors there appeared to be a stronger positive staining in the Pre stage compared to the Late stage of tumor development, suggesting a decrease in connexin phosphorylation and degradation.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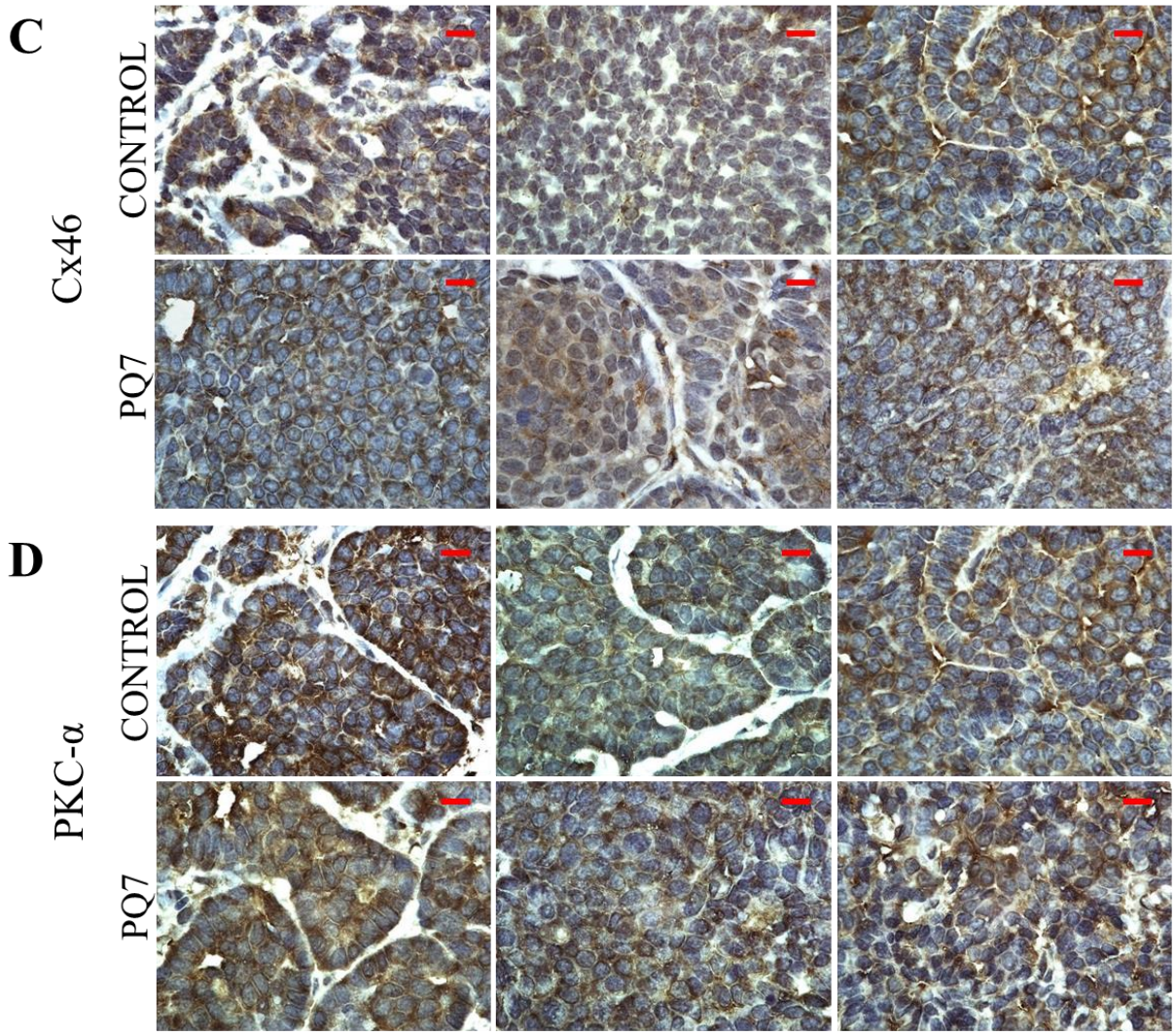

Figure 6.6 Immunohistochemistry of tumors from PyVT females. Paraffin-embedded tumor sections stained with A) H&E or antibodies against B) Cx43, C) Cx46, and D) PKCα from PyVT females treated with DMSO (control) or PQ7 (25 mg/kg) via 7 IPs at either Pre, Early, or Late stage of tumor development. Proteins staining: brown, counterstaining: blue (hematoxylin). Images represent only 1 of n = 6 per group at a 100X magnification. Scale bar = 10 μm.

6.4 Discussion

Effective use of antineoplastic drugs depends on the ability to balance the killing of tumor cells against the inherent toxicity to the host. Antineoplastic agents that act primarily on

rapidly dividing and growing cells produce multiple side effects and are dose limiting. The first generation gap junction enhancer was shown previously to have a low toxicity in healthy animals administered via oral gavage [5]. An intraperitoneal injection was used in this study to ensure a systemic exposure of a consistent amount of PQ7 to all the mice. Exposure of C57B/6J and PyVT mice to either a single or multiple doses, respectively, of PQ7 systemically showed no detrimental effects to any of the vital organs. Uptake of the compound into a specific tissue depends on the availability of the compound in the blood supply and the extent of vascularization. The highest levels of PQ7 were detectable in the liver, heart, lung, and uterus, which may be due to the fact that these are highly vascularized tissues. Interestingly PQ7 is detectable is the brain, indicating good penetration of the blood brain barrier. The 25 mg/kg PQ7 administered to approximately 20 gram mice was equivalent to 9.56 mM. Results indicate that a 9.5 mM concentration of PQ7 can be distributed to all the vital organs and metabolized in C57B/6J mice.

Exposure of C57B/6J and PyVT mice to either a single or multiple doses, respectively, of PQ7 systemically showed no detrimental effects to any of the vital organs, indicating a low toxicity. The total amount of PQ7 detected in the tissue after six hours was only 8.1%. This suggests that the majority of the parent compound is metabolized and/or eliminated in less than six hours. The half-life of PQ7 in the liver appeared to be about 6 hours, suggesting complete metabolism or elimination by 48 hours. The optimal time between injections for treatment purposes would therefore be 48 hours, which has been shown to be effects in tumor bearing mice [6].

The levels of PQ7 measured in the intestinal tract had a high variability, however the compound was detectable at the highest level in this organ 36 hours post exposure. The intestinal

mucosal layer accumulates lipids and hydrophobic compounds, which have an increased permeability in the intestinal tract. This suggests that PQ7 may be secreted into the gastrointestinal tract through the bile duct for fecal excretion and potentially reabsorbed into the intestinal mucosa due to its lipophilicity. This is supported by the lack of PQ7 detected in the plasma or kidney after 24 hours, indicating that urinary excretion of the parent compound is complete by 24 hours post injection. Collectively these results suggest that PQ7 treatment may be useful in targeting neoplasias of the gastrointestinal tract.

Cell proliferation and apoptosis are important factors in carcinogenesis [12], and GJIC is a key factor in carcinogenic process. Reduced GJIC in preneoplastic and neoplastic tissue can lead to excessive cell proliferation, abnormal differentiation, and inhibited apoptosis, leading to the loss of homeostasis. More than 100 non-mutagenic and mutagenic carcinogens were reported to inhibit GJIC *in vitro* and *in vivo* [13-15]. These compounds are chemically diverse, including pharmaceuticals, polyaromatic hydrocarbons, plant products, and pesticides. The inhibition of GJIC correlates best with carcinogenicity in multiple *in vitro* tests [16]. This shows that the carcinogenic mechanism of multiple agents involves the down-regulation of GJIC. Therefore a compound that restores GJIC is vital for cancer prevention and treatment. The ability to normalize GJIC in neoplastic cell could restore homeostasis and prevent further tumor development.

Many tumor promoters down-regulate GJIC to allow the initiated cell to proliferate and evade apoptosis [17]. The down-regulation of GJIC is a reversible process, indicating that intervention that enhanced GJIC could prevent promotion and progression of the neoplastic tissue. Previously PQ7 was shown to increase the expression of connexins and enhance GJIC [3, 4]. The data presented here shows that PQ7 delays the development of mammary carcinomas,

suggesting it could be utilized as a primary chemopreventive compound for breast cancer. The PyVT mouse has a genetic alteration that predisposes them to the development of mammary carcinomas, however with PQ7 treatment during a pre-cancerous stage, the development of these malignancies was delayed significantly. The use of chemical intervention before an initiated cell becomes independent of the promoter stimuli could induce regression of the neoplastic tissue which is a process of chemoprevention.

Cancer is a prominent disease throughout the world, despite the increasing knowledge of carcinogenesis and treatment options. More effective cancer therapies are needed. Due to the fact that GJIC is involved in the development of cancer and metastasis, it is a promising target for new therapies. The enhancement of GJIC has been shown to increase the efficacy of multiple types of cancer therapies through the bystander effect [18-24]. GJIC could increase the distribution of chemotherapeutic compounds in tissues that are poorly vascularized and have impaired drug delivery, this is especially important for hydrophilic compounds that are unable to pass through the cell membrane [25]. Additionally, up-regulation of GJIC has been shown to increase the sensitivity of cancer cells to conventional chemotherapeutics [26, 27]. Though PQ7 is not an effective anticancer compound on its own during later stages of tumor development, it could be used in combination with multiple types of chemotherapeutic options to enhance killing of the neoplastic cells.

Molecular analysis of the protein expression demonstrated a general increase of expression of Cx43 and Cx46 during tumor development. Cx46 is a hypoxia-specific gap junction protein in mammary tissue suggested to have pro-tumor effects by preventing hypoxic death [28]. As a tumor grows in size, the neoplastic cells in the center of the mass may upregulate Cx46 to survive more hypoxic conditions. Additionally an increase in Cx43

expression typically correlates with metastatic potential [29, 30]. Interestingly expression of Cx43 in PQ7 treated animals has a reciprocal relationship with control Cx43 expression, while Cx46 expression in treated tissue remains low despite the stage of development. PQ7 affects the expression of each connexin differently during tumor development. Importantly the decrease in Cx46 and increase in Cx43 observed during the Pre stage of development with PQ7 treatment may be the key for prevention or delay of tumor formation. Additional knowledge of the role of each connexin in tumorigenesis is needed.

The gap junction enhancer PQ7 is shown here to have no apparent side effects when systemically distributed to all the vital organs, and is capable of altering the development of a spontaneous mammary carcinoma. These results are promising in the development of a novel compound for chemoprevention or combinatory uses for breast cancer.

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Chapter 7 - The effect of antineoplastic drugs in a male spontaneous mammary tumor model

A research article of the following findings has been published in PLoS ONE (2013) 8(6): e64866. doi:10.1371/journal.pone.0064866.

7.1 Introduction

Male breast cancer accounts for 1% of all breast cancer cases in the United States, while it causes approximately 0.5% of all male cancer deaths [1]. Knowledge of this malignancy and appropriate therapies remain limited due to rarity of large cohorts of male breast cancer patients. Treatment for male breast cancer is therefore extrapolated from controlled clinical studies conducted in women [2]. Male breast cancer most commonly presents as late stage painless, firm masses in the subareolar location that become fixed to the pectoralis major muscle and the skin [3]. Male breast cancer is diagnosed in later stages than female breast cancer, leading to a tendency of small neoplasms to spread to the axillary lymph nodes. The 5-year survival rate for metastatic breast cancer in male patients is less than 20%, while the median survival is only about 15 months [4,5]. Prognosis of male breast cancer is similar to stage matched females.

Male breast cancer differs from female breast cancer in many aspects. Most notably male breast cancer is diagnosed at older ages, presents at higher stage, has a bimodal age-frequency [4,6], racial differences [6], distinct histological subtypes, immunophenotypic variations [4,6,7], low survival rates [4], and differential genetic mutations, such as CYP17 polymorphism [8], androgen receptor (AR) [9], and CHEK2 mutations [10]. Male breast cancer has also been shown to have a higher frequency of hormone receptor (HR), estrogen receptor (ER) and progesterone receptor (PR), expression (80-90%) compared to females (75%) [4,5]. It is unclear if there is a relationship between ER+ male breast carcinomas and patient survival [4,11,12]. This may be

due to differences in ER function in males as compared to females [13]. There is a relatively high expression of ER in males compared to females due to the naturally lower estrogen levels in the tissue microenvironment, leading to an increase in estrogen targets [14]. An example of this is Bcl-2, which is an inhibitor of apoptosis, and has also been found to be overexpressed in male breast cancer [15].

The molecular subtypes of male breast cancers are based on the expression of certain protein markers in the neoplastic tissue, which are used to evaluate their association with the observed pathological features and patient outcome. It is important to note that the HR positivity of male breast carcinomas may not have the same prognostic value as female breast cancer. It is unclear whether the human epidermal growth factor receptor 2 (HER2) plays a prognostic role in male breast cancer [16,17]. In a comparative study between male and female invasive breast carcinomas, the most common male breast tumor phenotype was luminal A (HR+/HER2-), while HR- and HER2+ were not identified in male patients [18]. In another study luminal A tumors were 82.8%, luminal B (HR-/HER2+) tumors found in 6.2%, and basal-like (HR-/HER2-) tumors were found in 9.6% of the male breast cancer cohort [19]. Contrary to these, another series reported to have no significant difference between tumor subtypes [20]. These studies show that the distribution of molecular subtypes in male breast cancer varies, but that it is also different compared to the female breast cancer cohorts. This is indicative of a pathological difference in carcinogenesis between males and females.

Certain populations have a higher risk of developing male breast cancer. The major risks are either genetic factors or hormone imbalance. Approximately 20% of males with breast cancer have a family history of breast or ovarian cancer [21]. Mutations in the *BRCA1* or *BRCA2* genes are the strongest known genetic risk factors for male breast cancer. More specifically the *BRCA2*

gene mutation has a 7% lifetime risk of male breast cancer [22], which is a greater risk than females with a genetic predisposition of this disease. A change in the ratio of estrogen and testosterone is also an important factor contributing to male breast cancer. Individuals with Klinefelter's syndrome have low testosterone levels, increasing the lifetime risk of developing male breast cancer to approximately 5% [21].

The commonly used therapeutic approach involves mastectomy with axillary lymph node evaluation and hormonal therapy, with potentially additional adjuvant chemotherapy. Hormonal therapy, primarily tamoxifen, is the mainstay of treatment for male breast cancer and is considered to be the first line treatment for metastatic male breast cancer, with an overall response rate of 49% [23]. In another study [24], it was reported that tamoxifen increased the 5-year actuarial survival (61% verse 44%) and disease free survival (55% verse 28%) rates of 39 male breast cancer patients compared with the historical control group [25]. Men in general tolerate tamoxifen well, with the most common side effects reported as decreased libido, weight gain, hot flashes, mood alteration and depression. However, it is important to note that there have been no randomized trials to evaluate the real response rates or the toxicity of tamoxifen in men. The role of other antineoplastic drugs commonly used for female breast cancer treatment has yet to be determined in male patients.

Advancements in diagnosis and treatment of female breast cancer have resulted in a steady decline in incidence and clinical outcome, while male breast cancer has been on a steady rise in incidence over the past several decades and a much slower improvement on clinical outcome [26]. The treatment for male breast cancer is extrapolated from female clinical trials, despite distinct clinical and pathological differences, as well as a lack of clinical improvement in patient outcome. There is a need for new clinical management of male breast cancer. The major

hormonal differences between male and female patients regarding endocrinology and the breast carcinomas' response, suggests a need to explore alternate treatment options.

The PyVT mouse is a novel *in vivo* model of mammary tumor formation and metastasis. Studies show that even at early stages of mammary development the mammary fat pads were clearly abnormal with irregular growth of side branches, enlarged terminal buds, and large tumorous masses. Male animals generally develop mammary tumors by 15 weeks of age and reach maximum tumor burden around 25 weeks of age. This study focuses on utilizing the *in situ* generation of male mammary tumors by the PyVT model to determine the efficacy of antineoplastic drugs, cisplatin, paclitaxel, and tamoxifen in attenuating tumor growth. The potential benefits of each treatment option are revealed for a male mammary tumor model.

7.2 Materials and methods

7.2.1 Mouse model

PyVT mice were purchased from the Jackson Laboratory (Bar Harbor, Maine). The mice were monitored every other day to check for the appearance of tumors. The tumor size was measured in two dimensions with calipers. Tumor volume was determined by the equation: Volume = ½(Length)*(Width)^2. Mice were observed for abnormal behavior, appearance or weight loss. If the animals showed any signs of pain, extreme tumor growth (greater than 1.5 cm) or loss of body condition, they were humanely euthanized before the end of the experimentation period. At different time points (10, 15, 20 weeks of age) the male mice were sacrificed to examine mammary epithelium and tumor formation. All treatments were conducted on male mice determined to be in an early tumor development stage, which was 15 weeks of age. Average weight of this age group was 28-32 grams. Male mice were randomly divided into four experimental groups: (1) control animals given the vehicle solvent (DMSO); (2) animals treated

with 3.5 mg/kg cisplatin; (3) animals treated with 10 mg/kg paclitaxel; and (4) animals treated with 40 mg/kg tamoxifen. All treatments were administered as an intraperitoneal injection.

7.2.2 Antibodies

Primary antibodies to Survivin (sc-8807, goat polyclonal), Caspase 3 (sc-56046, mouse monoclonal), Cyclin D1 (sc-8396, mouse monoclonal), Bcl-2 (sc-492, rabbit polyclonal), ERα (sc-8002, mouse monoclonal), ERβ (sc-8974, rabbit polyclonal) and PR (sc-166170, mouse monoclonal) from Santa Cruz Biotechnology (200 µg/ml; Santa Cruz, CA); anti-GAPDH (2118, rabbit monoclonal; 24 µg/ml) and anti-HER2 (4290, rabbit monoclonal; 22 µg/ml) from Cell Signaling (Boston, MA) were used for both western blot and immunohistochemistry (IHC).

7.2.3 Immunohistochemistry

All tumors were fixed in a solution of 10% formaldehyde and embedded into paraffin prior to sectioning them onto slides at a 5 µm thickness. Paraffin sections (5 µm) were dried at 60°C for 25 minutes. Deparaffinization was performed with 100% xylene and 100%, 90%, 75%, 50% ethanol. Antigen retrieval was performed in 1× Citrate buffer solution and steam for 20 minutes. Endogenous peroxidase was blocked using 3% hydrogen peroxide. Slides were then incubated overnight at room temperature with a polyclonal antibody (1:50 dilution). After washes in PBS, slides were successively incubated with biotinylated secondary antibodies (1:1000) for 15 minutes. Slides were washed and immunostains were amplified by incubation with Avidin Biotin Complex (ABC) for 10 minutes accordingly. Cells were visualized with 3,3-diaminobenzidine (DAB) followed by a hematoxylin counterstain. The sections were viewed and the images captured with a Nikon 80i microscope.

7.2.4 Western blot analysis

Mammary gland tumor tissue were homogenized in 500 mL of lysis buffer (20 mM Tris pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 0.5% Triton X-100) at 1:1000 dilution of protease inhibitors (Sigma-Aldrich, Saint Louis, MO). Tissue was homogenized using the OMNI Bead Ruptor 24 at a speed of 5.65 m/s for 45 seconds, followed by centrifugation at 13,000 rpm for 30 minutes at 4˚C. Twenty-five micrograms of whole-cell extract was resolved by 10% SDS polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membrane (Midwest Scientific, Saint Louis, MO). Nitrocellulose membranes were blocked with 0.5% milk in Tris-Buffered Saline and Tween 20 (TBST) using a SNAP i.d. device (Millipore) at room temperature. Membranes were then incubated with primary antibody at a 1:1000 dilution, followed by HRP-linked secondary antibodies (1:2000). Western blots were detected by enhanced chemiluminescence detection reagents (Pierce, Rockford, IL) and visualized by Fluorochem E imaging system (ProteinSimple, Santa Clara, CA).

7.2.5 Statistical analysis

Significance was considered at a p-value ≤ 0.05 using Student's t-test analysis. All data are presented as mean ± 95% confidence interval of a minimum of three samples for molecular analysis and six samples for animal studies.

7.3 Results

7.3.1 Characterization of the PyVT mouse model and effects of treatment on hormone receptor expression

Male PyVT mice developed tumors as early as 14 weeks of age. All 10 mammary pads developed tumors with the maximum tumor burden achieved around 25 weeks of age. Tumor development in this spontaneous model was divided into 3 stages based on the extent of tumor

size, the frequency of tumor formation, and whether it has metastasized to the lungs. Pre stage of male PyVT tumor development occurred from 10-13 weeks of age and consisted of a pre-cancerous condition where no tumors were palpated and the mammary tissue appeared normal on gross observation. The Early stage of development represents solid tumor formation within the breast tissue at 15-18 weeks of age. This stage consisted of the gross observation of 1-2 solid tumors. The Late stage consisted of the presence of all 10 primary mammary tumors and secondary lung metastasis, which occurred after 20 weeks of age. The presence of metastases was confirmed by hematoxylin and eosin (H&E) staining of representative sections of the lung and histopathological review. This report focused on the Early stage of tumor formation and examined the effect of antineoplastic drugs on tumor growth at this stage.

The mammary tumors were isolated from each treatment group to determine hormone receptor (ER, PR, and HER2) expression. Immunohistochemistry of the control tumor sections had weak positive staining of HER2 and strong positive staining of ERβ (Figure 7.1A). Tamoxifen treatment resulted in an increase in positive nuclear staining of ERα in the tumors isolated, while decreasing the positive staining of ERβ. Western blot analysis was conducted to determine quantifiable expression levels of each hormone receptor in treated tissue. Control tumors expressed ERα, ERβ, PR, and HER2 (Figure 7.1B). Tamoxifen treated animals had an increased expression of ERα (p-value = 0.0021) and PR (p-value = 0.0300; Figure 7.1B), while inducing a significant decrease in HER2 (p-value = 0.0002) and ERβ expression (p-value = 0.0002). Mice receiving paclitaxel treatment had a significant reduction in ERα and ERβ expression compared to control mice (p-value = 0.0201 and 0.0219, respectively), with no change in PR and HER2 expression. Interestingly animals treated with cisplatin showed no

change in ERα, ERβ, PR, or HER2 expression, suggesting that treatment does not affect expression of the molecular markers.

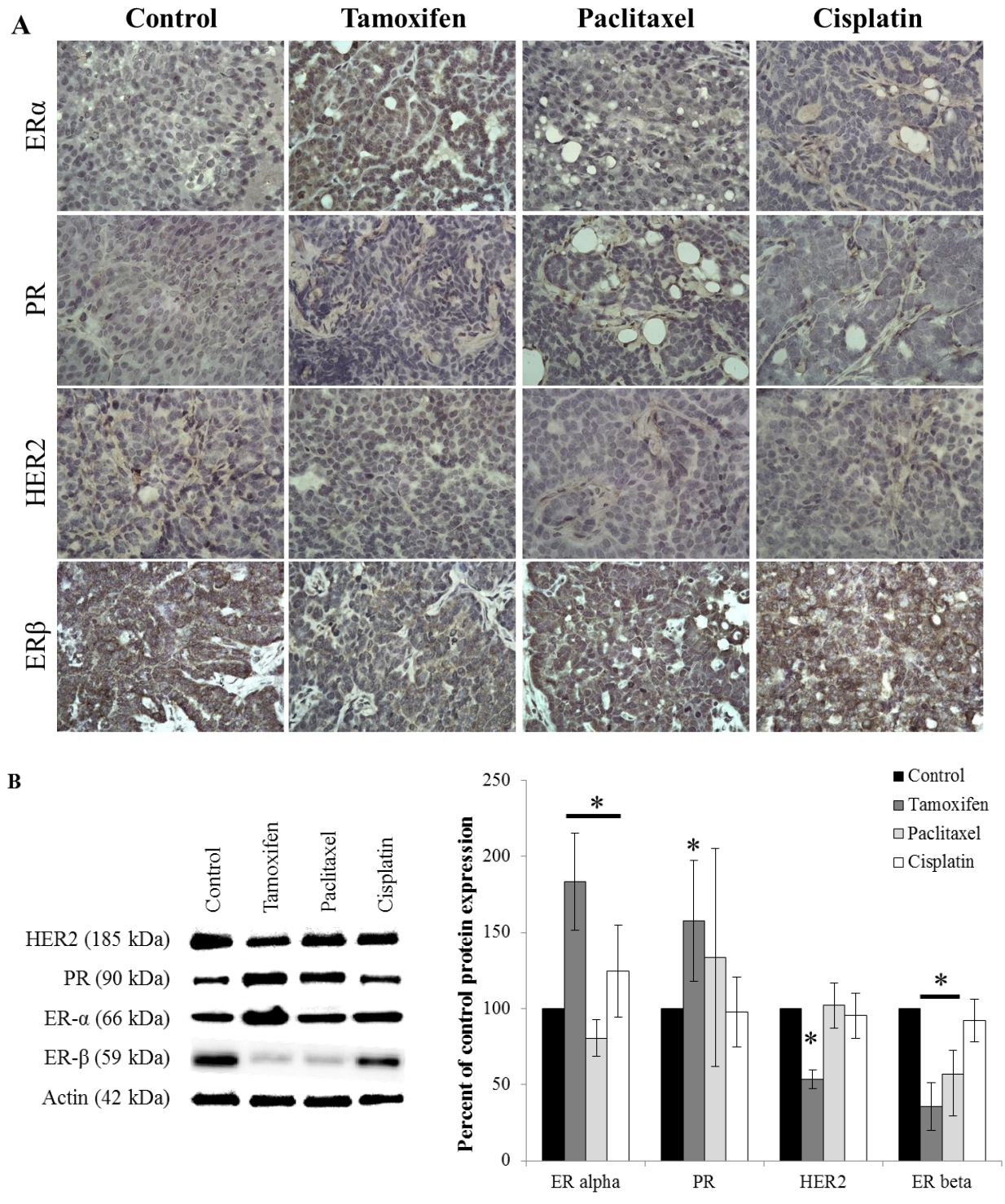

Figure 7.1 Male PyVT phenotype during early tumor development. A) Immunohistochemistry of tumor phenotype from PyVT males during tumor development. Paraffin-embedded sections stained with antibodies against estrogen receptor (ERα and β), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER-2) at the early stage of development. Proteins staining: brown, counterstaining: blue (hematoxylin). Images represent only 1 of 6 animals per group at a 60X magnification. B) Representative Western blot (n=1) for hormone receptor expression in tumors isolated from male PyVT. Graphical representation shows the percent of control protein expression determined by pixel intensity of ERα, PR, HER2, and ERβ in PyVT male tumors treated with DMSO (control), tamoxifen (40 mg/kg), paclitaxel (10 mg/kg) or cisplatin (3.5 mg/kg) via 7 IPs during early tumor development. n = 4. * *P*-value < 0.05 compared to control.

7.3.2 Effect of cisplatin on early development of PyVT mice

Tumor growth over a 14 day period with cisplatin treatment every other day indicates a significant effect of treatment on neoplastic development during the Early stage of tumor formation (Figure 7.2). The initial tumor volume for all mice was 66.86 ± 21.99 mm^3. There was a significant difference in tumor volumes between DMSO and cisplatin treated mice from day 8 to day 14 (Figure 7.2A). The final tumor volume for the control DMSO treated mice was 293.33 ± 71.39 mm^3, while the cisplatin treated mice had a final volume of 100.18 ± 105.78 mm^3. The change in tumor volume over the 14 day period shows a significant reduction of 215.59 mm^3 with cisplatin treatment compared to control (*p*-value = 0.00044; Figure 7.2B). This is a 90.71% difference between the overall changes in tumor growth after treatment with cisplatin.

Control mice have a total of 10 mammary fat pads that developed tumors during the treatment period. Treatment with cisplatin significantly reduced the number of tumors developed compared to the control group (*p*-value < 0.0001; Figure 7.2C). A total of 4 tumors developed with cisplatin treatment during the 14 day period.

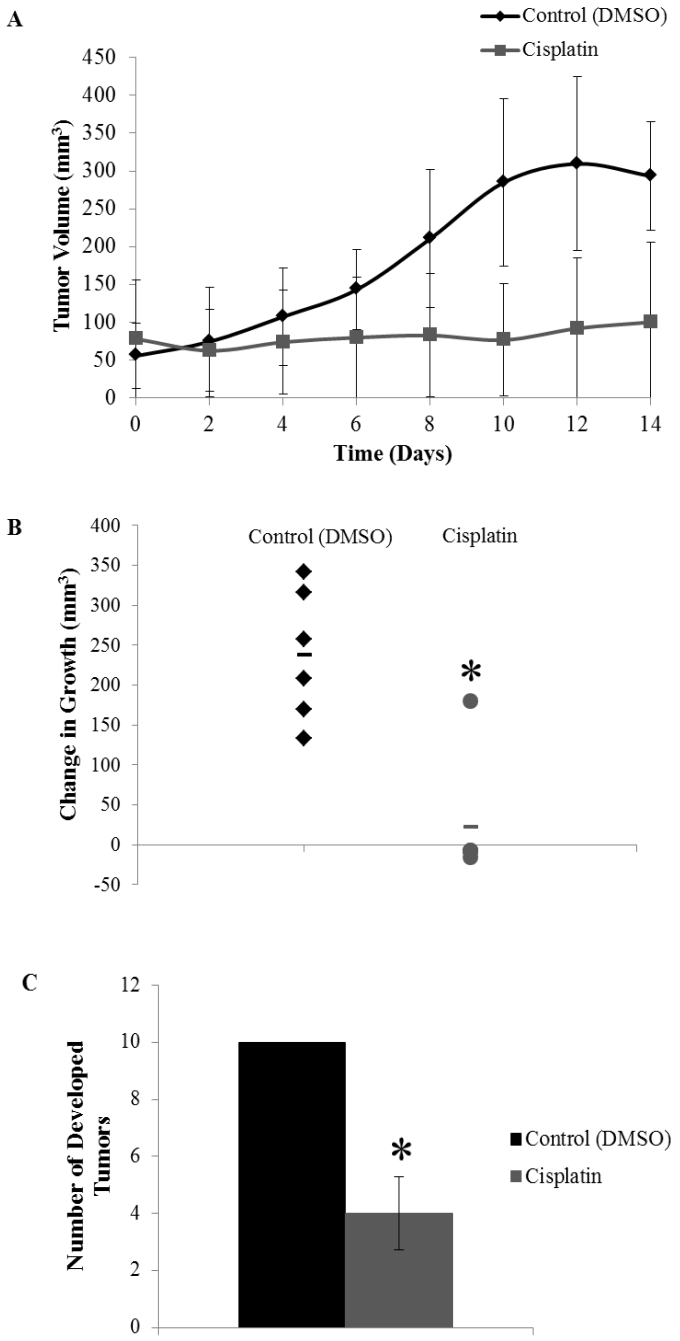

Figure 7.2 Tumor growth (mm^3) in PyVT male mice treated with cisplatin. Tumors measured in two dimensions with calipers every 2 days prior to administration of treatment during early tumor development. A) The tumor size is expressed over the 14 day treatment period for the DMSO (control) and cisplatin (3.5 mg/kg) treated PyVT mice. Days 0-12 represent the days of the 7 IP injections, day 14 represents the end of the study with measurements prior to tissue harvest. B) The overall change in tumor size after treatment with

DMSO (control) or cisplatin (3.5 mg/kg) via 7 IP injections. C) Number of developed tumors per PyVT male mouse during development. Tumors identified grossly during the early stage of tumor development after a 14 day period with either treatment with DMSO (control) or cisplatin (3.5 mg/kg) via 7 IP injections. n = 6. * *P*-value < 0.05 compared to control.

7.3.3 Effect of paclitaxel on early development of PyVT mice

The initial tumor volume for all mice treated with either paclitaxel or DMSO was 137.34 ± 93.05 mm^3. There was not a significant difference in tumor volumes between treatment groups at any time during the 14 day treatment period (Figure 7.3A). The change in tumor growth over the 14 day treatment period indicated that paclitaxel did not significantly attenuated tumor growth (*p*-value = 0.029, Figure 7.3B). The control mice had an overall tumor growth of 237.68 ± 65.78 mm^3, while those mice treated with paclitaxel grew by an additional 75.10 ± 134.57 mm^3. Paclitaxel treatment did significantly reduced the tumor burden by an average of 2.5 tumors (*p*-value = 0.00022, Figure 7.3C).

7.3.4 Effect of tamoxifen on early development of PyVT mice

The tamoxifen treated mice and respective control mice began treatment with an initial tumor volume of was 127.91 ± 122.53 mm^3. Tamoxifen treatment did not affect tumor growth compared to the control animals during the treatment period (Figure 7.4A and 7.4B). There was, however, a significant reduction in tumor burden by approximately 4 tumors (*p*-value = 0.00478, Figure 7.4C).

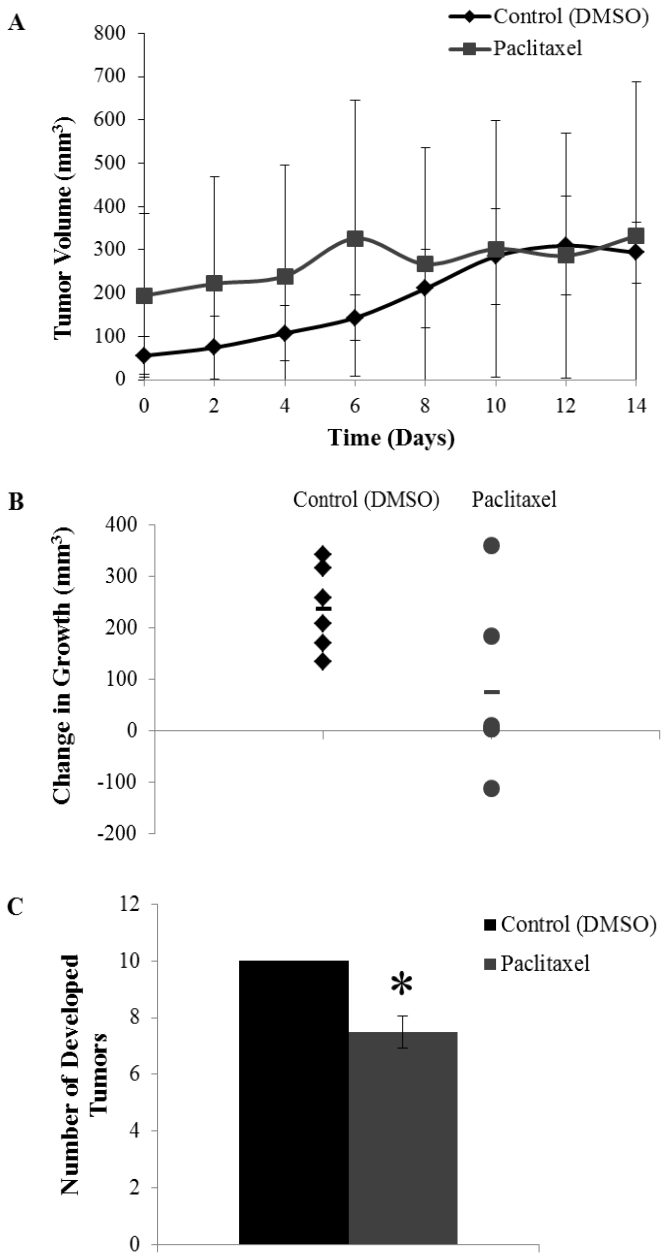

Figure 7.3 Tumor growth (mm^3) in PyVT male mice treated with paclitaxel. Tumors measured in two dimensions with calipers every 2 days prior to administration of treatment during early tumor development. A) The tumor size is expressed over the 14 day treatment period for the DMSO (control) and paclitaxel (10 mg/kg) treated PyVT mice. Days 0-12 represent the days of the 7 IP injections, day 14 represents the end of the study with measurements prior to tissue harvest. B) The overall change in tumor size after treatment with DMSO (control) or paclitaxel (10 mg/kg) via 7 IP injections. C) Number of developed tumors per PyVT male mouse during development. Tumors identified grossly during the early stage of

tumor development after a 14 day period with either treatment with DMSO (control) or paclitaxel (10 mg/kg) via 7 IP injections. n = 6. * *P*-value < 0.05 compared to control.

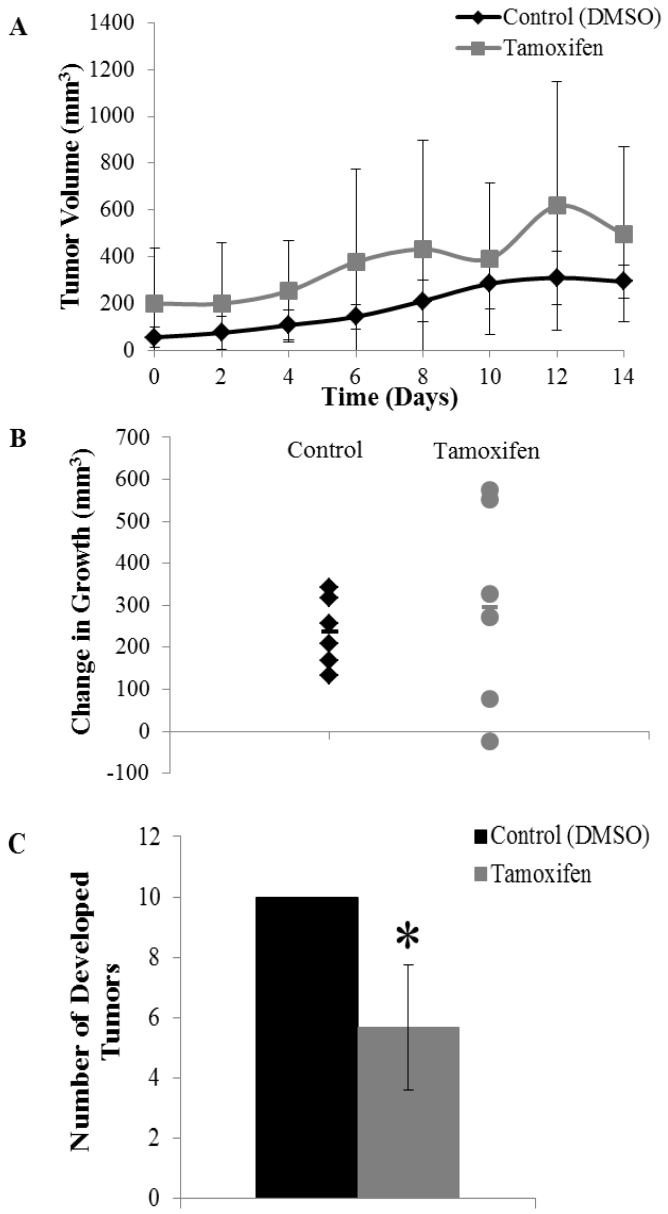

Figure 7.4 Tumor growth (mm^3) in PyVT male mice treated with tamoxifen. Tumors measured in two dimensions with calipers every 2 days prior to administration of treatment during early tumor development. A) The tumor size is expressed over the 14 day treatment period for the DMSO (control) and tamoxifen (40 mg/kg) treated PyVT mice. Days 0-12 represent the days of the 7 IP injections, day 14 represents the end of the study with measurements prior to tissue harvest. B) The overall change in tumor size after treatment with DMSO (control) or tamoxifen (40 mg/kg) via 7 IP injections. C) Number of developed tumors per PyVT male mouse during development. Tumors identified grossly during the early stage of

tumor development after a 14 day period with either treatment with DMSO (control) or tamoxifen (40 mg/kg) via 7 IP injections. n = 6. * *P*-value < 0.05 compared to control.

7.3.5 Protein expression in isolated PyVT tumors

Immunoblot analysis was conducted to determine the expression of molecular markers for male breast cancer, including Bcl-2, caspase-3, survivin, and cyclin D1. Bcl-2 has been shown to correlate with low mitotic cell count and lower grade tumors, suggesting it can be an important biomarker in male breast cancer pathogenesis [27]. Tamoxifen increased Bcl-2 by 65% compared to control (*p*-value = 0.0131; Figure 7.5B). Paclitaxel significantly reduced the expression of Bcl-2 by 22% (*p*-value = 0.0346). There was an insignificant decrease of 17% in Bcl-2 expression with cisplatin treatment. Immunohistochemistry indicates strong positive staining of Bcl-2 in control and treated tumors (Figure 7.5A). Tamoxifen treated tumors appear to have a stronger staining of Bcl-2, confirming western blot analysis.

The ability to induce apoptotic signaling in the tumor cells was determined by analysis of caspase expression. Cisplatin increased caspase 3 expression by 55% (*p*-value < 0.0001) compared to control tumors (Figure 7.5B). Tamoxifen and paclitaxel treatment did not change the expression of caspase 3. Control tumors have very weak positive staining for caspase 3 (Figure 7.5A). All antineoplastic compounds show an increase in positive staining compared to control, but cisplatin and tamoxifen have the strongest positive staining for caspase 3.

Tumor cells are highly proliferative; therefore we explored the expression of cyclin D1 as a biomarker for cell proliferation. Cyclin D1 is a key cell cycle regulator in which over expression results in rapid progression from G1 to S phase in mitosis [28]. Analysis of cyclin D1 expression indicated that tamoxifen significantly increased expression by 96% (P-value = 0.0115), while paclitaxel and cisplatin did not significantly alter expression levels (Figure 7.5B).

Control tumors had weak positive staining for cyclin D1 (Figure 7.5A). Tamoxifen treated tumors had a strong positive staining, while paclitaxel and cisplatin treated tumors had weak positive staining for cyclin D1 (Figure 7.5A).

Proteins that inhibit apoptosis provide protection for tumor cells against cytotoxic compounds. Survivin is a member of the inhibitors of apoptosis protein family that is expressed during embryogenesis and in tumor cells as an anti-apoptotic protein that is capable of regulating mitosis [29-31]. Survivin is highly expressed in a range of tumors and its expression correlates with both accelerated relapse and chemotherapy resistance [32]. Immunohistochemistry of the control tumors showed strong positive staining for survivin (Figure 7.5A). Tumors treated with tamoxifen and cisplatin had weak positive staining for survivin compared to paclitaxel and control tissue. Tamoxifen and cisplatin treatment significantly reduced survivin expression by 77% (p-value = 0.0019) and 48% (p-value < 0.0001), respectively (Figure 7.5B).

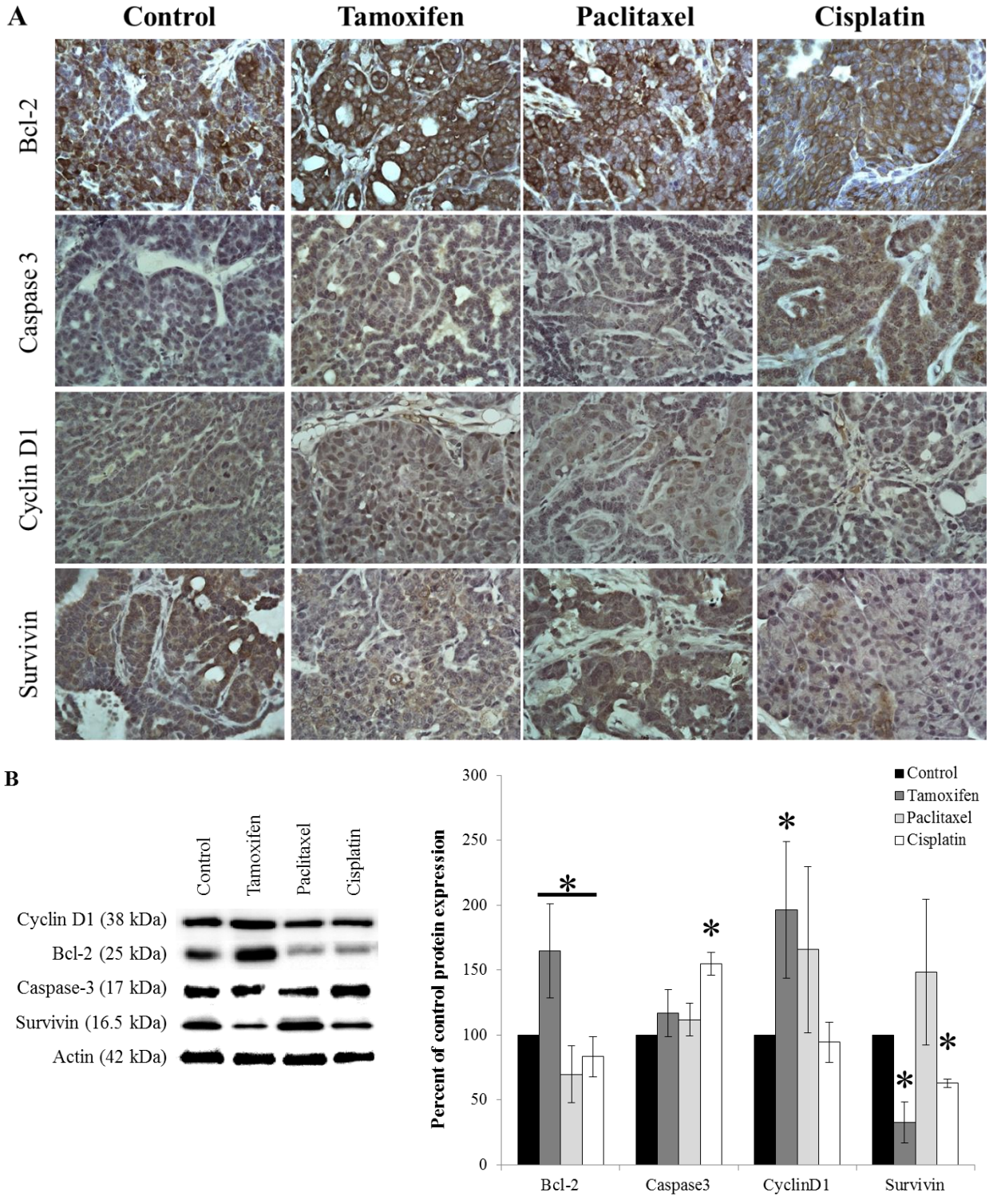

Figure 7.5 Expression of molecular markers Bcl-2, caspase 3, Cyclin D1, and survivin. Tumors isolated from PyVT males treated with DMSO (control), tamoxifen (20 mg/kg), paclitaxel (10 mg/kg), or cisplatin (3.5 mg/kg). A) Representative Western blot (n=1) of protein

expression in tumors isolated from male PyVT. Graphical representation shows the percent of control protein expression determined by pixel intensity of Bcl-2, caspase 3, Cyclin D1, and survivin in PyVT male tumors during early tumor development. n = 4. * *P*-value < 0.05 compared to control. B) Immunohistochemistry of tumors from PyVT males. Paraffin-embedded sections stained with antibodies against Bcl-2, caspase 3, Cyclin D1, or survivin from PyVT males. Proteins staining: brown, counterstaining: blue (hematoxylin). Images represent only 1 of 3 samples per group at a 60X magnification.

7.3.6 Pathological review of male mammary tumors

Pathological review of the mammary tumors was conducted for each treatment group. The tumors were categorized as either early carcinoma or late carcinoma. An early carcinoma consisted of a moderately demarcated neoplasm with closely packed nests and acini of proliferative neoplastic epithelial cells with cellular atypia and invasion of the basement membrane. The late carcinoma featured poorly demarcated neoplasms composed of sheets of tightly packed nest/acini of neoplastic epithelial cells separated by fibrovascular stroma with a loss of mammary architecture, increased proliferation, and more extensive invasion.

Control tumors were characterized by focal hyperplasia with normal lymph nodes and adipose tissue or early carcinoma lesions. Treatment with cisplatin and paclitaxel had no significant change in the histopathology, and tumors remained characteristic of early carcinoma. Mice treated with tamoxifen developed tumors characteristic of late carcinoma, suggesting an increase in malignancy due to treatment.

7.4 Discussion

The transgenic PyVT model was used here for translational studies of male breast cancer due to its clinically relevant pathology and protein expression profile. Models for male breast cancer are limited, but the lack of clinical patients make this transgenic mouse model vital to

gain an understanding about the pathological differences in male breast cancer compared to the female counterparts. The male PyVT model has provided an opportunity to address the efficacy of treatment using the antineoplastic drugs: tamoxifen, paclitaxel, and cisplatin. Tamoxifen is better known as a selective estrogen receptor modulator (SERM) because of its multiple activities [33]. Due to the high hormone receptor positivity in male breast cancer, tamoxifen is the standard adjuvant therapy. Paclitaxel, approved by the Food and Drug Administration (FDA) to treat ovarian and breast cancer, is a first-line treatment of female metastatic breast cancer. Paclitaxel promotes the stable assembly of microtubules and inhibits their de-polymerization [34], therefore interfering with the normal function of microtubules and preventing the progression of the cell cycle [35]. Cisplatin is a platinum based chemotherapy drug used to treat a variety of cancers through the formation of platinum-DNA adducts that induce cell cycle arrest [36, 37]. These compounds have drastically different modes of action. Here we determined the efficacy of each in attenuating mammary tumor growth in a male model.

This study shows that early male mammary tumor formation is significantly attenuated by cisplatin treatment, while tamoxifen and paclitaxel have no effect on male PyVT tumor growth. This suggests that treatment options may need to be reconsidered for male breast cancer patients. Tamoxifen is the current primary treatment, but results indicate that it does not efficiently attenuate tumor growth in male PyVT mice. Cisplatin was shown to be the more efficient antineoplastic tested, suggesting a switch in compounds for primary treatments of male breast cancer patients. Interestingly all three antineoplastic compounds, cisplatin, paclitaxel, and tamoxifen reduced the total number of developed tumors, indicating they could have a chemopreventive property.

Hormone receptor expression is the primary way to profile mammary carcinomas. The male PyVT mouse tumors were shown to be ERα/β+, PR+, and HER2+. Tamoxifen treatment increased the expression of both ERα and PR, while resulting in a decreased expression of HER2 and ERβ, indicating an inverse relationship between the ER isoforms due to tamoxifen treatment. Hormone sensitive tumors are typically based on the expression of only ERα. The role of ERβ in the pathology and treatment of breast cancer remains largely unknown. The functions of these two estrogen receptors are drastically different in response to both estrogen and anti-estrogenic compounds [38, 39]. Multiple reports show that estrogen exposure to ERα expressing breast cancer cells lead to an increase in proliferation, while exposure to ERβ expressing cells, either alone or in combination with ERα, results in inhibition of cellular proliferation [40-42]. This suggests that ERβ may function more as a tumor suppressor than a tumor promoter. The expression of ERβ has been found in 47% of breast tumors classified as ERα negative [43]. Interestingly paclitaxel also reduced the expression of ERβ, but did not affect expression of ERα, PR, or HER2. The clinical relevance of ERβ expression is uncertain, multiple studies indicate a correlation with improved survival [44, 45], while others suggest little correlation or worse prognosis [46, 47]. These findings emphasize the need to further elucidate the function of ERβ in the pathology and treatment of breast cancer.

Tumor regression occurs when the rate of cellular proliferation is less than the rate of cellular death. To determine the apoptotic signaling due to the antineoplastic treatments, caspase 3 expression levels were measures in all tumors. Cisplatin was the only compound to induce a significant increase in caspase 3, indicating induction of apoptosis due to treatment. This is observed grossly by a significant reduction in tumor size. Tamoxifen and paclitaxel did not have an observable apoptotic effect. The inhibitor of apoptosis, survivin, was measured in tumor tissue

after treatment with the antineoplastic compounds. There was a significant decrease in the expression of survivin after tamoxifen or cisplatin treatment. This indicates that both treatments reduce the anti-apoptotic signals, thus promoting cellular death. Cisplatin treatment therefore promotes and induces apoptosis, resulting in a decreased tumor volume. Tamoxifen treatment only promotes apoptosis, thus sensitizing the cell for apoptotic signaling, but not directly leading to cellular death.

Bcl-2 is another regulator of apoptosis, but has been shown to be an important biomarker in male breast cancer pathogenesis, correlating with low mitotic cell count and smaller tumors with lower histological grade [27]. Bcl-2 is also a critical biomarker for female breast cancer in predicting patient survival [48]. In this study tamoxifen was shown to increase Bcl-2 expression, while paclitaxel and cisplatin decreased expression levels compared to control tumors. The expression of survivin with other anti-apoptosis genes like Bcl-2 reduces apoptosis of cancer cells [49]. Bcl-2 expression is expected to correlate with survivin, which was observed with cisplatin treatment in which both proteins have a reduced expression compared to control tumors. Interestingly tamoxifen treated tumors show an inverse relationship between Bcl-2 and survivin. Bcl-2 proteins are found as dimers in the outer mitochondrial membrane [50]. The physiological role of Bcl-2 expression and control of homeostasis in normal breast tissue is suggested to involve upregulation by estradiol and down-regulation by progesterone [51]. Additionally in breast cancer cells estradiol was shown to stimulate, while progesterone inhibited Bcl-2 protein expression [52]. This suggests Bcl-2 regulation through the hormone receptors, specifically the upregulation of Bcl-2 by ERα and down-regulation by PR. Here we show that tamoxifen treatment increases expression of ERα and decreases ERβ, which may lead to the increased expression of Bcl-2.

Highly proliferative neoplastic cells and high histological grade tumors have been associated with increased expression of cellular markers for proliferation. Abnormal cyclin D1 expression is common in female breast cancer [53, 54]. Prognostic relevance of the proliferative proteins cyclin D1 and Ki67 have not been confirmed in male breast cancer patients. Cyclin D1 has been shown to be overexpressed in 77% of male breast cancer [27]. Interestingly, in a cohort of male breast cancer patients cyclin D1 overexpression was predicative of better patient survival, while high levels of cyclin A and B expression increase the risk for breast cancer related death by 2-3 fold [55]. In male PyVT mice, treatment with tamoxifen increased cyclin D1 expression without significantly altering tumor growth. This conflicts with the findings from Nilsson *et al.* while affirming that cyclin D1 may not be a suitable molecular marker for male breast cancer.

The differences in the physiology of female and male patients with breast cancer warrant a different treatment approach, specifically with regards to hormonal therapy. More research is needed to determine the role of anti-estrogenic compounds such as tamoxifen in male breast cancer treatment. Scattered reports are insufficient to recommend treatment guidelines. Based on the known differences in the biology of male and female breast cancer, it is only practical to consider treatment options that do not alter the hormonal signaling or at least not as a single treatment agent of breast cancer in men.

This study offers a unique opportunity to study the effects of certain antineoplastic drugs in a male mammary tumor model. The results show the effects of treatment with three accepted antineoplastic drugs that have not been effectively assessed in the male system due to low clinical occurrence rates of male breast cancer. The results of this study provide valuable

information toward the better understanding of male breast cancer and may help guide treatment decisions.

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Chapter 8 – General Discussion

8.1 Summary

GJIC and connexin expression are inversely correlated with neoplastic development. The synthesis of the substituted quinolines PQ1 and PQ7 has introduced a new class of anticancer compound that function as gap junction enhancers. In vitro modeling introduced these compounds as potent inhibitors of neoplastic cell proliferation and reduced viability. Here we introduced PQs to mouse models of mammary carcinoma to determine their efficacy in a biological system.

PQs were first used to examine the effect of a combinational treatment with antineoplastic compounds cisplatin, paclitaxel, and tamoxifen. These gap junction enhancers were previously shown to increase cellular communication; therefore, it was hypothesized that they could be utilized to increase the efficacy of commonly used antineoplastic compounds through the bystander effect. Tamoxifen was observed to have severe side effects in the xenograft model, and was then removed from this study. The efficacy of cisplatin was increased when used in combination with PQ1, but not PQ7, while both PQs increased the efficacy of paclitaxel when used in combination. Interestingly PQs alone were shown to be effective anticancer treatments in the xenograft model. Isolation of the T47D tumor showed that PQ treatment induced an upregulation of connexin and active caspase expression, and a reduction in proliferative marker expression.

In search for a new model of mammary carcinoma development to use as a preclinical tool, the female PyVT mouse was characterized. This model proved to be both similar to human female breast cancer in morphology and molecular marker expression (i.e. ER), while having multiple stages of tumor development and consistent lung metastasis. Using the antineoplastic

compounds cisplatin, paclitaxel, and tamoxifen, the efficacy of currently marketed drug treatments were tested in this model system. Paclitaxel and cisplatin were both ineffective treatment options at all stages of development, while tamoxifen significantly attenuated tumor growth in the Pre and Early stages of tumor formation. This may be due to the fact that the MMTV promoter sequence of the transgene is an estrogen responsive element and tamoxifen inhibits estrogen signaling.

To determine if the first generation substituted quinoline was an effective anticancer drug in the PyVT model; mice at each stage of development were treated via 7 intraperitoneal injections at 25 mg PQ/kg body weight for a period of 14 days. PQ1 was shown to attenuate tumor growth and reduce tumor burden at all stages of development, as well as reduce the frequency of metastasis. This indicates PQ1 is a promising chemotherapeutic compound for the treatment of breast cancer. At the molecular level, PQ1 was able to modulate connexin expression profiles during tumorigenesis, possibly playing a role in its efficacy as an anticancer compound.

The substituted quinoline PQ7 was then tested for its bioavailability via intraperitoneal injection in normal healthy mice. From a single injection of 25mg PQ/kg body weight, this compound was systemically distributed to all organs and was present within the system even at 36 hours post injection. Additionally there was no change in morphology of the tissue. Interestingly there was a temporary reduction in Cx43 after exposure in a majority of the tissue, which was normalized by 36 hours post injection. From these results we concluded that treatment every 48 hours would be appropriate for continual exposure. It is still uncertain if the Cx43 reduction observed in the normal tissue is detrimental to the animal.

The work was continued to test the effects of PQ7 treatment in the PyVT model. Again mice at each stage of development were treated via 7 IP injections at 25 mg PQ/kg body weight for a period of 14 days. PQ7 successfully attenuated tumor growth at the Pre stage of tumor formation. There was no significant effect on tumor growth during the Early or Late stages. This observation suggests that PQ7 could be used as a chemoprotective compound rather than a chemotherapeutic compound. Interestingly PQ7 was also able to alter the connexin profile during tumorigenesis, but differently than previously seen with PQ1 treatment.

The PyVT model was further utilized to test the efficacy of antineoplastic treatment on male mammary carcinoma development. Due to the lack of large cohorts of male breast cancer patients to study therapeutic responses, treatment options are dependent of female studies. Here was show that the male PyVT mice can be utilized as a preclinical model for male breast cancer. At the Early stage of tumor development paclitaxel and tamoxifen were ineffective chemotherapeutic options, while cisplatin effectively reduced tumor burden and attenuated tumor growth. Tamoxifen was shown to alter the hormone receptor expression profile of the tumors, and increase the expression of the proliferative marker Ki-67. This indicates that endocrine therapy for male breast cancer may exacerbate the disease rather than providing relief. Cisplatin treatment was shown to upregulate the expression of caspase-3, while reducing survivin expression leading to an increase in apoptotic signaling. These results suggest that the current treatment options for male breast cancer should be revised.

8.2 Discussion

Proper preclinical models are necessary for drug development. Here the utilization of xenograft model of female breast cancer in nude mice and a transgenic model of spontaneous mammary carcinoma development were studied. Both models provide valuable information

about the efficacy of treatment options for breast cancer patients. Xenografts are commonly used as preclinical models, while the PyVT model has only previously been used in genetic studies. This transgenic mouse may be a vital preclinical tool to test chemopreventive mechanisms and new anticancer compounds for multiple stages of tumor development. It is advantageous to have a model system that develops not only the primary tumor, but also metastasizes frequently and predictably. The PyVT mouse model studies above indicate that with an increase in malignancy, there is an epithelial-mesenchymal transition, predictable changes in expression of anti-apoptotic proteins (survivin), proliferative markers (Ki-67 or cyclinD1), and connexins. This model may be used in an array of new molecular and therapeutic studies, such as determining the role of connexins in tumorigenesis.

Current antineoplastic drugs were tested for their efficacy at attenuating tumor growth in both models. Xenograft tumors were most significantly affected by cisplatin treatment, while paclitaxel was ineffective and tamoxifen produced fatal side effects. In the PyVT model the therapeutic benefits of each compound were determined in both the male and female mice. Interestingly the antineoplastic that significantly attenuated tumor growth varied between the sexes despite the tumors being genetically similar. This indicates that the difference in endocrinology between males and females may be vital in determining the response to drug treatment.

From bioavailability studies, PQs appear to be well distributed through the tissue via oral gavage and intraperitoneal injection. Pathological review of the normal tissues from PQ treated mice in all the studies indicated a limited acute toxicity. Additionally the observed reduction in Cx43 seen with exposure of normal tissue to PQ did not appear to affect functionality or morphology. Due to the short period of exposure and monitoring post exposure, long term

toxicity was not determined. Future research should identify the long term effects of treatment and the effects of chronic exposure to low levels of the compounds.

Treatment with the gap junction enhancers PQ1 and PQ7 altered the connexin expression of the neoplastic cells. In the T47D xenograft model Cx26, Cx32, and Cx43 were upregulated, which was not surprising due to in vitro results previously reported. In the PyVT model there was a change in the connexin profile in normal tumorigenesis due to PQ treatment. Both PQs reduced the expression of Cx46 in all stages of development. Interestingly PQ1 treatment induced an upregulation of Cx43 expression during all stages, while PQ7 treatment elevated Cx43 in the Pre stage and reduced it in the Late stage of tumor development. This difference in connexin modulation may provide insight into why these two compounds differ in efficacy in this model. The therapeutic response to PQ1 seen in the Early and Late stages may be due to the increased expression of Cx43, which is not observed in PQ7 treated mice. The PQ induced effects on Cx43 expression may be key to the efficacy of these compounds in vivo.

This dissertation has provided information on the effects of PQs in animal models of breast cancer, and the therapeutic efficacy of cisplatin, paclitaxel, and tamoxifen in a spontaneous model of mammary carcinoma. Xenograft and transgenic models both show PQ1 and PQ7 are effective anticancer compounds, which increase connexin expression and activate the apoptotic signaling pathway. The exact mechanism of action for PQs is not known. Future research will focus on determining the signaling pathways activated or inhibited by PQ treatment, specifically those that lead to an increase in active caspase-3 and the upregulation of connexin expression.

8.3 Future direction

Future studies will determine mechanism of action of PQ1 in mammary carcinoma cell lines. Two cell lines have already been established from the late stage of male and female PyVT mice, named MMC2 and FMC2u respectively. Although a number of breast cancer cell lines have been established, no breast cancer cell lines have been reported derived from a transgenic mouse model of spontaneous mammary carcinomas. Additionally there is no male breast cancer cell line for in vitro research. The establishment of such lines will provide another method to study tumor growth and therapeutic effects despite genetic predisposition for cancer formation.

Appendix A - Combinational treatment of PQs and paclitaxel

A.1 Results

A.1.1 T47D xenograft tumor growth in nude mice

Mice were implanted with 17ß-estradiol (1.7 mg/pellet) before the injection of 1 x 10^7 T47D breast cancer cells subcutaneously into the inguinal region of mammary fat pad. Seven days post cell injection, animals was randomly assigned to each treatment group. Animals were treated intraperitoneally with DMSO as a control of drug solvent, paclitaxel, PQ1, PQ7, or a combining treatment of paclitaxel and PQ in a total volume of 100 µl. All PQ treatments significantly reduced tumor size (**Figure A.1**) compared to control and paclitaxel alone. Paclitaxel alone did not significantly reduce mammary tumor growth. Combinational treatment of paclitaxel with PQ1 and PQ7 showed a 2.3- and a 2.2-fold increase in efficacy compared to paclitaxel alone, respectively (p-value < 0.0001). PQ1 treatment alone led to a 2.5-fold decrease in tumor growth compared to paclitaxel treatment alone (p-value < 0.0001). Combinational treatment of paclitaxel with PQs showed greater reductions in tumor volume compared to paclitaxel alone.

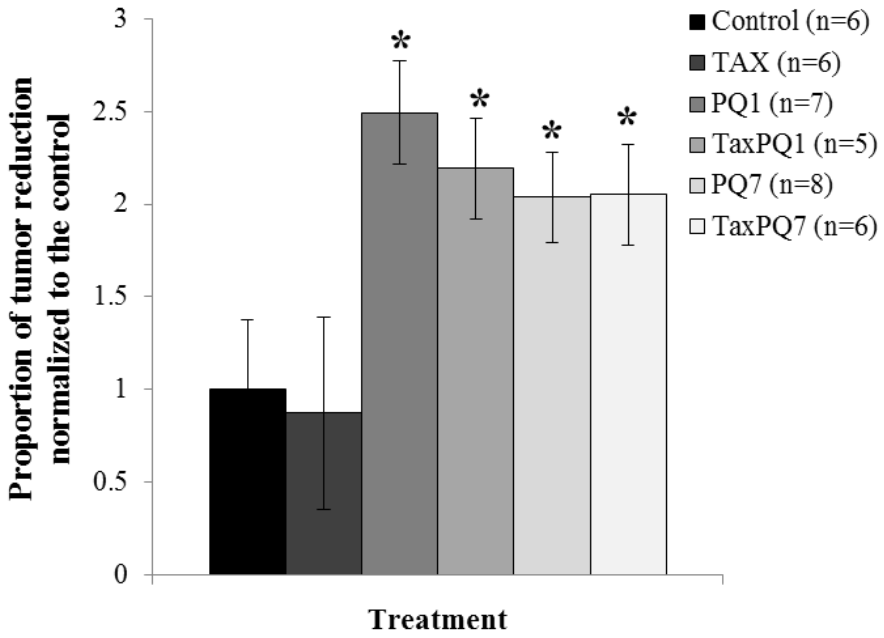

Figure A.1 Xenograft tumor growth in nude mice. The graphical presentation shows the proportion of tumor reduction normalized to control after 7 IP injections of DMSO, paclitaxel (TAX; 10mg/kg), PQ1 (25mg/kg), PQ7 (25mg/kg), or a combination of paclitaxel and PQ. * *P*-value is <0.05 compared to control and paclitaxel treatments.

A.1.2 Protein expression of xenograft tumors

Adjacent cells are able to exchange small molecules to maintain homeostasis, such as anti-growth signals and apoptotic factors, through gap junctions. There are three major connexin proteins that are expressed in the human breast tissue: Cx43, Cx32, and Cx26 [1]. Immunoblot analysis and immunohistochemistry were conducted on T47D xenograft tumors harvested from mice after 7 intraperitoneal injections of DMSO, paclitaxel, PQ1, PQ7, or a combining treatment of paclitaxel and PQ. Tumors treated with PQ showed an increase in connexins (Cx 43, 32, and 26), compared to controls (**Figure A.2A**). PQ1 and PQ7 treatment increased Cx43 expression in T47D xenografts by a 1.9- (*p*-value = 00111) and 2.9-fold (*p*-value = 0.0042) increase compared to control, respectively. (*p*-value = 0.0117).

The apoptotic signaling pathway induced by the treatment was determined by analysis of caspase expression. There are two major signaling pathways that lead to apoptosis: 1) intrinsic activation through the release of pro-apoptotic effects (i.e. caspase-9) [2] and 2) extrinsic activation through the interaction of death receptors with associated proteases, which leads to the activation of caspase-8 [3]. Data indicate there is an increase in the expression of the apoptotic proteins, caspase-3, with treatment compared to control (**Figure A.2B**). Paclitaxel treatment increased capase-9 expression by 5.1-fold (*p*-value= 0.0003) and caspase-8 expression by 3.1-fold (*p*-value= 0.0019) compared to control. PQ treatment alone increased the expression of caspase-8 and caspase-9. The combinational treatment of PQ1 and paclitaxel led to a reduction in caspase-3 and caspase-8 expression compared to PQ1 alone (*p*-value = 0.0375 and 0.0039, respectively), which may explain why this combination did not upregulate caspase-9. Interestingly PQ7 in combination with paclitaxel did not alter caspase-8, possibly due to high variability.

Cyclin D1 is a key cell cycle regulator in which over expression results in rapid progression from G1 to S phase in mitosis [4]. From immunoblot analysis paclitaxel treated mice developed tumors with an upregulation in expression of Cyclin D1 (**Figure A.2C**). Xenografts isolated from PQ7 and both combinational treatment groups did not show a change in Cyclin D1 expression compared to control tissue due to treatment. Cyclin D1 expression in T47D xenografts was significantly reduced with PQ1 treatment compared to control by 20% (*p*-value = 0.0032). This indicates that PQ1 treatment downregulates and paclitaxel treatment upregulates the expression of the proliferative protein Cyclin D1 in xenograft tumors.

Molecular analysis indicates that PQs are more effective anticancer compounds than paclitaxel. PQs are gap junction enhancers that are able to upregulate connexin expression, while

activating both intrinsic and extrinsic apoptotic pathways. Paclitaxel was also able to induce upregulation of caspases, accompanied by an increase in cellular proliferation, and resulting in a lack of therapeutic response. In combinational treatment, PQs and paclitaxel significantly attenuate tumor growth. The hypothesis is that this is due to an increase in GJIC which is suggested by the upregulation of connexin protein induced by PQ treatment, which leads to an increase in the distribution of paclitaxel due to the bystander effect.

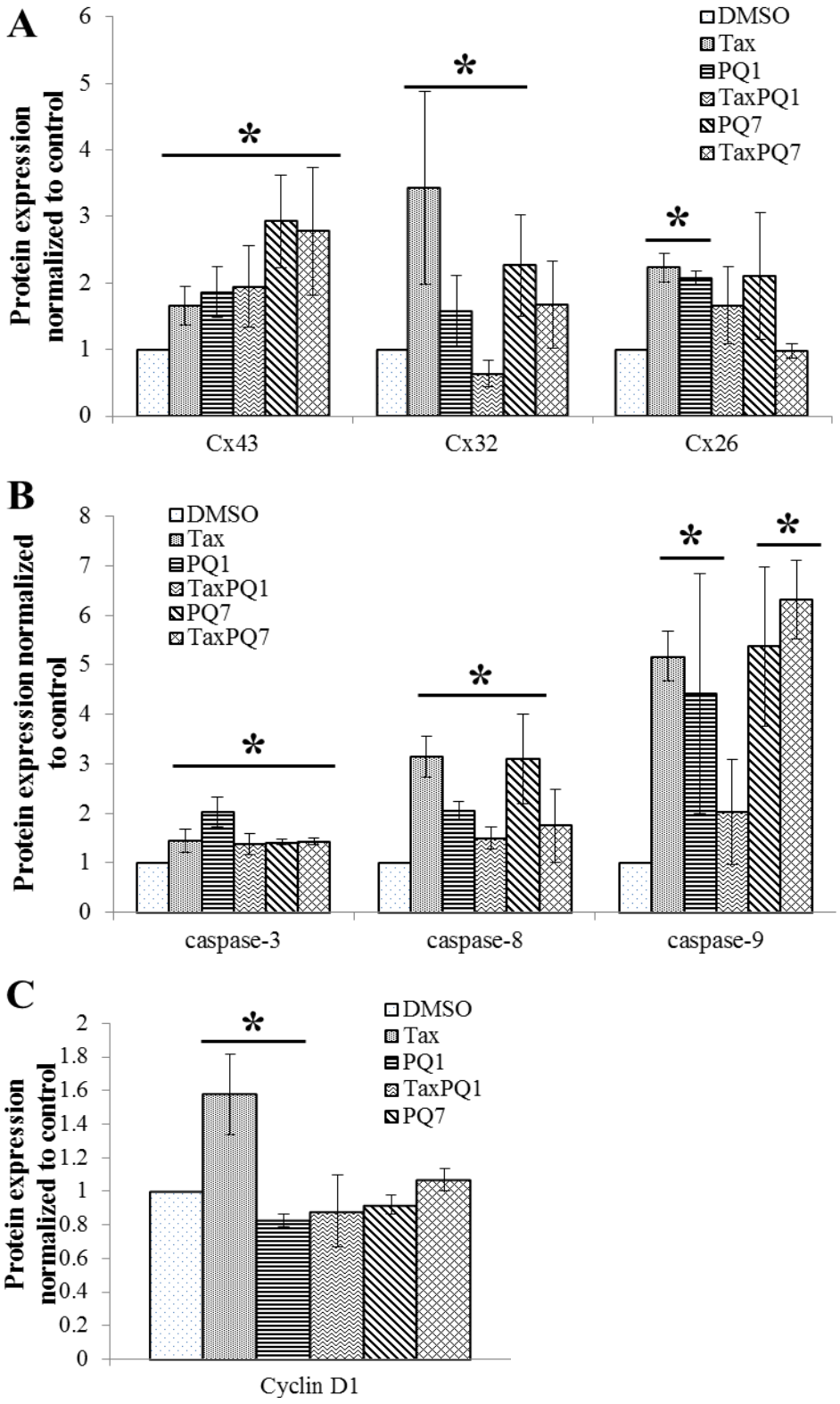

Figure A.2 Analysis of T47D xenograft tumors isolated from nude mice. Raw and graphical representation of protein expression in tumors from Western blot analysis. Fold-pixel intensity of

A) connexins (Cx43,32,26) , B) caspases (-3,-8,-9), and C) Cyclin D1 normalized to loading control in T47D xenograft tumors treated with DMSO (control), paclitaxel (10 mg/kg), PQs (25 mg/kg) or a combination of PQ and paclitaxel via 7 IPs. n = 3. * *P*-value < 0.05 compared to control.

A.1.3 Histological review of organs

Histological examination of the vital organs from xenografted mice showed no significant difference in morphology due to the treatment received.

A.2 Discussion

Here we investigated the influence PQs have on the cytotoxicity of paclitaxel in T47D xenografts. The results indicated that through the combinational treatment of T47D xenograft tumors, the gap junction enhancers were able to increase the efficacy of paclitaxel to attenuate tumor growth. The bystander effect is mediated by GJIC and has previously been shown to play an important role in transferring toxic effects [5, 6]. PQs were able to increase the expression of connexin proteins, suggesting that the attenuation of tumor growth is GJIC-dependent. This suggests that PQs are able to increase the efficacy of paclitaxel through the bystander effect. In addition it has been shown that paclitaxel treatment is related to the impairment of GJIC [7]. The reduced GJIC due to paclitaxel treatment may prevent the cytotoxic molecules or signals from spreading throughout the tumor. Therefore treatment options that regulate gap junctions would be useful in increasing paclitaxel's efficacy.

In epithelial cells the formation of functional gap junctions is dependent on the integrity of the microtubule network [7]. Paclitaxel is a microtubule stabilizer that disrupts membrane organization, membrane trafficking [8, 9], and alters signal transduction [10, 11]. This indicates a

potential disruption of GJIC and connexin transport, which may disturb gap junction assembly and induce an overexpression of connexin proteins.

PQs are promising agents in a gap junction based anticancer therapy. PQ treatment alone reduced tumor size significantly compared to control and paclitaxel treatment. The combinational treatments did not induce a greater therapeutic response than the PQs alone. This suggests that PQs are more effective anticancer compounds than paclitaxel.

This study showed that the combinational treatment of PQs and paclitaxel had a synergistic effect on apoptosis by activating both the intrinsic and extrinsic pathways of apoptosis. This may be due to both the bystander effect and a GJIC-independent mechanism. One hypothesis is that the PQs restore gap junctions therefore allowing more paclitaxel to induce a cytotoxic response and pass to the neighboring cells. This increases the distribution of the antineoplastic through the tumor, triggering apoptosis in more cells by a GJIC-dependent mechanism.

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