ROLE OF GAP JUNCTIONS IN BREAST CANCER

by

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B.V.Sc & A.H., Punjab Agricultural University, Punjab, India, 2002
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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

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2009

Abstract

Gap junctional intercellular channels allow the cells to communicate with each other. A breach in gap junctional intercellular communication (GJIC) affects cell growth and proliferation. In addition, many neoplastic cells exhibit a decrease in GJIC. Many factors that decrease GJIC have been shown to potentiate cancer formation. 2,3,7,8 tetrachlorodibenzo-p-dioxin (TCDD), an environmental pollutant, is a carcinogen; however, its mechanism of carcinogenicity is unclear. Therefore, we examined the effect of TCDD on GJIC in MCF-7, a human breast cell line and normal mammary epithelial cells (HMEC). TCDD showed a decrease in GJIC in MCF-7 cells caused by increased phosphorylation of gap junctional protein, Cx43. PKCα-mediated phosphorylation of Cx43 was confirmed by inhibitor studies using calphostin C. Interestingly, TCDD affected GJIC in HMEC through a novel pathway involving redistribution of Cx43 to the perinuclear membrane. Our studies suggest that TCDD causes decrease in GJIC which could potentially lead to cancer. This also indicates that if GJIC is restored it could decrease cell growth and proliferation. Therefore, we investigated the role of substituted quinolines (PQ1), shown to bind with gap junctional proteins by computational docking. The results showed that indeed PQ1 significantly increases GJIC and exerts anti-tumor effect in human breast cancer cells compared to control without treatment or HMEC. We found an increase in GJIC, growth attenutation and increased apoptosis in T47D human breast cancer cell line. Our studies suggest that PQ1 is a novel gap junctional activator causing a decrease in tumor growth. Since PQ1 alone is effective in decreasing tumor growth in breast tumors, we proposed to test its efficacy with the current drug of choice for breast cancer, tamoxifen. The combinational treatment of tamoxifen and PQ1 showed a significant decrease in cell viability, increase in BAX (Bcl2-associated X), and, increase in caspase 3 activation compared to individual treatments. Hence, combinational treatment of PQ1 and tamoxifen can potentiate decrease in tumor growth. In conclusion, downregulation of gap junctions can potentiate tumor growth while restoration of GJIC can induce apoptosis and decrease tumor growth.

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Thu Annelise Nguyen

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Dedication

I would like to dedicate my dissertation to my loving family- Dad, Mom, Sonu, Bunny, Jiju and the youngest but dearest “Dhruv”. Dhruv, my angel will be always remembered and loved.

Chapter 1 - Review of Literature

1.1.1 Introduction

Human body is a complex network of cells forming tissues/organs and performing specific functions desired at a specific time. The coordinated movements in the body are a balancing act to achieve homeostasis. In a multi-cellular organism, homeostasis is mechanistically governed by three major communication processes – extracellular-communication via hormones, growth factors, neurotransmitters and cytokines which trigger intracellular-communication via alterations in second messages (e.g., Ca^{2+}, diacylgycerol, pH, ceramides, nitric oxide, c-AMP, reactive oxygen species) and activated signal transduction systems to modulate intercellular-communication governed by gap junction channels (Figure 1.1). Cell adhesion and cell-matrix interactions are considered a subclass of intercellular communication molecules (Trosko and Ruch, 1998).

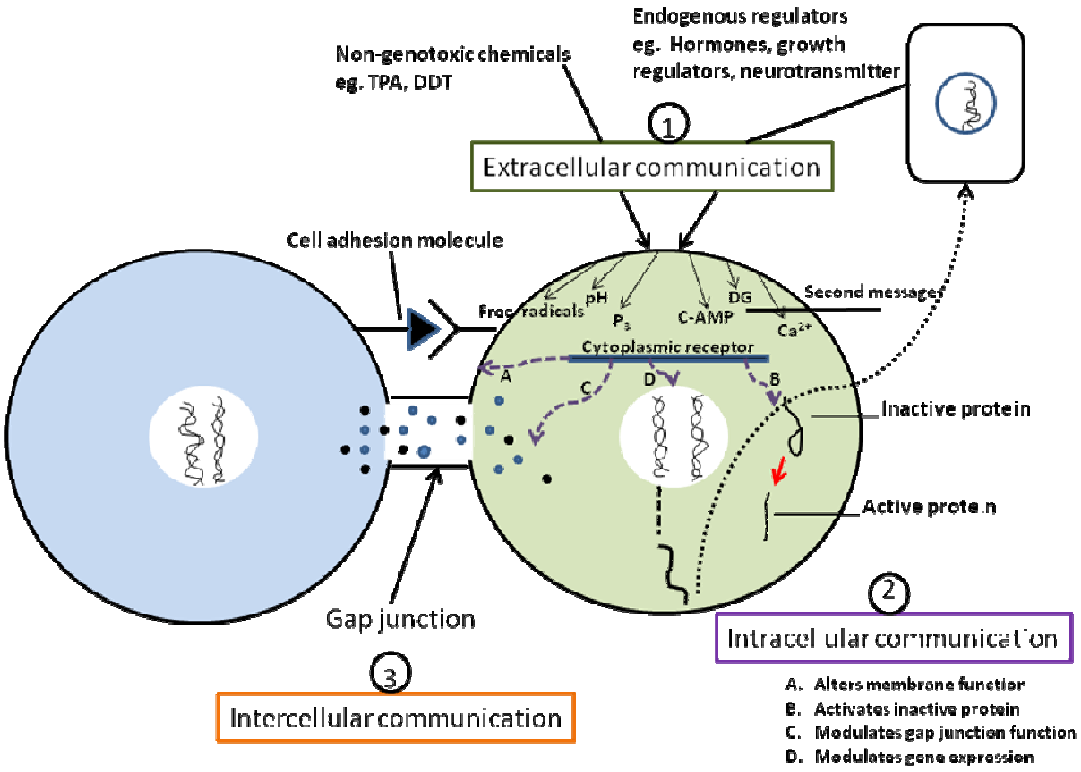


Figure 1.1 Scheme of the postulated link between extracellular communication and gap junctional intercellular communication via various intracellular signal transducing mechanisms (second message) mechanisms.

Diagram illustrates how exogenous nongenotoxic agents can either interfere with, or mimic, endogenous extracellular signals. TPA= 12-O-tetradecanoyl-phorbol-13-acetate, DG= Diacylglycerol, DDT= Dichlorodiphenyltrichloroethane, P3= Phosphate, c-AMP= Cyclic-adenosine monophosphate.

All of these communication processes in a multicellular organism are intimately interconnected to maintain its normal development and health. In effect, these communication processes must control a cell's ability to proliferate, differentiate, apoptose and respond adaptively. Disruption of any one of these three forms of communication could lead to increased or decreased proliferation, abnormal differentiation, increased or decreased apoptosis and abnormal adaptive responses of differentiated cells.

1.2 Intercellular communication

Intercellular communication is governed by linking cells together via intercellular junctions (tight, desmosomes, adherens and gap junctions). These junctions are mostly present in epithelial cells. Desmosomes and adherens both belong to one class of junctions called anchoring junctions.

1.2.1 Tight Junctions

Cells such as epithelial adhere tightly to each other via junctions present on the cell membranes forming a tight seal to provide compartmentalization in the body. Tight junctions are one type of specialized intercellular junctional complex which provides a selectively permeable barrier to diffusion through intercellular space (Figure 1.2). These junctions tightly regulate the diffusion of proteins and macromolecules between the apical and the basolateral surface of the cell. Therefore, tight junctions regulate cell polarity, proliferation, and differentiation. The junctions consist of transmembrane proteins that mediate direct contact between cells, peripheral

membrane proteins that anchor transmembrane proteins to the cytoplasmic proteins (Shin *et al.*, 2006). The three major transmembrane proteins are occludins, claudins and junctional adhesion molecules. Transmembrane proteins associate with peripheral membrane proteins such as zonula occludens which anchor to the cytoplasmic proteins such as actin. Tight junctions are mostly impermeable to macromolecules but allowing the passage of small molecules such as inorganic ions, Ca^{2+}, Na^{+}. However, the permeability to various small molecules varies greatly in different tissues. Tight junctions in the epithelium lining the small intestine, for example, are 10,000 times more permeable to inorganic ions, such as Na^{+}, than the tight junctions in the epithelium lining the urinary bladder. These differences reflect differences in tight junction proteins that form the junctions (Alberts, 2002).

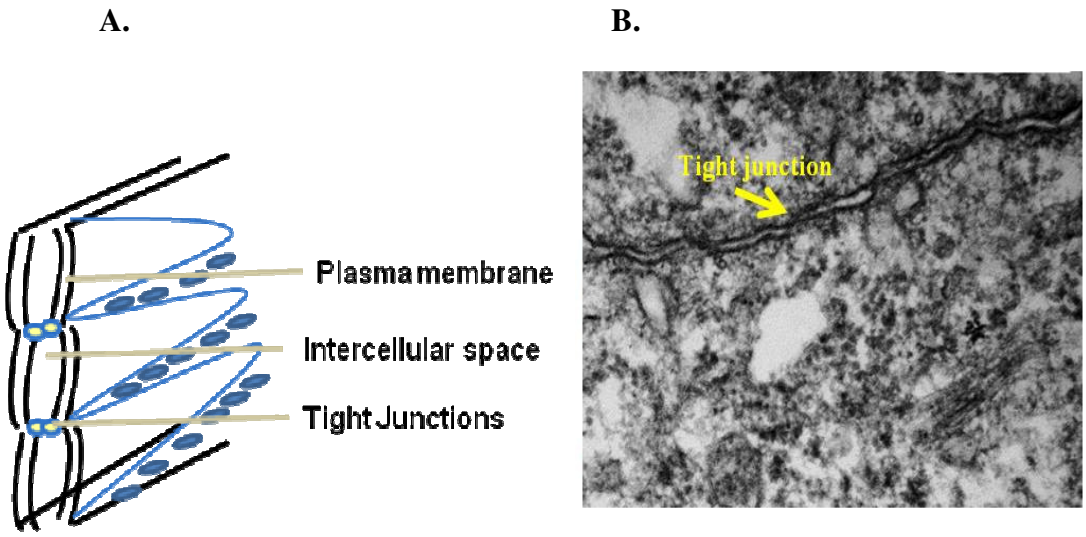


Figure 1.2 Tight junctions.

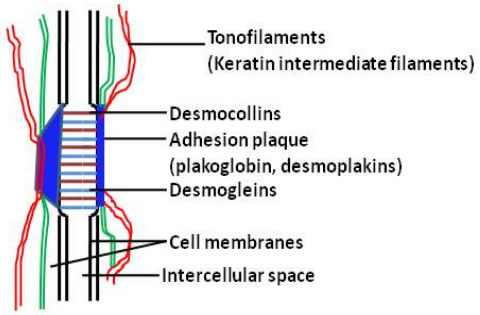
A) Tight junctions do not have space in between them thereby allowing selective permeability. **B)** An electron micrograph of tight junctions in T47D human breast cancer cells.

1.2.2 Desmosomes

Desmosomes are buttonlike junctional complexes which join cell adhesion proteins such as cadherins with the intracellular intermediate filaments. Inside the cell, they serve as anchoring sites for ropelike intermediate filaments, which form a structural framework of great tensile

strength. The particular type of intermediate filaments attached to the desmosomes depends on the cell type such as keratin filaments in most epithelial cells and desmin filaments in heart muscle cells. The junction has a dense cytoplasmic plaque composed of a complex of intracellular anchor proteins (plakoglobin and desmoplakin) that are responsible for connecting the cytoskeleton to the transmembrane adhesion proteins (desmoglein and desmocollin) (Alberts, 2002). Figure 1.3A is a diagram showing the different proteins involved in desmosomes and Figure 1.3B shows an electron micrograph of desmosomes in the heart tissue of a mouse.

A.



Desmosome

B.

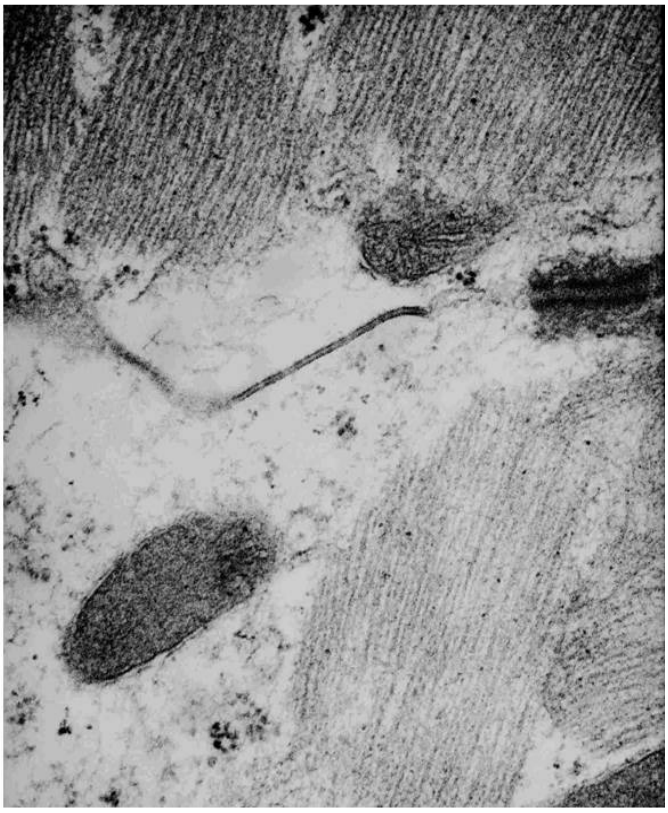
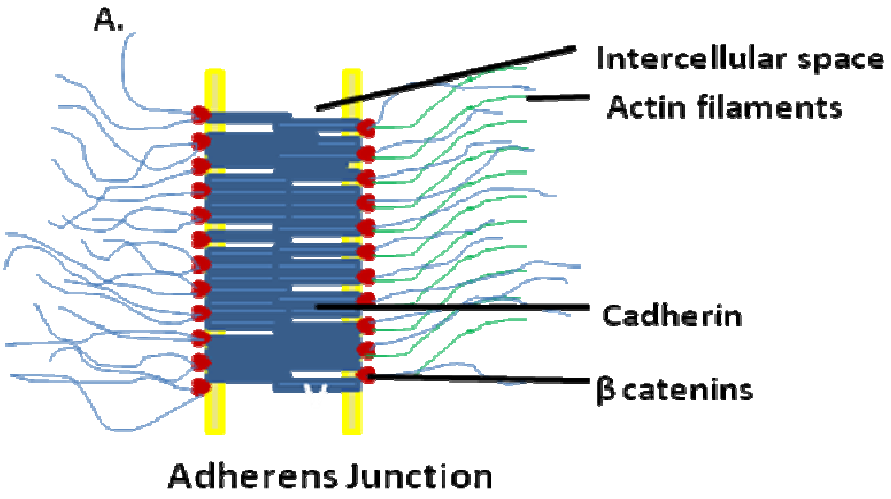


Figure 1.3 Desmosomes.

A) Diagram showing different proteins involved in forming a desmosome. **B)** An electron micrograph of desmosomes in the heart tissue of a mouse. The main function of desmosomes is to hold the plasma membrane of two cells together.

1.2.3 Adherens Junctions

Adherens junction is a type of anchoring junctions which anchor cells together through their cytoplasmic actin filaments. It occurs in various forms such as small punctate or streak-like attachments. But the prototypical examples of adherens junctions occur in epithelia, where they often form a continuous adhesion belt (or zonula adherens) just below the tight junctions, encircling each of the interacting cells in the sheet. Figure 1.4A shows a diagrammatic view of adherens junctions while Figure 1.4B is an electron micrograph of adherens junctions in a heart muscle of a mouse. The transmembrane proteins are composed of cadherins, E-cadherins, N-cadherins, and integrins. Cadherins, E-cadherins, and N-cadherins are proteins that anchor to other cells while integrins function as anchor to extracellular matrix. It plays an important role in the morphogenesis of organs, mediating the folding of epithelial cell sheets into tubes and other related structures.



B.

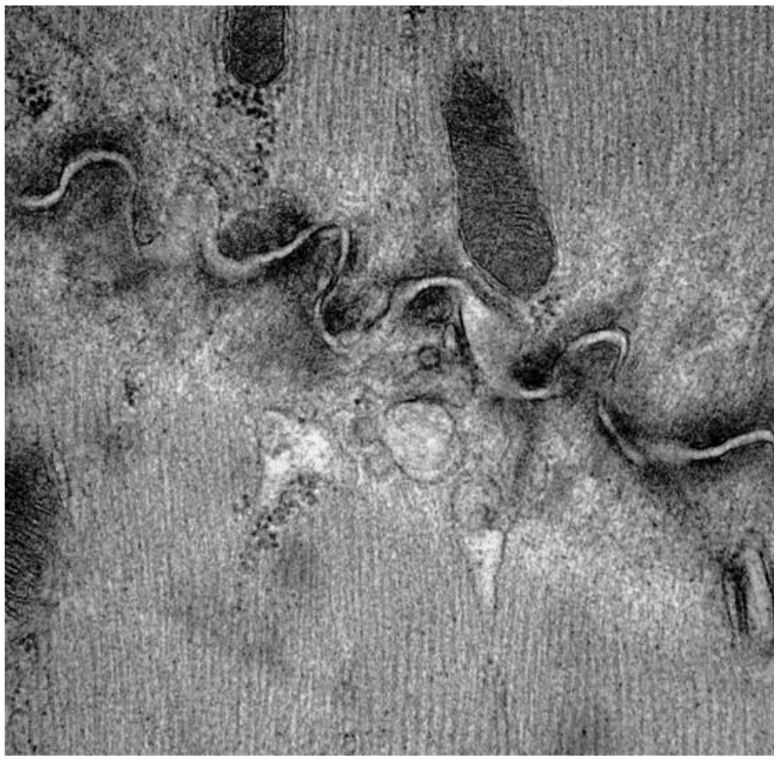


Figure 1.4 Adherens junctions.

A) Diagram showing the proteins involved in forming adherens junctions. **B)** An electron micrograph showing adherens junctions in the heart tissue of a mouse.

1.2.4 Gap junctions

A gap junction is the only intercellular communication which provides a direct connection between the cytoplasmic spaces of the two neighboring cells. It forms a bridge between the cells allowing the passage of molecules from one cell to another. Gap junctions play an important role in cell growth, differentiation and maintaining homeostasis. My dissertation will focus on gap junctions and their significance in our body.

1.3 Gap junctions

Gap junctions are transmembrane hydrophilic channels allowing the passage of molecules less than 1200 Daltons (Da) between the cells through the intracytoplasmic space.

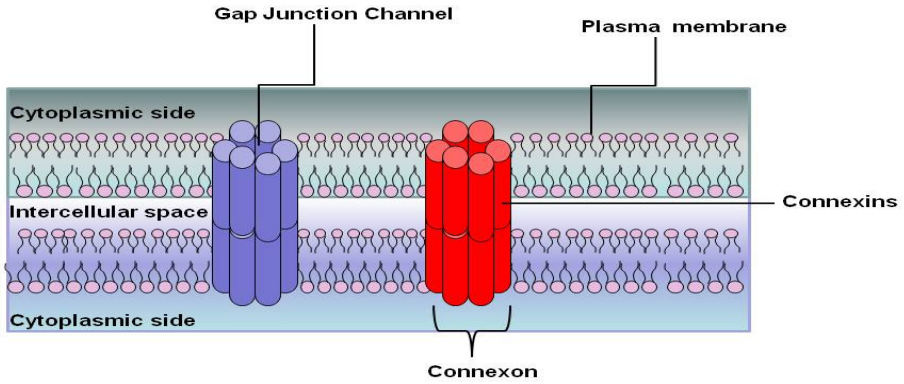


Figure 1.5 Structure of a gap junction.

The figure shows that two connexons (in blue and red) on different plasma cell membrane appose together to form a channel of 1.5 nm diameter. Each connexon is composed of six connexin proteins.

Gap junctions are formed when one connexon (a hemichannel consisting of hexameric subunits of connexins) of one cell docks with a connexon of an adjacent cell as shown in Figure 1.5 (Steel, 1998). The passage of small molecules through gap junctions suggests that the maximal functional pore size for the channel is about 1.5 nm in diameter in mammalian cells (Oyamada, 1998). Small molecules such as cAMP, inositol triphosphate, glucose, and calcium ions can pass while large molecules such as proteins or complex sugars cannot pass through the gap junctions (Figure 1.6) (Alexander and Goldberg, 2003). Gap junctions are the only specialization of the cell membranes which allows the communication between the adjacent cells (Dagli and Hernandez-Blazquez, 2007).

Several hundreds of gap junction channels in the contact regions of the adjacent cells forms an aggregation known as gap junctional plaques (Werner, 1998a). Each gap junctional plaque consists of a large number of connexons. Bukauskas *et al.* showed that the formation of gap junctional plaques is a prerequisite for the functional gap junctions (Bukauskas *et al.*, 2000).

They found that coupling was absent in small plaques but present in larger plaques. Extensive analysis of the exchange of a range of small fluorescent dyes injected into cells has become a popular technique to establish whether cells are functionally coupled by gap junctions. These studies have uncovered charge and size discrimination of the channel within a 0.2 ± 1.0 KDa envelope, mainly in HeLa cells expressing recombinant gap junction channels constructed of various connexin isoforms (Elfgang *et al.*, 1995; Cao *et al.*, 1998; Nicholson *et al.*, 2000).

1.3.1 Structure of connexin

Intercellular junctions between the cells have been given different names as nexus, macula communicans, and gap junction. But the term gap junctions by Revel and Karnovsky got the scientific acceptance despite the contradiction between the function and the morphological characteristics it denotes (Revel and Karnovsky, 1967). The name could be justified by the revelation of the gap observed between the adjacent plasma membranes by electron microscopy (Ogawa, 1993).

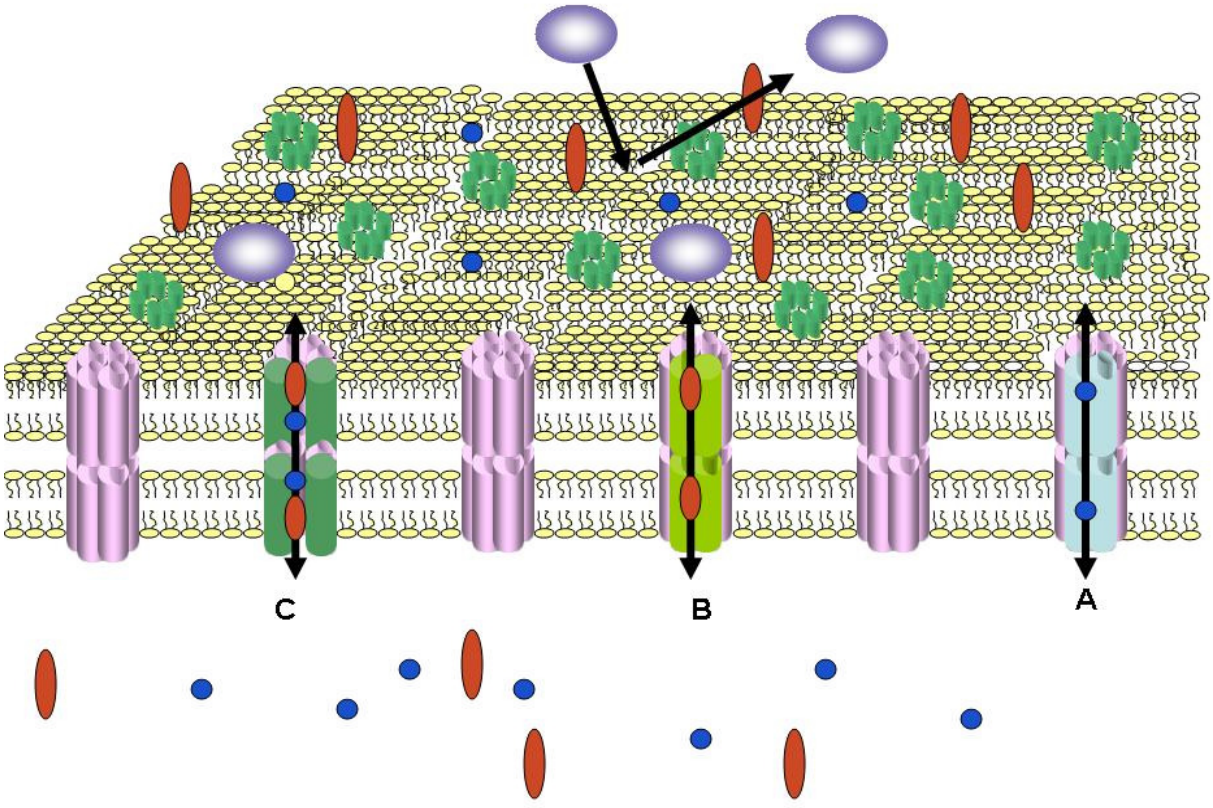


Figure 1.6 Schematic diagram illustrating the selective trans-junctional properties of gap junctions in intercellular communication.

Gap junction channels can be permeable to – **(A)** small molecules **(B)** small molecules with elongated shapes or **(C)** combinations of both molecular shapes. It is also important to note that the charge of the trans-junctional molecule also governs permeability characteristics. Gap junctions are typically not permeable to molecules exceeding 1 KDa (purple) (Laird, 2006).

In 1967, Revel and Karnovsky identified that the cross-sectional profile of gap junctions is displayed as two plasma membranes separated by an intercellular gap, observed by infiltration of lanthanum or horseradish peroxidase between the gap (Revel and Karnovsky, 1967). Freeze-fracture replication and negative staining further showed that gap junctions are patchy arrays of closely packed membrane channels in a hexagonal or polygonal matrix forms. An exact analysis of the structure of gap junction channels was identified through *in vitro* isolation from the liver by sucrose gradient centrifugation and subsequent detergent treatment (Dermietzel *et al.*, 1990). X-ray diffraction experiments performed on the isolated gap junctions suggest that the portion of gap junction channel contributed by each connexon is composed of six subunits, connexins (Cx), together possessing a hexameric structure (Goodenough, 1976). The amino acids derived from the cloned cDNAs helped in predicting the structure of connexins (Hertzberg, 2000). Each connexin consists of four hydrophobic transmembranes and two extracellular and three cytoplasmic loops (Figure 1.7). The N and C termini face the cytoplasmic side of the cell while the extracellular side contains two extracelluar loops (Sosinsky, 1996). The most conserved regions in the connexin are localized to the extracellular loops followed by less conserved region of four membrane-spanning domains and carboxy-terminus, amino-terminus and cytoplasmic loops exhibit variable region. Connexins differ markedly from each other in sequence and length mainly based on their carboxy termini and the cytoplasmic loop (Hertzberg, 2000).

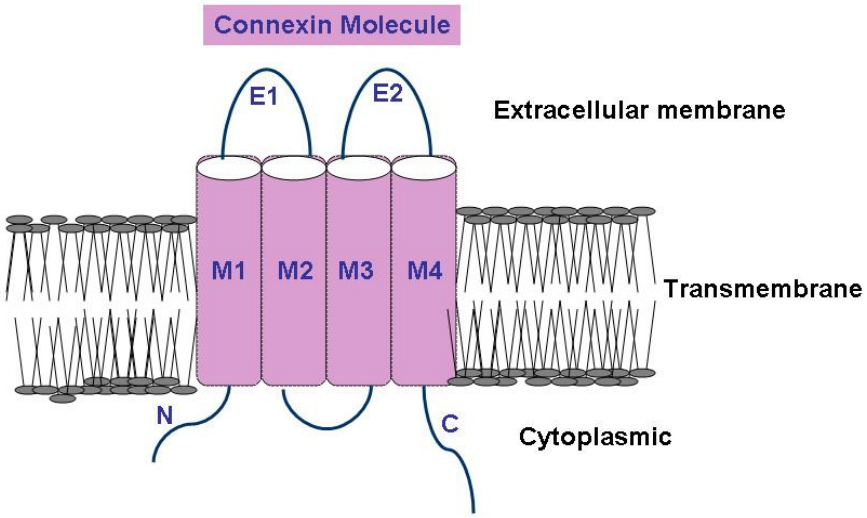


Figure 1.7 Connexin structure.

The cylinders represent transmembrane domains (M1-M4). The loops between the first and second, as well as the third and fourth, transmembrane domains are predicted to be extracellular (E1 and E2, respectively). The carboxy-terminus has many phosphorylation sites (Korkiamaki, 2002).

1.3.2 Nomenclature of connexins

The diverse and ubiquitous distribution of gap junctions is possible due to the fact that the connexin family consists of 20 members in the mouse and 21 members in humans (Laird, 2006). However, connexins are highly related (50-80%) to each other, differing in the cytoplasmic portion sequences (Goodenough *et al.*, 1996). They have molecular masses between 25 and 62 KDa; thus the size of each connexin is used for the nomenclature of the proteins (Evans *et al.*, 2006). In some instances, a prefix including the species name is added while describing the protein. For example, the most abundant and prominent connexin in rat heart is a 43,036 Da protein, hence called rat Cx43. The finding that two or more connexins have the same molecular mass has lead to a decimal point to distinguish them, for example mouse Cx30.3 or mouse Cx31.1 (Saez *et al.*, 2003). Another nomenclature is also used to describe connexins also; however, less commonly used. This nomenclature is based on the degree of relatedness of different connexins. The analysis for the sequence relation and phylogeny of connexin

polypeptide sequences categorize connexins into two groups: α and, β. The α group includes Cx33, Cx37, Cx38, Cx40, Cx43, Cx45, Cx46, Cx50, and Cx56 while β comprises Cx26, Cx30, Cx30.3, Cx31.1, and Cx32 (Kumar and Gilula, 1992; Hertzberg, 2000).

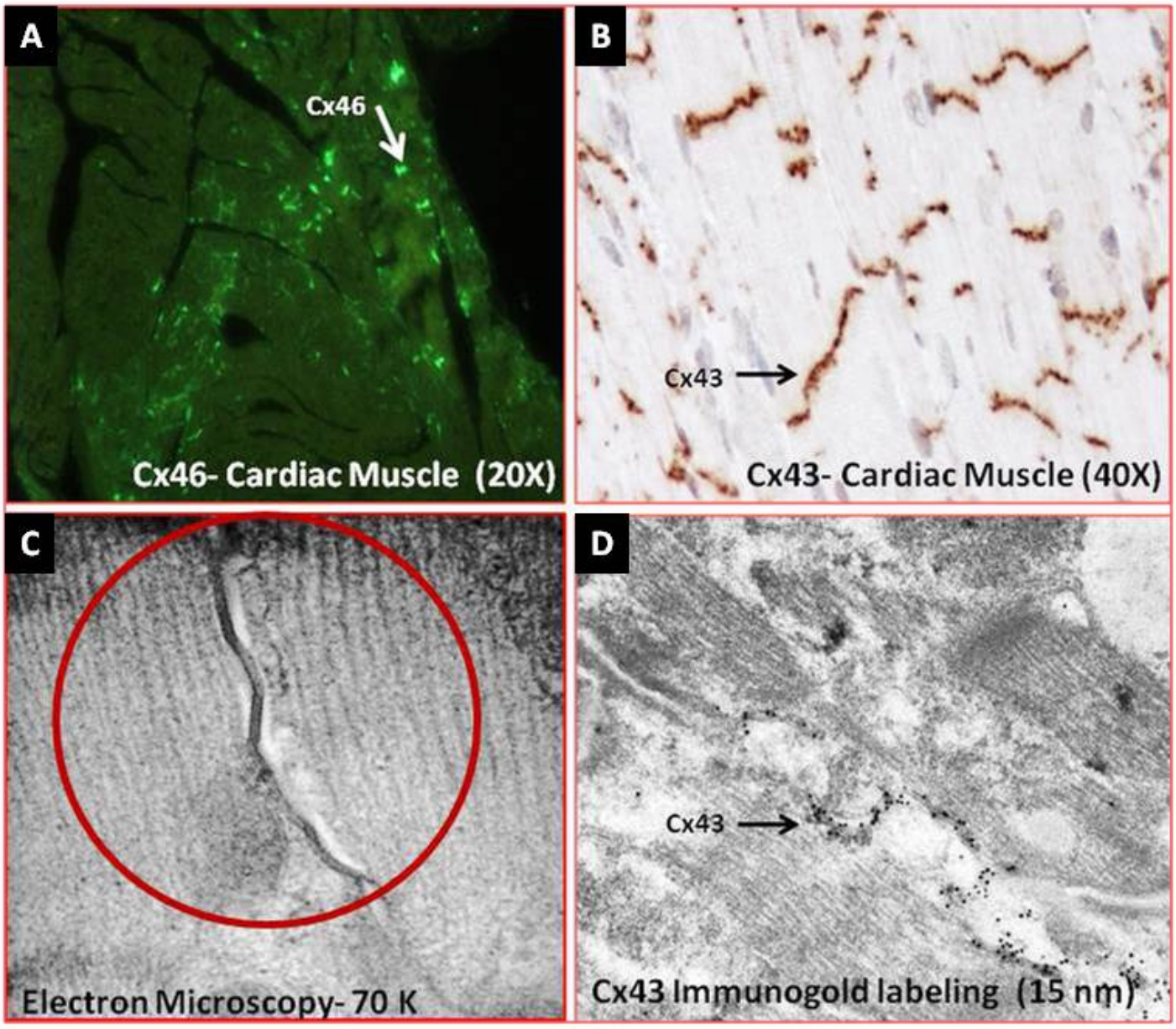


Figure 1.8 (A) Immunofluorescence of Cx46 in a mouse heart tissue. (B) DAB staining of Cx43 in a mouse heart tissue (C) Electron Microscopic view of gap junctions in a mouse heart tissue. (D) Immunogold labeling of Cx43 in a mouse heart tissue (70,000 X).

The function of gap junctions in cell is of utmost importance because of their presence in nearly every mammalian cell type. Gap junctions appear as plaques of varying size, as these unit channels accrete laterally in the plasma membrane and have been studied by electron microscopy of immunogold labelled freeze-fracture replicas (Rash and Yasumura, 1999), by immunocytochemistry (Dupont *et al.*, 2001) and by fluorescently tagged connexins (Bukauskas *et al.*, 2000; Falk, 2000; Rutz and Hulser, 2001). Figure 1.8 shows the expression of Cx46 and Cx43 in mouse heart tissue by different methods.

1.3.3 Tissue specific expression of connexins

Gap junctions are found in very primitive invertebrates like hydra, jellyfish and similar structures known as plasmodesmata are found in plants (Trosko and Ruch, 1998). In vertebrates, gap junctions are found in all cell types except red blood cells, platelets, spermatozoa, and skeletal muscle fibres. Nevertheless, gap junctions are present in the progenitors of these cell types (Rosendaal *et al.*, 1994; Constantin *et al.*, 1997; Proulx *et al.*, 1997; Mok *et al.*, 1999). Moreover, it has been observed that different connexins are present in different tissues. Most cells express two or more connexins (Saez *et al.*, 2003). Presence of different connexins in different tissues suggests specific roles of different connexins. For example, keratinocytes express at least Cx26, Cx30, Cx30.3, Cx31, Cx31.1, and Cx43. Cardiomyocytes express Cx43, Cx40, and Cx45 and hepatocytes express Cx26 and Cx32 (Table 1.1) (Laird, 2006). Collectively, co-expression of multiple connexin family members within the same cell type allows for possible compensatory mechanisms to overcome the loss or mutation of one connexin family member (Laird, 2006). Different connexins do show selective permeability. Cx26 has been shown to form smaller pore size gap junction channels than Cx32. Cx43 gap junctions were shown to readily pass lucifer yellow dye than Cx45 gap junctions (Steinberg *et al.*, 1994). Cx43 appears to be the most predominant connexin in terms of the number of tissues expressing it and the concentration

present in tissues (Hertzberg, 2000). Cx43 mRNA is particularly abundant in heart, relatively lower in mammary gland, ovary, lens epithelium, kidney, and uterus (Hertzberg, 2000).

1.3.4 Factors regulating gap junctions

Gap junctional intercellular communication (GJIC) is regulated at many levels, ranging from gene regulation, gap junction assembly formation, gating at the plasma membrane and gap junction degradation. Two major kinetic courses of GJIC control have been discussed – fast control (millisecond range) and the long term control (hour range).

Mouse connexins	Representative tissue/organ	Representative cell type
Cx23		
Cx26	Liver, skin	Hepatocytes, keratinocytes
CX29	Brain	Oligodendrocytes
Cx30	Skin	Keratinocytes
Cx30.2	Testis	Smooth-muscle cells
Cx30.3	Skin	Keratinocytes
Cx31	Skin	Keratinocytes
Cx31.1	Skin	Keratinocytes
Cx32	Liver, nervous	Hepatocytes, Schwann cells
Cx33	Testes	Sertoli cells
Cx36	Retina, nervous	Neurons
Cx37	Blood vessels	Endothelial cells
Cx39	Developing muscle	Myocytes
Cx40	Heart, Skin	Cardiomyocytes, Keratinocytes
Cx43	Heart, Skin	Cardiomyocytes, Keratinocytes
Cx45	Heart, Skin	Cardiomyocytes, Keratinocytes
Cx46	Lens	Lens fibre cells
Cx47	Nervous	Oligodendrocytes
Cx50	Lens	Lens fibre cells
Cx57	Retina, nervous	Horizontal cells

Table 1.1 Representative tissues and cell types where mouse connexin family members are found.

The half life of connexins is very short ranging from 1.5-2 hours. The fast control is referred to as gating which is affected by many factors like H^+, Ca^{2+}, pH, and phosphorylation. Among these actions, phosphorylation at the carboxy termini has been widely studied. Phosphorylation of the N-terminal region of connexins that is cytoplasmically located is not reported (Lampe and Lau, 2000). Most connexin family members are phosphoproteins, except Cx26. Several kinases are known to target connexin proteins, including MAPK (Mitogen-activated protein kinase), PKC (Protein kinase C), PKA (Protein kinase A), and CK1 (Casein kinase 1). Connexin phosphorylation has been implicated in the regulation of intercellular communication through a number of mechanisms, including connexin biosynthesis, trafficking, assembly, membrane insertion, channel gating, internalization and degradation. Cx43 has been the most studied connexin in terms of modification by many kinases (Saez *et al.*, 2003). The different phosphorylation sites identified on the carboxyl terminus are: Ser-368 and Ser-372 phosphorylated by PKC; and Ser-255, Ser-279, and Ser-282 phosphorylated by MAPK (Figure 1.9). Many studies describe the changes in the state of phosphorylation of Cx43 by PKC-dependent pathways. Ser-368 of Cx43 appears to be an important site since mutation of Ser-368 partially prevents the cellular uncoupling induced by phosphorylation caused by PKC in the presence of phorbol ester (TPA) (Liu and Johnson, 1999; Lampe *et al.*, 2000). The closure of gap junctions is either induced by cell trauma (sudden drop in pH, increase in Ca^{2+} level or decrease in the voltage) or physiological regulators like phosphorylation (Werner, 1998b).

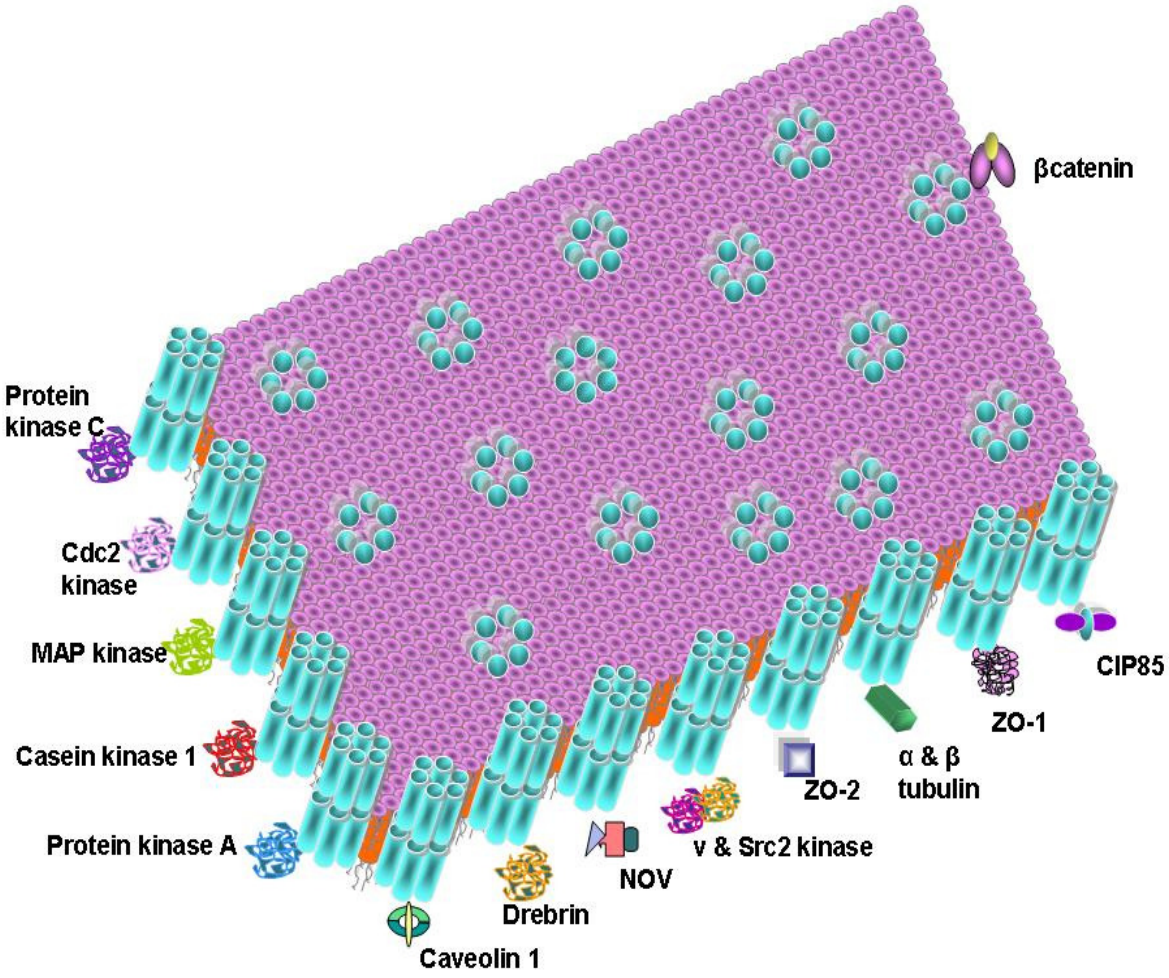


Figure 1.9 Cx43 binding proteins.

Protein kinases known to phosphorylate Cx43 are shown along the top of a schematic diagram of gap junction plaque. A number of scaffolding proteins and proteins of unknown function that have been shown to bind directly or indirectly to Cx43 are shown along the bottom of the gap junction plaque. It is important to note that it is not necessarily expected that all proteins shown here bind to Cx43 while it is a resident of the gap junction plaque. MAPK; CIP85, Cx43-interacting protein of 85 KDa (Laird, 2006).

1.3.5 Functional assays to measure gap junctions

Studies to assess the functionality of gap junctions can be divided into two groups:

A) Measurement of dye transfer

B) Measurement of metabolic cooperation and electrical conductance

A) Measurement of dye transfer

Dye transfer involves measuring the movement of nontoxic dyes from one cell to another. Molecules should be small enough in the range of < 1 KDa to cross through the gap junctions. The techniques to measure dye transfer are:

Microinjection: Microinjection of non-permeable, nontoxic tracers has been the first technique used to identify cell-cell communication (Abbaci *et al.*, 2008). Individual cells are injected with a fluorescent dye in a micropipette having a diameter of 0.2 µ, using appropriate pressure. Since the onset and duration of the tracer is well controlled, kinetic studies can be performed showing the rate of transfer of the tracer from one cell to another (Bruzzone, 1999). Lucifer yellow is the most widely used fluorescent dye for this technique, after replacing fluoroscein as it was shown to cross nonjunctional membranes.

Advantages: It allows selective loading of tracer molecule which permits correlation of the morphological and functional data from an individual cell. The onset and the duration of the tracer injection can be accurately controlled, allowing the measurement of dye transfer from one cell to another.

Limitations: It requires a special instrument to inject the tracer in an individual cell without damaging the cell. Also, this technique is not useful if the tracer is going to be injected in many cells at one time. Results obtained by this method vary with the investigator's experience in impaling cells without causing too much damage and discriminating stained from unstained (autofluorescent) cells.

Scrape Load/Dye Transfer (SL/DT): Scrape load dye transfer is a very simple technique in which monolayers of adherent cells are scraped in the presence of a membrane-permeable dye, which becomes incorporated into the cells at the line of cut by mechanical perturbation of the

cell membrane. The distance at which the fluorescent dye diffuses during a certain period away from the scrape line is indicative of GJIC. Lucifer yellow (MW=443 Da), diameter of 0.5-0.7 nm is the most commonly used dye for SL/DT. Rhodamine dextran with a molecular weight of 10,000 Da is used as a control.

Advantages: This technique does not require any special equipment. It allows the measurement of GJIC in a large number of cells. It is a fast and a simple technique, relatively short period from the time of preparation to obtaining the results.

Limitations: The technique is hardly adequate to investigate small cells or cell assemblies (such as pairs), as well as low density cultures. It is also complicated when the extent of junctional coupling is small or when selected cells have to be individually screened for coupling. This technique is not well suited to three-dimensional systems because this invasive method may introduce uncertainities in quantifying dye transfer rates due to variations in cell-staining intensity after scrape loading (Dakin *et al.*, 2005).

Electroporation: This technique was introduced by Raptis *et al.* proposing the introduction of a non-permeable dye into the adherent cells on a partly conductive slide (Raptis *et al.*, 1994). Cells are grown on a glass slide, half of which is coated with electrically conductive, optically transparent, indium-tin oxide. An electric current which opens the pores is applied on the conductive side of the slide in the presence of Lucifer yellow. The transfer of the dye to the cells on the non-conductive side is measured.

Advantages: The gap junctional permeable dye, Lucifer yellow, can enter into large number of cells with minimal disturbance to the cellular metabolism. It is a satisfactory method for loading a narrow longitudinal strip of cells for subsequent studies on the temporal and spatial spread of fluorescent dyes via gap junctions.

Limitations: This technique cannot be used for non-adherent cells. If cells are grown to high confluency, chances of cells getting detached from the slide when electrode is removed are higher.

Gap-FRAP (Fluorescence recovery after photobleaching): Fluorescent molecules in a small region of the cell are irreversibly photobleached using a high-powered laser beam, and subsequent movement of surrounding nonbleached fluorescent molecules into the photobleached

area is recorded at low power laser over time (Lippincott-Schwartz *et al.*, 2001). FRAP data requires that the bleach event is much shorter than the recovery time and preferably as short as possible (Lippincott-Schwartz *et al.*, 2003).

Advantages: It is a non-invasive technique compared to microinjection and SL/DT. Therefore, the risk of cell injury is minimal to negligible. It enables to quantify and compare GJIC capacity between characterized cell types. Kinetic curves can be established to precisely measure the functionality of GJIC (Abbaci *et al.*, 2008).

Limitations: The gap-FRAP technique requires a laser beam coupled with an epifluorescence microscope or, commonly, a confocal laser-scanning microscope, all of which are sophisticated and expensive instruments. To avoid photochemical and/or thermal cell injury, a highly sensitive light detector should be used; the minimization of illumination thereby reduces the amount of energy absorbed by the specimen (Abbaci *et al.*, 2008).

Preload assay or Parachute assay: This method consists of preloading cells with a gap junctional-permeable dye, such as calcein-AM (green dye), and then letting suspended loaded and unloaded cells form a confluent monolayer together. As suspended loaded cells are added to the monolayer, the parachute assay requires formation of gap junction channels in order to measure subsequent intercellular communication. Depending on the cells and the technique used, the loaded cells/donor cells form gap junctions in 15 min-3 hr with the recipient cells (Goldberg *et al.*, 1995; Ziambaras *et al.*, 1998). To distinguish recipient cells from the donor cells after the passage of calcein-AM, the recipient cells can be labeled with other non-gap junctional dye (Vybrant-DiD, red dye). Therefore, the recipient cells will be having both green and red dye.

Advantages: The parachute technique is a non-invasive method since the integrity of the cells is maintained. Flow cytometry enables simultaneous analysis of a large number of cells (at least 10^4 cells) (Jongen *et al.*, 1991). It ensures objectivity and statistically reliable data.

Limitations: This method is not applicable to the vast majority of adherent cell types in culture (Meda, 2001). By flow cytometry, nonspecific dye transfer of calcein has been observed in many studies (Koval *et al.*, 1995; Fonseca *et al.*, 2004). The preloading assay is also a time-consuming method that is influenced by the section studied (cells in different areas of the dish can behave differently), the resolution, and sensitivity of the microscope equipment (Czyz *et al.*, 2000).

B) Measurement of metabolic cooperation and electrical conductance

Many molecules, exchanging *in vivo* by coupled cells, have various dimensions and charges and hence may be restricted or facilitated in their passage through gap junction channels, depending on the type of connexins (Koval *et al.*, 1995; Meda, 2001). Some investigators have examined the transfer of endogenous compounds by metabolic cooperation (Larson and Sheridan, 1985; Saez *et al.*, 1989; Charles *et al.*, 1992).

Metabolic cooperation by radiolabel nucleotides: A population of donor cells is incubated in the presence of an excess of a radiolabeled precursor (typically uridine) and then co-cultured with unlabeled recipient cells. Under such conditions, quantitative autoradiography enables evaluation of the transfer of the resulting metabolites from loaded to unloaded cells as a function of time. In this type of experiment, coupling is demonstrated by the autoradiographic labeling of the cytoplasm of recipient cells due to the incorporation in their ribonucleic acid of radiolabeled nucleotide synthesized within donor cells and transferred across gap junction channels (Meda, 2001).

Advantages: Direct transfer of radioactive nucleotides allows the evaluation of the permeability of gap junction channels in a highly sensitive way. Contrary to the synthetic dyes, radioactive nucleotides can better represent the transfer of biological molecules across the cell membrane.

Limitations: The measurement of labeled nucleotides and nucleic acids from a damaged donor cell to the recipient cell could be possible. Safety must be rigorously practiced especially dealing with aerosol particles produced during cell sorting procedures, incubation and metabolic labeling.

Measuring electrical conductance by dual patch clamp technique: The dual patch clamp technique is a powerful method for quantitative determination of junctional conductance (Van Rijen, 2001). A freshly made glass pipette with a tip diameter of only a few µm is pressed gently on the cell membrane to form a gigaseal. The membrane breaks when the suction is applied leading to the mixing of the cytoplasm and pipette solution. Currently, the most widely used

dual-voltage clamp method is the double whole-cell voltage clamp with one suction pipette on each cell of a cell pair (Wilders and Jongsma, 1992). The method consists of separately controlling the membrane potential of each cell and measuring the corresponding currents.

Advantages: This method is the most sensitive technique to detect functional gap junctions: a single gap junction channel can be recorded, in contrast to dye transfer. This is the most common method to assay the electrical properties of gap junctions. The technique has been used to study ionic permeability and selectivity of gap junction channels. Measurements of electrical conductance between cells can be used to determine the number of gap junction channels that are open within a given period of time (Alexander and Goldberg, 2003).

Limitations: Analysis of electrical conductance is a slow, labor-intensive, and expensive technique. Obtaining reliable and reproducible measurements of junctional ionic permeability can be difficult since the integrity of coupled cells can be disrupted due to the change of internal ionic environment.

1.4 Breast Cancer

1.4.1 Breast cancer and factors affecting breast cancer

More than 80-95% cases of breast cancer are ductal whereas 8% cases are lobular. Both these types of breast cancers are invasive. Invasive or infiltrating breast cancer also includes another type of breast cancer – inflammatory breast cancer. For 2007, the estimates by American Cancer Society are that 178,480 women in the United States will be diagnosed of invasive breast cancer while an additional 62,030 women will be diagnosed with ductal carcinoma *in situ* (DCIS). For 2008, the estimated US breast cancer cases by American Cancer Society was 179,920 while deaths caused by breast cancer was 40,729.

The three most significant risk factors for breast cancer are – being a woman (male breast cancer occurs at 1% the rate of female breast cancers), age and hereditary susceptibility. Hormonal stimulation (estrogen and progesterone) of the breast tissue co-relates with being a woman, long exposure to exogenous carcinogens co-relates with living, and mutations in the genes corresponds to the hereditary susceptibility. These three risk factors are integrated in life

process that cannot be controlled. The other risk factors include – early menarche, late menopause, nulliparity, postmenopausal obesity, environmental pollutants and hormone replacement therapy. Protective factors are the factors which help in reducing the risk of getting breast cancer. These include – decreasing the lifetime exposure to estrogen (prolonged lactation, ovariectomy, and exercise), maintaining a healthy lifestyle by avoiding intake of alcohol, eating a low-fat diet. Hereditary factors include mutations in the two breast cancer genes – BRCA1 and BRCA2, accounting for less than 10% of the breast cancer cases (Gray, 2009). Women with an inherited mutation in BRCA1 and BRCA2 have 60-82% chance in getting breast cancer in the lifetime (King *et al.*, 2003). This suggests that even beyond the identified mutations, there are other factors like lifestyle and environment which affect the development of breast cancer. For example, female BRCA1 carriers born after 1940 have nearly twice as much breast cancer by ages 40 and 50 as those born earlier (King *et al.*, 2003). In a large twin study conducted, inherited genes were found to contribute 27%, shared environmental factors 6%, and non-shared environmental factors 67% of the breast cancer risk (Lichtenstein *et al.*, 2000).

Hormonal factors accounts for a quarter of breast cancer cases (Seidman *et al.*, 1982). Clearly, being a woman as one of the risk factors points to the steroid hormones circulating in a female body. Estrogen and progesterone are the two major steroid hormones in a female endocrine system. These hormones are required for the normal development of the female body; however, the action of estrogen in breast tissue is quite varied. The most studied mode of action is mediated through the interaction with the estrogen receptor α (ERα). ERα is a nuclear receptor localized inside the nucleus of certain type of cells (endometrial, breast). Upon binding of estradiol or estradiol-like molecules, there is a change in the structure of ERα leading to either homo or hetero- dimerization of the receptor. The ER-complex binds to estrogen-responsive elements in the regions of DNA which regulate the expression of a range of proteins that collectively promote the growth of mammary epithelium. Among the downstream mediators of estrogen action are epidermal growth factor (EGF), its ligand amphiregulin, transforming growth factor α and β (TGF-α and β), platelet derived growth factor (PDGF), and the insulin-like growth factors (IGF-I and IGF-II).

1.4.2 Role of environmental pollutants in breast cancer

One of the risk factors for breast cancer is exposure to environmental chemicals with endocrine property. An estimated 80,000 synthetic chemicals are used today in the United States, and another 1,000 or more are added each year (Seidman *et al.*, 1982). Yet complete toxicological screening data are available for just 7% of these chemicals and more than 90% have never been tested for their effects on human health (Devesa *et al.*, 1995). Mammary gland is largely composed of adipose tissue; therefore, lipophilic compounds are under frequent investigation as these can get accumulated in the breast tissue for years (Warshawsky, 2006). A list of chemicals has been registered by the International Agency for Research on Cancer (IARC) as carcinogens and has also received ratings by regulatory agencies regarding induction of human breast and animal mammary tumors. Some of the chemicals included in the list are bisphenol A, polyaromatic hydrocarbons, tobacco smoke, dioxins, alkylphenols, metals, phthalates, parabens, pesticides such as dieldrin, aldrin, atrazine, benzene, polyvinyl chloride, 1,3-butadiene, ethylene oxide and aromatic amines (Gray, 2008). Some of these chemicals are found in maternal blood, placental tissue, breast milk samples from pregnant women, and mothers who have recently given birth, indicating that maternal burdens of environmental contaminants are being passed on to their young during pregnancy and breastfeeding (Van der Ven *et al.*, 1992; Anderson and Wolff, 2000; Chen *et al.*, 2006; Shen *et al.*, 2007).

A wide variety of chemicals including plastic additives, industrial solvents, pesticides, herbicides, and chemical byproducts of combustion or industrial manufacturing processes, can mimic or alter the activities of the natural hormones, especially the estrogens. The xenoestrogens are members of a larger class of synthetic chemicals known as endocrine disruptors. Endocrine disruptors mimic or disturb the activity of a much wider group of hormones, including the androgens, adrenal hormones, and thyroid hormones. The term “endocrine disruptor” is used to reflect compounds that affect the endocrine system (Gray, 2009). Few examples of endocrine disruptors are dioxin and dioxin like compounds, organochlorine compounds such as DDT, diethylstilbesterol (DES), polychlorinated biphenyl (PCBs), and some other pesticides. Some chemicals, particularly pesticides and plasticizers, such as Bisphenol A are suspected endocrine disruptors based on animal studies (Sciences, 2007).

1.4.3 TCDD and cancer

2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD, also known as dioxin) is an aryl hydrocarbon receptor agonist. TCDD was first listed in the *Second Annual Report on Carcinogens* as reasonably anticipated to be a human carcinogen in 1981 which later on in 2001 was supported by both epidemiological and mechanistic studies indicating causal relationship between TCDD and human cancer. TCDD is very persistent in the environment, but it can be slowly degraded by sunlight (ATSDR, 1998; HSDB, 2003). It has a half-life of 5.8 to 11.3 years in humans (Olson, 1994); therefore, TCDD accumulates in human tissue at a higher rate when compared to most experimental animals following chronic low-dose exposure. Polychlorinated dibenzo-*p*-dioxins (CDDs), including TCDD, are inadvertently produced by paper and pulp bleaching, by incineration of municipal, toxic, and hospital wastes, in PCB-filled electrical transformer fires, in smelters, and during production of chlorophenoxy herbicides (Silkworth, 1996; Schecter, 1997; Schecter *et al.*, 1997). The greatest unintentional production of CDDs occurs from waste incineration, metal production, and fossil fuel and wood combustion (ATSDR, 1998). World Health Organization (WHO), National Institute for Occupational Safety and Health (NIOSH), and the U.S. Environmental Protection Agency (EPA) concluded that dioxin increases the risk for all cancers (ATSDR, 1998). However, the mechanism by which TCDD can promote cancer is unclear.

1.4.4 TCDD and gap junctions

The abilities to stimulate cellular proliferation and inhibit GJIC and apoptosis are thought to be essential properties of promoters (Koblyakov, 1998). The ability of the initial non-metabolized polyaromatic hydrocarbon molecule to stimulate cell proliferation and to inhibit GJIC provides evidence to consider that polyaromatic hydrocarbon mediate the promotion stage in the initial form during carcinogenesis induced by this group of compounds (Sharovskaja *et al.*, 2004). In the present study, we examined the effect of TCDD on gap junctions in human breast cancer cells (MCF-7) and human mammary epithelial cells (HMEC). Our research sheds light on the mechanism of action of TCDD on GJIC in human breast tissue via cell model system.

1.5 Cancer and Gap Junctions

1.5.1 Gap junction in human mammary gland

Gap junctions are present in all cell types of vertebrates, except few dynamic cells (spermatozoa, red blood cells, platelets) and mature skeletal muscle fibers (Abrams *et al.*, 2003). Out of the various organs, heart and mammary gland, have been studied in greater depth for gap junctions. Cx43 has been shown to be widely present in heart and mammary gland. The mammary gland undergoes growth, development and differentiation at many stages of life. Therefore, it requires a precise intercellular communication for its proper development and differentiation. One such communication is gap junction. So far, the human mammary gland has been shown to express only Cx43 and Cx26. The basal myoepithelial cells are consisted of Cx43 while luminal epithelial cells express Cx26 and Cx43 (McLachlan *et al.*, 2007). Studies have speculated that in the resting human mammary gland Cx43 is required to maintain myoepithelial differentiation and more variable Cx26 presence could have a dynamic role in luminal cell proliferation (Lee *et al.*, 1992).

1.5.2 Aberrant gap junctions and cancer

Pioneering studies by Lowenstein and Kanno in 1966 suggested that cancer cells have decreased cellular communication than normal cell confirmed by junctional membrane resistance (Loewenstein, 1966). The lack of gap junctional intercellular communication in cancer could be due to two main reasons – the lack of expression of connexin genes or the aberrant localization of connexin proteins. The lack of expression of genes could be due to lack of transcription caused by hypermethylation of the CpG islands. The downregulation of Cx32 gene expression by hypermethylation of the CpG island of the Cx32 gene has been observed in human renal carcinoma cell line and human renal carcinomas (Yano *et al.*, 2004).

1.5.3 Gap junctions in breast tumors

Cancer is a complex and evolving disease with the formation of defects at multiple genetic steps in a cell. Cancer was the first pathology associated with the defects in gap

junctions. In 1966, Lowenstein and Kanno associated GJIC with cell growth control (Loewenstein, 1966). They observed a decrease in electrical coupling in rat hepatomas compared to normal liver cells. A series of molecular mechanism showed that cancer phenotype is related to loss of coupling (Cronier *et al.*, 2009). Since the most obvious observation in tumor cells phenotype is deregulated growth, the assumption of all the studies is that gap junctions are involved in cell growth control (Mesnil, 2002). Recently, gap junction deficiency has been defined from either lack of gap junctional plaques observed by ultrastructure approaches (electron microscopy and freeze-fracture) or by decrease in GJIC (Mesnil *et al.*, 2005). It is a debatable question that how far connexins are from the primary insult and whether they play a key role in carcinogenesis or simply is it a secondary effect to cancer (McLachlan *et al.*, 2007).

Deficiency of Cx43 gap junction can be used as an independent marker for breast tumors (Laird *et al.*, 1999). Early studies showed downregulation of Cx26 and Cx43 in the primary cells derived from human breast tumor (Lee *et al.*, 1992), rat mammary tumors (Laird *et al.*, 1999), and breast cancer cell lines ((Laird *et al.*, 1999; Singal *et al.*, 2000). Since, the promoter of Cx26 is located within a CpG island, it is speculated that the methylation of these sites could lead to the repression of the gene (McLachlan *et al.*, 2007). Investigational studies by Singal *et al*. found hypermethylation of Cx26; however, inhibition of a DNA methyltransferase did not induce the expression of the gene (Singal *et al.*, 2000). On the contrary, Tan *et al*. found that only one in eight breast cancer cell lines tested was hypermethylated in the Cx26 promoter region but that, in this case, it correlated with a complete loss of mRNA that was recovered after treatment with a DNA methyltransferase inhibitor (Tan *et al.*, 2002). Furthermore, the Cx26 promoter was found to be methylated in >50% of patient tissue samples tested, albeit heterogeneously. These conflicting results suggest that Cx26 may indeed be a tumor suppressor that is inactivated by methylation, but this is likely not the only mechanism to downregulate expression (McLachlan *et al.*, 2007). Upregulation of phosphorylated forms of Cx43 was observed in both myoepithelial cells and transformed luminal cells of *in situ* carcinomas and all cells of invasive breast carcinomas (Gould *et al.*, 2005). Connexins are mostly present on the cell membrane but in some tumors despite an increase in connexins, they are typically retained in the intracellular compartments. Kanczuga-Koda *et al.* report that the level of Cx43 expression, which was cytoplasmic in 90% of the tumors, was positively correlated with advanced histological grade of the tumor (Kanczuga-Koda *et al.*, 2005).

1.5.4 Effect of restoration of gap junctions on cancer

Connexins were described as class II tumor suppressor because the genes encoding connexin protein is not mutated but the protein expression is affected (Lee *et al.*, 1992). Specifically, Cx26 was first deemed as a tumor suppressor in 1991. Mehta *et al.* showed that the transfection of a gene for Cx43 protein in mouse transformed 10T1/2 cells having low GJIC leads to a decrease in the cell growth, reduced saturation density and focus formation suppression (Mehta *et al.*, 1991). Transformed dog kidney epithelial cells after transfection with Cx43 showed restored GJIC, became flatter in cell morphological appearance, decreased expression of cell cycle-regulatory genes such as cyclin A, D1, D2, and cyclin D kinase 5 and 6 (Chen *et al.*, 1995). Transfection of C6 glioma cells with Cx43 (Zhu *et al.*, 1991) and of human hepatoma cell line SKHep1 with Cx32 (Eghbali *et al.*, 1991) provides preliminary evidence that the growth of transfectants and tumors in nude mice decreases. HUVEC endothelial cells were transfected with Cx37 showed increased GJIC and increase in apoptosis measured by caspase assay and DNA fragmentation. Many studies have also shown a decrease in cell or tumor growth in the presence of Cx43 despite forming GJIC (Koffler *et al.*, 2000; Qin *et al.*, 2002). Transfection of Cx26 and not Cx32 in HepG2 cells caused an increase in dye transfer, decrease in the saturation density and tumor growth. HepG2 cells also showed a change in the morphological appearance of the cells from multilayer to monolayer formation (Yano *et al.*, 2001). In another study, transfection of MDA-435C cell line with either hCx43 or hCx26 gene caused a restoration in GJIC, decrease cell growth and smaller size tumor formation in mice (Hirschi *et al.*, 1996). All these studies suggest a potential role of connexin genes as tumor suppressors.

1.5.5 Design and synthesis of quinolines

In search of new activators that enhance GJIC, we collaborated with Dr. Duy H. Hua in the Department of Chemistry at Kansas State University who examined the potential interactions of a number of substituted quinolines (code name **PQs**) with the partial crystal structure of connexon (Makowski *et al.*, 1977; Foote *et al.*, 1998; Fleishman *et al.*, 2006) using Autodock

computational docking software (Goodsell and Olson, 1990; Morris *et al.*, 1996; Morris, 1998). Scientists in Dr. Hua's lab observed binding of PQs to the inert pore of the hexameric hemichannel of gap junctions. The connexon structure was constructed by using electron cryomicroscopy to derive a three dimensional density map at 5.7 Å in plane and 19.8 Å vertical resolution, and analysis of evolutionary conservation and compensatory mutations in connexin evolution to identify the packing interfaces between the helices (Fleishman *et al.*, 2006). In one of the minimum energy (-0.7 kcal/mol) bound structures, interactions (closed contact) between CF3 group of PQ1 and NH of Leu144 of connexin (2.5 Å), OCH3 group of PQ1 and CH2 of Phe81 of connexin (2.0 Å), and NH3 + of PQ1 and –O2CGlu146 of connexin are found. Consequently, they synthesized this class of quinolines and studied their GJIC and anticancer activities (Gakhar, 2008). Since PQ1 (one of the substituted quinolines) is predicted to have a high binding affinity for connexin via computational docking (Figure 1.10), we tested PQ1 and its synthetic precursors effect on the GJIC in T47D breast cancer cells.

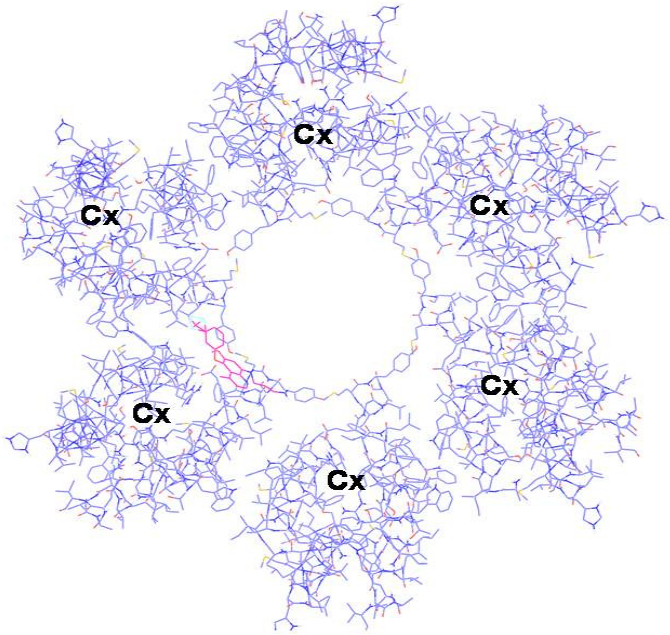


Figure 1.10 Docking of PQ1 with connexin.

Six connexins form a connexon. The figure illustrates docking of PQ1 (pink) with the connexin protein. The computational docking software docks the PQ1 based on the lowest energy levels. The diagram hypothetically depicts one PQ1 molecule binding to the connexin protein; however,

it is not feasible to know how many PQ1 molecules can bind to the connexin protein based on the docking software.

1.6 Treatment

1.6.1 Tamoxifen treatment studies for breast cancer

Tamoxifen is a nonsteroidal triphenylethylene derivative which competitively blocks the binding of estrogen to the estrogen receptor, thus considered to be an antiestrogen. Studies were conducted to test the efficacy of tamoxifen against breast cancer. A small randomized clinical trial of 3 years of tamoxifen versus no treatment demonstrated a survival advantage for ER^+ patients who received tamoxifen (Delozier *et al.*, 1986). In 1977, a pilot study was conducted to determine whether patients could tolerate 5 years of adjuvant therapy and whether metabolic tolerance would occur during long-term use of tamoxifen. No unusual side effects were observed and blood levels of tamoxifen and its metabolites remained stable (Tormey, 1984; Tormey, 1987). In 1987, a Scottish trial showed an increase in survival rate of recurrent patients for breast cancer receiving tamoxifen treatment compared to controls. A 10-year study conducted by Nolvadex Adujvant Trial Organization (NATO) found a significant effect of 2 years of tamoxifen treatment on the disease free survival rate in patients with early breast cancer (Jordan, 1994). From 1992-1997, the National Breast and Bowel Project (NSABP) randomized 13,388 women into the Breast Cancer Prevention Trial (NSABP BCPT; P-1) who were at increased risk of breast cancer to either tamoxifen or placebo. The study showed a 49% reduction in overall invasive breast cancer risk and a 50% reduction in non-invasive breast cancer risk in women who received tamoxifen (Rashid, 2006). In 1998, tamoxifen was approved as a chemoprevention drug for breast cancer by the Food and Drug Administration (FDA), after NCI released the results of the Breast Cancer Prevention Trial (BCPT), a 6-year study of the drug. In BCPT, tamoxifen was found to reduce the incidence of breast cancer by 49 percent. Based on that study, FDA approved the drug for women at high risk of developing invasive breast cancer. High risk was defined as women of age 35 and older who have a 5-year risk of at least 1.67% (NCI, 2003).

Tamoxifen was introduced as an antiestrogen and hence, it was used against breast cancer as estrogen potentiates breast cancer. But later studies were conducted to find if tamoxifen

affects pathways other than mediated through prevention of estrogen binding with estrogen-responsive elements. Clinical response in breast cancer patients receiving tamoxifen daily for at least 3 months showed increased apoptosis and decreased proliferation using surrogate markers of apoptosis (Bcl-2) and mitosis (Ki-S1) (Keen *et al.*, 1997; Cameron *et al.*, 2000) Zhang and Shapiro found that tamoxifen induces apoptosis in MCF-7 cells by regulating p38 mitogen activated protein kinase (MAPK) (Zhang and Shapiro, 2000). These studies suggest that tamoxifen might be exerting effect on many proapoptotic pathways besides being an antiestrogen.

1.6.2 Side effects of Tamoxifen

In the breast, the antitumor effects of tamoxifen are due to ER antagonist activity, competitively blocking the binding of estrogen to ERs. Estrogen deprivation due to tamoxifen can also lead to unwanted side effects (Perez, 2007). Endometrial cancer risk was associated with tamoxifen therapy for breast cancer (odds ratio = 1.52; 95% confidence interval [CI] = 1.07-2.17). Women with more than 5 years of exposure to tamoxifen had 4.06-fold greater odds of developing endometrial cancer than nonusers (95% CI = 1.74-9.47). Risk associated with tamoxifen use was stronger among heavier women than among thinner women, although trends did not differ statistically (P = .10) (Bernstein *et al.*, 1999). A case-control study done at Netherlands found that the median time between the diagnoses of breast cancer and endometrial cancer was 34 (5-201) months and the relative risk of endometrial cancer for women who had ever been treated with tamoxifen compared with those who had not was 1.3 (Van Leeuwen *et al.*, 1994). Patients treated with tamoxifen experience hot flashes, night sweats, vaginal discharge, vaginal bleeding, gall stone formation (Day R, 1999; Day, 2001; Day *et al.*, 2001; Vogel *et al.*, 2006). In premenopausal women, tamoxifen may result in bone loss. In a British study of tamoxifen on bone mineral density (BMD), it was found that BMD of premenopausal women decreased progressively in lumbar spine ($p < .001$) and in hip ($p < .05$) for women on tamoxifen compared to the placebo group (O'Regan *et al.*, 2002). These more severe side effects contribute to the adverse risk: benefit profile associated with longer-term tamoxifen use, which limits adjuvant tamoxifen to 5 years (Fisher *et al.*, 1996).

1.6.3 Combinational studies of tamoxifen for breast cancer

Tamoxifen acts by inhibiting the binding of estrogen with the estrogen receptor, whereas aromatase inhibitors block the conversion of androgens to estrogen by aromatase enzyme, thereby causing estrogen deprivation. The third-generation aromatase inhibitors (AIs), letrozole, anastrozole and exemestane, have demonstrated superior efficacy to tamoxifen in large, randomized clinical trials in postmenopausal women with HR^+ (hormone receptor positive) early breast cancer (Coombes *et al.*, 2004; Boccardo *et al.*, 2005; Howell A, 2005; Jakesz *et al.*, 2005; Thurlimann *et al.*, 2005). Analysis of the ATAC trial at a median follow-up time of 47 months demonstrated that after an additional follow-up period, anastrozole continued to exhibit superior efficacy than tamoxifen alone and in combination with anastrozole. This demonstrated by disease free survival (DFS), time to recurrence (TTR), and reduction in the incidence of contralateral breast cancer (CLBC) (Baum *et al.*, 2003). The 5 year disease-free survival was significantly greater in the letrozole group (77.9%) than in the tamoxifen group (71.4%, hazard ratio for the primary end point, 0.81; 95% confidence interval), especially reducing recurrence at distant sites (hazard ratio, 0.73; 95% confidence interval). The 5-year estimates of disease-free survival were 84.0% in the letrozole group and 81.4% in the tamoxifen group (Thurlimann *et al.*, 2005). In another study the outcome showed that switching patients to adjuvant treatment with exemestane after 2 to 3 years of tamoxifen therapy was associated with a statistically and clinically significant improvement in disease-free survival, which included a reduction in the incidence of metastatic disease. DFS after 3 years of randomization was 91.5% (95% confidence interval, 90.0 to 92.7) in the exemestane group and 86.8% (95% confidence interval, 85.1 to 88.3) in the tamoxifen group (Coombes *et al.*, 2004). Overall, many studies indicate that aromatase inhibitors might gradually displace tamoxifen as the gold standard for adjuvant endocrine therapy (Perez, 2007).

Raloxifene hydrochloride is a selective estrogen receptor modulator like tamoxifen that has antiestrogenic effects on breast and endometrial tissue and estrogenic effects on bone, lipid metabolism, and blood clotting (Cummings *et al.*, 1999). Trials of tamoxifen and raloxifen were done separately and a 33% and 59% reduction, respectively, in the relative risk of breast cancer was found; however, both the treatments cannot be compared because the participants in the tamoxifen and raloxifen study were pre-menopausal women and post-menopausal women, respectively (Cummings *et al.*, 2009). A study of tamoxifene and raloxifene (STAR) program

was conducted to estimate the efficacy of raloxifene compared to tamoxifen. The study showed that both the drugs reduced the risk of developing invasive breast cancer by about 50% and women who took raloxifene and followed for 4 years showed 36% fewer uterine cancers and 29% fewer blood clots (NCI, 2007).

All these studies suggest that tamoxifen, despite being a drug of choice for the treatment of endocrine-responsive breast cancers, has some serious side effects. The treatment of tamoxifen depends on the risk:benefit ratio in an individual. However, combination of tamoxifen with another drug causing an increase in apoptosis of the cancer cells could be a potential therapy for the breast cancer. The combinational therapy could either decrease the 5-year treatment with tamoxifen or could help in negating the effects of tamoxifen. In the present study, we combined PQ1, a gap junctional activator with tamoxifen. Studies conducted with PQ1 alone showed an increase in GJIC, decrease in cell proliferation and colony growth assay in breast cancer cells. Furthermore, PQ1 also showed a 70% decrease in tumor growth in nude mice. Therefore, we investigated the effect of combinational treatment of tamoxifen and PQ1.

1.7 References

Abbaci, M., Barberi-Heyob, M., Blondel, W., Guillemin, F., and Didelon, J. (2008). Advantages and limitations of commonly used methods to assay the molecular permeability of gap junctional intercellular communication. *Biotechniques* **45**, 33-52, 56-62.

Abrams, C. K., Freidin, M., Bukauskas, F., Dobrenis, K., Bargiello, T. A., Verselis, V. K., Bennett, M. V., Chen, L., and Sahenk, Z. (2003). Pathogenesis of X-linked Charcot-Marie-Tooth disease: differential effects of two mutations in connexin 32. *J Neurosci* **23**, 10548-10558.

Alberts, B. J., Alexander; Lewis, Julian; Raff, Martin; Roberts, Keith; Walter, Peter (2002). Molecular Biology of the cell. In *Molecular Biology of the Cell*. Garland science, New York and London.

Alexander, D. B., and Goldberg, G. S. (2003). Transfer of biologically important molecules between cells through gap junction channels. *Curr Med Chem* **10**, 2045-2058.

Altieri, D. C. (2003). Survivin in apoptosis control and cell cycle regulation in cancer. *Prog Cell Cycle Res* **5**, 447-452.

Anderson, H. A., and Wolff, M. S. (2000). Environmental contaminants in human milk. *J Expo Anal Environ Epidemiol* **10**, 755-760.

ATSDR (1998). Toxicological profile for chlorinated dibenzo-p-dioxins. Update. (Final Report), pp. 729. Agency for Toxic Substances and Disease Registry, Atlanta, GA.

Baum, M., Buzdar, A., Cuzick, J., Forbes, J., Houghton, J., Howell, A., and Sahmoud, T. (2003). Anastrozole alone or in combination with tamoxifen versus tamoxifen alone for adjuvant treatment of postmenopausal women with early-stage breast cancer: results of the ATAC (Arimidex, Tamoxifen Alone or in Combination) trial efficacy and safety update analyses. *Cancer* **98**, 1802-1810.

Bernstein, L., Deapen, D., Cerhan, J. R., Schwartz, S. M., Liff, J., McGann-Maloney, E., Perlman, J. A., and Ford, L. (1999). Tamoxifen therapy for breast cancer and endometrial cancer risk. *J Natl Cancer Inst* **91**, 1654-1662.

Berstein, L. M., Zheng, H., Yue, W., Wang, J. P., Lykkesfeldt, A. E., Naftolin, F., Harada, H., Shanabrough, M., and Santen, R. J. (2003). New approaches to the understanding of tamoxifen action and resistance. *Endocr Relat Cancer* **10**, 267-277.

Boccardo, F., Rubagotti, A., Puntoni, M., Guglielmini, P., Amoroso, D., Fini, A., Paladini, G., Mesiti, M., Romeo, D., Rinaldini, M., Scali, S., Porpiglia, M., Benedetto, C., Restuccia, N., Buzzi, F., Franchi, R., Massidda, B., Distante, V., Amadori, D., and Sismondi, P. (2005). Switching to anastrozole versus continued tamoxifen treatment of early breast cancer: preliminary results of the Italian Tamoxifen Anastrozole Trial. *J Clin Oncol* **23**, 5138-5147.

Broker, L. E., Kruyt, F. A., and Giaccone, G. (2005). Cell death independent of caspases: a review. *Clin Cancer Res* **11**, 3155-3162.

Bruzzone, R., Giaume, C. (1999). Methods in Molecular Biology: Connexin Methods and Protocols. In *Connexin Methods and Protocols: Methods in Molecular Biology* (P. Meda, Ed.), pp. 204-206. Humana Press, Totowa, NJ.

Bukauskas, F. F., Jordan, K., Bukauskiene, A., Bennett, M. V., Lampe, P. D., Laird, D. W., and Verselis, V. K. (2000). Clustering of connexin 43-enhanced green fluorescent protein gap junction channels and functional coupling in living cells. *Proc Natl Acad Sci U S A* **97**, 2556-2561.

Cameron, D. A., Keen, J. C., Dixon, J. M., Bellamy, C., Hanby, A., Anderson, T. J., and Miller, W. R. (2000). Effective tamoxifen therapy of breast cancer involves both antiproliferative and pro-apoptotic changes. *Eur J Cancer* **36**, 845-851.

Cao, F., Eckert, R., Elfgang, C., Nitsche, J. M., Snyder, S. A., DF, H. u., Willecke, K., and Nicholson, B. J. (1998). A quantitative analysis of connexin-specific permeability differences of gap junctions expressed in HeLa transfectants and Xenopus oocytes. *J Cell Sci* **111 (Pt 1)**, 31-43.

Charles, A. C., Naus, C. C., Zhu, D., Kidder, G. M., Dirksen, E. R., and Sanderson, M. J. (1992). Intercellular calcium signaling via gap junctions in glioma cells. *J Cell Biol* **118**, 195-201.

Chen, J. W., Wang, S. L., Yu, H. Y., Liao, P. C., and Lee, C. C. (2006). Body burden of dioxins and dioxin-like polychlorinated biphenyls in pregnant women residing in a contaminated area. *Chemosphere* **65**, 1667-1677.

Chen, S. C., Pelletier, D. B., Ao, P., and Boynton, A. L. (1995). Connexin43 reverses the phenotype of transformed cells and alters their expression of cyclin/cyclin-dependent kinases. *Cell Growth Differ* **6**, 681-690.

Clarke, R., Leonessa, F., Welch, J. N., and Skaar, T. C. (2001). Cellular and molecular pharmacology of antiestrogen action and resistance. *Pharmacol Rev* **53**, 25-71.

Clarke, R. B., Laidlaw, I. J., Jones, L. J., Howell, A., and Anderson, E. (199 3). Effect of tamoxifen on Ki67 labelling index in human breast tumours and its relationship to oestrogen and progesterone receptor status. *Br J Cancer* **67**, 606-611.

Constantin, B., Cronier, L., and Raymond, G. (1997). Transient involvement of gap junctional communication before fusion of newborn rat myoblasts. *C R Acad Sci III* **320**, 35-40.

Coombes, R. C., Hall, E., Gibson, L. J., Paridaens, R., Jassem, J., Delozier, T., Jones, S. E., Alvarez, I., Bertelli, G., Ortmann, O., Coates, A. S., Bajetta, E., Dodwell, D., Coleman, R. E., Fallowfield, L. J., Mickiewicz, E., Andersen, J., Lonning, P. E., Cocconi, G., Stewart, A., Stuart, N., Snowdon, C. F., Carpentieri, M., Massimini, G., Bliss, J. M., and van de Velde, C. (2004). A randomized trial of exemestane after two to three years of tamoxifen therapy in postmenopausal women with primary breast cancer. *N Engl J Med* **350**, 1081-1092.

Cronier, L., Crespin, S., Strale, P. O., Defamie, N., and Mesnil, M. (2009). Gap junctions and cancer: new functions for an old story. *Antioxid Redox Signal* **11**, 323-338.

Cummings, S. R., Eckert, S., Krueger, K. A., Grady, D., Powles, T. J., Cauley, J. A., Norton, L., Nickelsen, T., Bjarnason, N. H., Morrow, M., Lippman, M. E., Black, D., Glusman, J. E., Costa, A., and Jordan, V. C. (1999). The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. Multiple Outcomes of Raloxifene Evaluation. *JAMA* **281**, 2189-2197.

Cummings, S. R., Tice, J. A., Bauer, S., Browner, W. S., Cuzick, J., Ziv, E., Vogel, V., Shepherd, J., Vachon, C., Smith-Bindman, R., and Kerlikowske, K. (2009). Prevention of breast cancer in postmenopausal women: approaches to estimating and reducing risk. *J Natl Cancer Inst* **101**, 384-398.

Czyz, J., Irmer, U., Schulz, G., Mindermann, A., and Hulser, D. F. (2000). Gap-junctional coupling measured by flow cytometry. *Exp Cell Res* **255**, 40-46.

Dagli, M. L., and Hernandez-Blazquez, F. J. (2007). Roles of gap junctions and connexins in non-neoplastic pathological processes in which cell proliferation is involved. *The Journal of membrane biology* **218**, 79-91.

Dakin, K., Zhao, Y., and Li, W. H. (2005). LAMP, a new imaging assay of gap junctional communication unveils that Ca2+ influx inhibits cell coupling. *Nat Methods* **2**, 55-62.

Day, R. (2001). Quality of life and tamoxifen in a breast cancer prevention trial: a summary of findings from the NSABP P-1 study. National Surgical Adjuvant Breast and Bowel Project. *Ann N Y Acad Sci* **949**, 143-150.

Day, R., Ganz, P. A., and Costantino, J. P. (2001). Tamoxifen and depression: more evidence from the National Surgical Adjuvant Breast and Bowel Project's Breast Cancer Prevention (P-1) Randomized Study. *J Natl Cancer Inst* **93**, 1615-1623.

Day R, G. P., Costantino JP, Cronin WM, Wickerham DL, Fisher B. (1999). Health-related quality of life and tamoxifen in breast cancer prevention: a report from the National Surgical Adjuvant Breast and Bowel Project P-1 Study. *Journal of Clinical Oncology* **17**, 2659-2669.

Delozier, T., Julien, J. P., Juret, P., Veyret, C., Couette, J. E., Graic, Y., Ollivier, J. M., and de Ranieri, E. (1986). Adjuvant tamoxifen in postmenopausal breast cancer: preliminary results of a randomized trial. *Breast Cancer Res Treat* **7**, 105-109.

Dermietzel, R., Hwang, T. K., and Spray, D. S. (1990). The gap junction family: structure, function and chemistry. *Anat Embryol (Berl)* **182**, 517-528.

Devesa, S. S., Blot, W. J., Stone, B. J., Miller, B. A., Tarone, R. E., and Fraumeni, J. F., Jr. (1995). Recent cancer trends in the United States. *J Natl Cancer Inst* **87**, 175-182.

Dupont, E., Matsushita, T., Kaba, R. A., Vozzi, C., Coppen, S. R., Khan, N., Kaprielian, R., Yacoub, M. H., and Severs, N. J. (2001). Altered connexin expression in human congestive heart failure. *J Mol Cell Cardiol* **33**, 359-371.

Eghbali, B., Kessler, J. A., Reid, L. M., Roy, C., and Spray, D. C. (1991). Involvement of gap junctions in tumorigenesis: transfection of tumor cells with connexin 32 cDNA retards growth in vivo. *Proc Natl Acad Sci U S A* **88**, 10701-10705.

Elfgang, C., Eckert, R., Lichtenberg-Frate, H., Butterweck, A., Traub, O., Klein, R. A., Hulser, D. F., and Willecke, K. (1995). Specific permeability and selective formation of gap junction channels in connexin-transfected HeLa cells. *J Cell Biol* **129**, 805-817.

Ellis, P. A., Saccani-Jotti, G., Clarke, R., Johnston, S. R., Anderson, E., Howell, A., A'Hern, R., Salter, J., Detre, S., Nicholson, R., Robertson, J., Smith, I. E., and Dowsett, M. (1997). Induction of apoptosis by tamoxifen and ICI 182780 in primary breast cancer. *Int J Cancer* **72**, 608-613.

Encarnacion, C. A., Ciocca, D. R., McGuire, W. L., Clark, G. M., Fuqua, S. A., and Osborne, C. K. (1993). Measurement of steroid hormone receptors in breast cancer patients on tamoxifen. *Breast Cancer Res Treat* **26**, 237-246.

Enomoto, T., Sasaki, Y., Shiba, Y., Kanno, Y., and Yamasaki, H. (1981). Tumor promoters cause a rapid and reversible inhibition of the formation and maintenance of electrical cell coupling in culture. *Proceedings of the National Academy of Sciences of the United States of America* **78**, 5628-5632.

Evans, W. H., De Vuyst, E., and Leybaert, L. (2006). The gap junction cellular internet: connexin hemichannels enter the signalling limelight. *Biochem J* **397**, 1-14.

Falk, M. M. (2000). Connexin-specific distribution within gap junctions revealed in living cells. *J Cell Sci* **113 (Pt 22)**, 4109-4120.

Fisher, B., Dignam, J., Bryant, J., DeCillis, A., Wickerham, D. L., Wolmark, N., Costantino, J., Redmond, C., Fisher, E. R., Bowman, D. M., Deschenes, L., Dimitrov, N. V., Margolese, R. G., Robidoux, A., Shibata, H., Terz, J., Paterson, A. H., Feldman, M. I., Farrar, W., Evans, J., and Lickley, H. L. (1996). Five versus more than five years of tamoxifen therapy for breast cancer patients with negative lymph nodes and estrogen receptor-positive tumors. *J Natl Cancer Inst* **88**, 1529-1542.

Fisher, B., Redmond, C., Legault-Poisson, S., Dimitrov, N. V., Brown, A. M., Wickerham, D. L., Wolmark, N., Margolese, R. G., Bowman, D., Glass, A. G., and et al. (1990). Postoperative chemotherapy and tamoxifen compared with tamoxifen alone in the treatment of positive-node breast cancer patients aged 50 years and older with tumors responsive to tamoxifen: results from the National Surgical Adjuvant Breast and Bowel Project B-16. *J Clin Oncol* **8**, 1005-1018.

Fitzgerald, D. J., Mesnil, M., Oyamada, M., Tsuda, H., Ito, N., and Yamasaki, H. (1989). Changes in gap junction protein (connexin 32) gene expression during rat liver carcinogenesis. *J Cell Biochem* **41**, 97-102.

Fleishman, S. J., Unger, V. M., and Ben-Tal, N. (2006). Transmembrane protein structures without X-rays. *Trends Biochem Sci* **31**, 106-113.

Fonseca, P. C., Nihei, O. K., Urban-Maldonado, M., Abreu, S., de Carvalho, A. C., Spray, D. C., Savino, W., and Alves, L. A. (2004). Characterization of connexin 30.3 and 43 in thymocytes. *Immunol Lett* **94**, 65-75.

Foote, C. I., Zhou, L., Zhu, X., and Nicholson, B. J. (1998). The pattern of disulfide linkages in the extracellular loop regions of connexin 32 suggests a model for the docking interface of gap junctions. *J Cell Biol* **140**, 1187-1197.

Gakhar, G., Ohira, Takahiro, Shi, Aibin, Hua, D.H., Nguyen, T.A. (2008). Antitumor effect of substituted quinolines in breast cancer cells. *Drug Development Research* **69**, 526-534.

Game, X., Allard, J., Escourrou, G., Gourdy, P., Tack, I., Rischmann, P., Arnal, J. F., and Malavaud, B. (2008). Estradiol increases urethral tone through the local inhibition of neuronal nitric oxide synthase expression. *Am J Physiol Regul Integr Comp Physiol* **294**, R851-857.

Gazzaniga, P., Gradilone, A., Giuliani, L., Gandini, O., Silvestri, I., Nofroni, I., Saccani, G., Frati, L., and Agliano, A. M. (2003). Expression and prognostic significance of livin, survivin and other apoptosis-related genes in the progression of superficial bladder cancer. *Ann Oncol* **14**, 85-90.

Gelmann, E. P. (1996). Tamoxifen induction of apoptosis in estrogen receptor-negative cancers: new tricks for an old dog? *J Natl Cancer Inst* **88**, 224-226.

Gibbs, L. K., Hickman, D. L., Lewis, A. D., and Colgin, L. M. (2007). Staphylococcus-induced urolithiasis in estrogen-treated ovariectomized nude mice. *J Am Assoc Lab Anim Sci* **46**, 61-65.

Goldberg, G. S., Bechberger, J. F., and Naus, C. C. (1995). A pre-loading method of evaluating gap junctional communication by fluorescent dye transfer. *Biotechniques* **18**, 490-497.

Goldhirsch A, G. R. (1989). In *Adjuvant therapy of primary breast cancer* (G. A. Senn H-J, Gelber RD, Osterwalder B, Ed.), pp. 153-162. Springer-Verlag.

Goodall, H., and Maro, B. (1986). Major loss of junctional coupling during mitosis in early mouse embryos. *J Cell Biol* **102**, 568-575.

Goodenough, D. A. (1976). In vitro formation of gap junction vesicles. *J Cell Biol* **68**, 220-231.
Goodenough, D. A., Goliger, J. A., and Paul, D. L. (1996). Connexins, connexons, and intercellular communication. *Annu Rev Biochem* **65**, 475-502.
Goodsell, D. S., and Olson, A. J. (1990). Automated docking of substrates to proteins by simulated annealing. *Proteins* **8**, 195-202.
Gould, V. E., Mosquera, J. M., Leykauf, K., Gattuso, P., Durst, M., and Alonso, A. (2005). The phosphorylated form of connexin43 is up-regulated in breast hyperplasias and carcinomas and in their neoformed capillaries. *Hum Pathol* **36**, 536-545.
Gray, J. (2008). State of the evidence 2008 Executive Summary.
Gray, J., Evans, Nancy, Taylor Brynn, Rizzo Jeanne, Walker, Marissa (2009). State of the evidence: the connection between breast cancer and the environment. *International Journal of Occupational Environmental Health* **15**, 43-78.
group, T. I. B. C. S. (1997). Effectiveness of adjuvant chemotherapy in combination with tamoxifen for node-positive postmenopausal breast cancer patients. *Journal of Clinical Oncology* **15**, 1385-1394.
Gunjan Gakhar, T. O., Aibin Shi, Duy H Hua, Thu Annelise Nguyen Antitumor effect of substituted quinolines in breast cancer cells. *Drug Development Research* **69**, 526-534.
Hertzberg, E. L. (2000). *Gap junctions*. Jai Press, Stanford, CT.
HH, H. (1988). Hereditary hydronephrosis. In *Urinary Systems* (M. U. Jones TC, Hunt RD, Ed.), pp. 273-275. Springer-Verlag, Berlin.
Hirschi, K. K., Xu, C. E., Tsukamoto, T., and Sager, R. (1996). Gap junction genes Cx26 and Cx43 individually suppress the cancer phenotype of human mammary carcinoma cells and restore differentiation potential. *Cell Growth Differ* **7**, 861-870.
Horton, C. E., Jr., Davisson, M. T., Jacobs, J. B., Bernstein, G. T., Retik, A. B., and Mandell, J. (1988). Congenital progressive hydronephrosis in mice: a new recessive mutation. *J Urol* **140**, 1310-1315.
Howell A, C. J., Baum M, Buzdar A, Dowsett M, Forbes JF, Hoctin-Boes G, Houghton I, Locker GY, Tobias JS, ATAC Trialists' Group (2005). Results of the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial after completion of 5 years adjuvant tretment for breast cancer. *Lancet* **365**, 60-62.
HSDB (2003). Hazardous Substances Database. National Library of Medicine. http://tox.net.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB.
Hull, D. F., 3rd, Clark, G. M., Osborne, C. K., Chamness, G. C., Knight, W. A., 3rd, and McGuire, W. L. (1983). Multiple estrogen receptor assays in human breast cancer. *Cancer Res* **43**, 413-416.
Jakesz, R., Jonat, W., Gnant, M., Mittlboeck, M., Greil, R., Tausch, C., Hilfrich, J., Kwasny, W., Menzel, C., Samonigg, H., Seifert, M., Gademann, G., Kaufmann, M., and Wolfgang, J. (2005). Switching of postmenopausal women with endocrine-responsive early breast cancer to anastrozole after 2 years' adjuvant tamoxifen: combined results of ABCSG trial 8 and ARNO 95 trial. *Lancet* **366**, 455-462.
Jensen, R., and Glazer, P. M. (2004). Cell-interdependent cisplatin killing by Ku/DNA-dependent protein kinase signaling transduced through gap junctions. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 6134-6139.
Jongen, W. M., Fitzgerald, D. J., Asamoto, M., Piccoli, C., Slaga, T. J., Gros, D., Takeichi, M., and Yamasaki, H. (1991). Regulation of connexin 43-mediated gap junctional

intercellular communication by Ca2+ in mouse epidermal cells is controlled by E-cadherin. *J Cell Biol* **114**, 545-555.

Jordan, V. C. (1994). *Long-Term tamoxifen treatment for breast cancer*. The University of Wisconsin Press, Madison, Wisconsin.

Kanczuga-Koda, L., Sulkowski, S., Tomaszewski, J., Koda, M., Sulkowska, M., Przystupa, W., Golaszewska, J., and Baltaziak, M. (2005). Connexins 26 and 43 correlate with Bak, but not with Bcl-2 protein in breast cancer. *Oncol Rep* **14**, 325-329.

Keen, J. C., Dixon, J. M., Miller, E. P., Cameron, D. A., Chetty, U., Hanby, A., Bellamy, C., and Miller, W. R. (1997). The expression of Ki-S1 and BCL-2 and the response to primary tamoxifen therapy in elderly patients with breast cancer. *Breast Cancer Res Treat* **44**, 123-133.

Kerr, J. F., Wyllie, A. H., and Currie, A. R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* **26**, 239-257.

King, M. C., Marks, J. H., and Mandell, J. B. (2003). Breast and ovarian cancer risks due to inherited mutations in BRCA1 and BRCA2. *Science* **302**, 643-646.

Koblyakov, V. A. (1998). Inducers of the cytochrome P-450 superfamily as promoters of carcinogenesis. *Biochemistry (Mosc)* **63**, 885-898.

Koffler, L., Roshong, S., Kyu Park, I., Cesen-Cummings, K., Thompson, D. C., Dwyer-Nield, L. D., Rice, P., Mamay, C., Malkinson, A. M., and Ruch, R. J. (2000). Growth inhibition in G(1) and altered expression of cyclin D1 and p27(kip-1)after forced connexin expression in lung and liver carcinoma cells. *J Cell Biochem* **79**, 347-354.

Korkiamaki, T. (2002). Intercellular calcium-mediated cell signaling in keratinocytes cultured from patients with NF1 or psoriasis. In *Anatomy and Cell Biology, Dermatology and Venereology*. Oulu, Finland.

Koval, M., Geist, S. T., Westphale, E. M., Kemendy, A. E., Civitelli, R., Beyer, E. C., and Steinberg, T. H. (1995). Transfected connexin45 alters gap junction permeability in cells expressing endogenous connexin43. *J Cell Biol* **130**, 987-995.

Kumar, N. M., and Gilula, N. B. (1992). Molecular biology and genetics of gap junction channels. *Semin Cell Biol* **3**, 3-16.

Kuroda, H., Kohrogi, T., Uchida, N., Imai, I., Terada, N., Matsumoto, K., and Kitamura, Y. (1985). Urinary retention induced by estrogen injections in mice: an analytical model. *J Urol* **134**, 1268-1270.

Laird, D. W. (2006). Life cycle of connexins in health and disease. *Biochem J* **394**, 527-543.

Laird, D. W., Fistouris, P., Batist, G., Alpert, L., Huynh, H. T., Carystinos, G. D., and Alaoui-Jamali, M. A. (1999). Deficiency of connexin43 gap junctions is an independent marker for breast tumors. *Cancer Res* **59**, 4104-4110.

Lampe, P. D., and Lau, A. F. (2000). Regulation of gap junctions by phosphorylation of connexins. *Arch Biochem Biophys* **384**, 205-215.

Lampe, P. D., TenBroek, E. M., Burt, J. M., Kurata, W. E., Johnson, R. G., and Lau, A. F. (2000). Phosphorylation of connexin43 on serine368 by protein kinase C regulates gap junctional communication. *J Cell Biol* **149**, 1503-1512.

Langan Fahey SM, J. V., Fritz NF, Robinson SP, Waters D, Tormey DC (1994). In *Long term tamoxifen treatment for breast cancer*
(V. C. Jordan, Ed.), pp. 27-56. University of Wisconsin Press, Madison.

Larson, D. M., and Sheridan, J. D. (1985). Junctional transfer in cultured vascular endothelium: II. Dye and nucleotide transfer. *J Membr Biol* **83**, 157-167.

Lee, S. W., Tomasetto, C., Paul, D., Keyomarsi, K., and Sager, R. (1992). Transcriptional downregulation of gap-junction proteins blocks junctional communication in human mammary tumor cell lines. *J Cell Biol* **118**, 1213-1221.

Levin-Allerhand, J. A., Sokol, K., and Smith, J. D. (2003). Safe and effective method for chronic 17beta-estradiol administration to mice. *Contemp Top Lab Anim Sci* **42**, 33-35.

Li, F., Ambrosini, G., Chu, E. Y., Plescia, J., Tognin, S., Marchisio, P. C., and Altieri, D. C. (1998). Control of apoptosis and mitotic spindle checkpoint by survivin. *Nature* **396**, 580-584.

Lichtenstein, P., Holm, N. V., Verkasalo, P. K., Iliadou, A., Kaprio, J., Koskenvuo, M., Pukkala, E., Skytthe, A., and Hemminki, K. (2000). Environmental and heritable factors in the causation of cancer--analyses of cohorts of twins from Sweden, Denmark, and Finland. *N Engl J Med* **343**, 78-85.

Lippincott-Schwartz, J., Altan-Bonnet, N., and Patterson, G. H. (2003). Photobleaching and photoactivation: following protein dynamics in living cells. *Nat Cell Biol* **Suppl**, S7-14.

Lippincott-Schwartz, J., Snapp, E., and Kenworthy, A. (2001). Studying protein dynamics in living cells. *Nat Rev Mol Cell Biol* **2**, 444-456.

Liu, T. F., and Johnson, R. G. (1999). Effects of TPA on dye transfer and dye leakage in fibroblasts transfected with a connexin 43 mutation at ser368. *Methods Find Exp Clin Pharmacol* **21**, 387-390.

Loewenstein, W. R., Kanno, Y (1966). Intercellular communication and the control of tissue growth: Lack of communication between cancer cells. *Nature* **209**, 1248-1249.

Makowski, L., Caspar, D. L., Phillips, W. C., and Goodenough, D. A. (1977). Gap junction structures. II. Analysis of the x-ray diffraction data. *J Cell Biol* **74**, 629-645.

Mandlekar, S., and Kong, A. N. (2001). Mechanisms of tamoxifen-induced apoptosis. *Apoptosis* **6**, 469-477.

Mandlekar, S., Yu, R., Tan, T. H., and Kong, A. N. (2000). Activation of caspase-3 and c-Jun NH2-terminal kinase-1 signaling pathways in tamoxifen-induced apoptosis of human breast cancer cells. *Cancer Res* **60**, 5995-6000.

Mannen, H., Tsuji, S., and Goto, N. (1993). Influence of chronic oestrogen treatment on severity of hydronephrosis in inbred DDD mice. *Lab Anim* **27**, 124-130.

Margulis, V., Lotan, Y., and Shariat, S. F. (2008). Survivin: a promising biomarker for detection and prognosis of bladder cancer. *World J Urol* **26**, 59-65.

Martin, G., Melito, G., Rivera, E., Levin, E., Davio, C., Cricco, G., Andrade, N., Caro, R., and Bergoc, R. (1996). Effect of tamoxifen on intraperitoneal N-nitroso-N-methylurea induced tumors. *Cancer Lett* **100**, 227-234.

McLachlan, E., Shao, Q., and Laird, D. W. (2007). Connexins and gap junctions in mammary gland development and breast cancer progression. *J Membr Biol* **218**, 107-121.

Meda, P. (2001). Assaying the molecular permeability of connexin channels. *Methods Mol Biol* **154**, 201-224.

Mehta, P. P., Hotz-Wagenblatt, A., Rose, B., Shalloway, D., and Loewenstein, W. R. (1991). Incorporation of the gene for a cell-cell channel protein into transformed cells leads to normalization of growth. *J Membr Biol* **124**, 207-225.

Mesnil, M. (2002). Connexins and cancer. *Biol Cell* **94**, 493-500.

Mesnil, M., Crespin, S., Avanzo, J. L., and Zaidan-Dagli, M. L. (2005). Defective gap junctional intercellular communication in the carcinogenic process. *Biochim Biophys Acta* **1719**, 125-145.

Mesnil, M., Fitzgerald, D. J., and Yamasaki, H. (1988). Phenobarbital specifically reduces gap junction protein mRNA level in rat liver. *Mol Carcinog* **1**, 79-81.

Mok, B. W., Yeung, W. S., and Luk, J. M. (1999). Differential expression of gap-junction gene connexin 31 in seminiferous epithelium of rat testes. *FEBS Lett* **453**, 243-248.

Morris, G. M., Goodsell, D. S., Huey, R., and Olson, A. J. (1996). Distributed automated docking of flexible ligands to proteins: parallel applications of AutoDock 2.4. *J Comput Aided Mol Des* **10**, 293-304.

Morris, G. M., Goodsell, D.S., Halliday R.S., Huey, R., Hart, W.E., Belew, R.K., Olson, A.J. (1998). Automated docking using Lamarckian genetic algorithm and an empirical binding free energy function. *Journal of Computational Chemistry* **19**, 1639-1662.

Mouridsen, H. T., Rose, C., Overgaard, M., Dombernowsky, P., Panduro, J., Thorpe, S., Rasmussen, B. B., Blichert-Toft, M., and Andersen, K. W. (1988). Adjuvant treatment of postmenopausal patients with high risk primary breast cancer. Results from the Danish adjuvant trials DBCG 77 C and DBCG 82 C. *Acta Oncol* **27**, 699-705.

Murray, A. W., and Fitzgerald, D. J. (1979). Tumor promoters inhibit metabolic cooperation in cocultures of epidermal and 3T3 cells. *Biochem Biophys Res Commun* **91**, 395-401.

NCI (2003). NCI Study Estimates More Than 2 Million Women Could Benefit from Tamoxifen, Bethesda, M.D.

NCI (2007). Study of tamoxifen and raloxifene (STAR) trial.

Nenci, I., Marchetti, E., and Querzoli, P. (1988). Commentary on human mammary preneoplasia. The estrogen receptor-promotion hypothesis. *J Steroid Biochem* **30**, 105-106.

Neveu MJ, H. J., Paul DL and Pitot HC (1989). Regulation of the expression of proto-oncogenes and the gene of the 27-KD gap junction protein during multistage hepatocarcinogenesis in the rat. *Proceedings of American Association Cancer Research* **30**, 207.

Nicholson, B. J., Weber, P. A., Cao, F., Chang, H., Lampe, P., and Goldberg, G. (2000). The molecular basis of selective permeability of connexins is complex and includes both size and charge. *Braz J Med Biol Res* **33**, 369-378.

O'Day, D. (2005). From cells to tissues: Cell Junctions, Univ of Toronto at Mississauga.

O'Regan, R. M., Gajdos, C., Dardes, R. C., De Los Reyes, A., Park, W., Rademaker, A. W., and Jordan, V. C. (2002). Effects of raloxifene after tamoxifen on breast and endometrial tumor growth in athymic mice. *J Natl Cancer Inst* **94**, 274-283.

Ogawa, K. a. B., Tibor (1993). *Electron Microscopic Cytochemistry and Immunocytochemistry in Biomedicine*. CRC Press, Boca Raton, Florida.

Olson, J. R. (1994). Pharmacokinetics of dioxins and related chemicals. In *Dioxins and Health* (A. Schecter, Gasiewicz, Thomas A, Ed.), pp. 163-167. John Wiley and Sons Inc., New York, NY.

Osborne, C., Elledge RM, Fuqua SAW (1996). Estrogen receptors in breast cancer therapy. *Science Medicine* **3**, 32-41.

Osborne, C. K. (1998). Tamoxifen in the treatment of breast cancer. *N Engl J Med* **339**, 1609-1618.

Otter, I., Conus, S., Ravn, U., Rager, M., Olivier, R., Monney, L., Fabbro, D., and Borner, C. (1998). The binding properties and biological activities of Bcl-2 and Bax in cells exposed to apoptotic stimuli. *J Biol Chem* **273**, 6110-6120.

Oyamada, M., Oyamada, Yumiko and Takamatsu, Tetsuro (1998). Gap junctions in health and disease. *Medical Electron Microscopy* **31**, 115-120.

Perez, E. A. (2007). Safety profiles of tamoxifen and the aromatase inhibitors in adjuvant therapy of hormone-responsive early breast cancer. *Annals of Oncology* **18**, 26-35.

Perry, R. R., Kang, Y., and Greaves, B. (1995). Effects of tamoxifen on growth and apoptosis of estrogen-dependent and -independent human breast cancer cells. *Ann Surg Oncol* **2**, 238-245.

PO Lague, H. D., H Rubin, C Tobias (1970). Electrical coupling: Low resistance Junctions between mitotic and interphase fibroblasts in tissue culture. *Science* **170**, 464-466.

Pritchard, K. I., Paterson, A. H., Fine, S., Paul, N. A., Zee, B., Shepherd, L. E., Abu-Zahra, H., Ragaz, J., Knowling, M., Levine, M. N., Verma, S., Perrault, D., Walde, P. L., Bramwell, V. H., Poljicak, M., Boyd, N., Warr, D., Norris, B. D., Bowman, D., Armitage, G. R., Weizel, H., and Buckman, R. A. (1997). Randomized trial of cyclophosphamide, methotrexate, and fluorouracil chemotherapy added to tamoxifen as adjuvant therapy in postmenopausal women with node-positive estrogen and/or progesterone receptor-positive breast cancer: a report of the National Cancer Institute of Canada Clinical Trials Group. Breast Cancer Site Group. *J Clin Oncol* **15**, 2302-2311.

Proulx, A., Merrifield, P. A., and Naus, C. C. (1997). Blocking gap junctional intercellular communication in myoblasts inhibits myogenin and MRF4 expression. *Dev Genet* **20**, 133-144.

Qin, H., Shao, Q., Curtis, H., Galipeau, J., Belliveau, D. J., Wang, T., Alaoui-Jamali, M. A., and Laird, D. W. (2002). Retroviral delivery of connexin genes to human breast tumor cells inhibits in vivo tumor growth by a mechanism that is independent of significant gap junctional intercellular communication. *J Biol Chem* **277**, 29132-29138.

Raptis, L. H., Brownell, H. L., Firth, K. L., and Mackenzie, L. W. (1994). A novel technique for the study of intercellular, junctional communication: electroporation of adherent cells on a partly conductive slide. *DNA Cell Biol* **13**, 963-975.

Rash, J. E., and Yasumura, T. (1999). Direct immunogold labeling of connexins and aquaporin-4 in freeze-fracture replicas of liver, brain, and spinal cord: factors limiting quantitative analysis. *Cell Tissue Res* **296**, 307-321.

Rashid, A., Elledge, R.M., and Chang, J.C. (2006). In *New Breast Cancer Research* (A. P. Yao, Ed.), pp. 1-25. Nova Science Publishers, New York.

Revel, J. P., and Karnovsky, M. J. (1967). Hexagonal array of subunits in intercellular junctions of the mouse heart and liver. *J Cell Biol* **33**, C7-C12.

Riggins, R. B., Lan, J. P., Zhu, Y., Klimach, U., Zwart, A., Cavalli, L. R., Haddad, B. R., Chen, L., Gong, T., Xuan, J., Ethier, S. P., and Clarke, R. (2008). ERRgamma mediates tamoxifen resistance in novel models of invasive lobular breast cancer. *Cancer Res* **68**, 8908-8917.

Riggins, R. B., Schrecengost, R. S., Guerrero, M. S., and Bouton, A. H. (2007). Pathways to tamoxifen resistance. *Cancer Lett* **256**, 1-24.

Rivkin, S. E., Green, S., Metch, B., Cruz, A. B., Abeloff, M. D., Jewell, W. R., Costanzi, J. J., Farrar, W. B., Minton, J. P., and Osborne, C. K. (1994). Adjuvant CMFVP versus tamoxifen versus concurrent CMFVP and tamoxifen for postmenopausal, node-positive, and estrogen receptor-positive breast cancer patients: a Southwest Oncology Group study. *J Clin Oncol* **12**, 2078-2085.

Robinson, D., and Cardozo, L. D. (2003). The role of estrogens in female lower urinary tract dysfunction. *Urology* **62**, 45-51.

Rosendaal, M., Green, C. R., Rahman, A., and Morgan, D. (1994). Up-regulation of the connexin43+ gap junction network in haemopoietic tissue before the growth of stem cells. *J Cell Sci* **107 (Pt 1)**, 29-37.

Rozenberg, S., Pastijn, A., Gevers, R., and Murillo, D. (2004). Estrogen therapy in older patients with recurrent urinary tract infections: a review. *Int J Fertil Womens Med* **49**, 71-74.

Rutz, M. L., and Hulser, D. F. (2001). Supramolecular dynamics of gap junctions. *Eur J Cell Biol* **80**, 20-30.

Saez, J. C., Berthoud, V. M., Branes, M. C., Martinez, A. D., and Beyer, E. C. (2003). Plasma membrane channels formed by connexins: their regulation and functions. *Physiol Rev* **83**, 1359-1400.

Saez, J. C., Connor, J. A., Spray, D. C., and Bennett, M. V. (1989). Hepatocyte gap junctions are permeable to the second messenger, inositol 1,4,5-trisphosphate, and to calcium ions. *Proc Natl Acad Sci U S A* **86**, 2708-2712.

Schecter, A., Cramer, P., Boggess, K., Stanley, J., and Olson, J. R. (1997). Levels of dioxins, dibenzofurans, PCB and DDE congeners in pooled food samples collected in 1995 at supermarkets across the United States. *Chemosphere* **34**, 1437-1447.

Schecter, A., Papke, O., Isaac, J., Hrimat, H., Neiroukh, F., Saf, J., El-Nahhal, Y. (1997). 2, 3, 7, 8-Chlorine substituted dioxin and dibenzofuran congeners in 2,4-D, 2,4,5-T and pentachlorophenol in Organohalogen compounds. In *Short papers from Dioxin* (R. Hites, Ed.), pp. 51-55, Indianapolis, IN.

Sciences, N. I. o. E. H. (2007). Endocrine Disruptors, NC.

Seidman, H., Stellman, S. D., and Mushinski, M. H. (1982). A different perspective on breast cancer risk factors: some implications of the nonattributable risk. *CA Cancer J Clin* **32**, 301-313.

Sharovskaja, J. J., Vaiman, A. V., Solomatina, N. A., and Kobliakov, V. A. (2004). Inhibition of gap junction intercellular communications in cell culture by polycyclic aromatic hydrocarbons (PAH) in the absence of PAH metabolism. *Biochemistry (Mosc)* **69**, 413-419.

Shen, H., Main, K. M., Virtanen, H. E., Damggard, I. N., Haavisto, A. M., Kaleva, M., Boisen, K. A., Schmidt, I. M., Chellakooty, M., Skakkebaek, N. E., Toppari, J., and Schramm, K. W. (2007). From mother to child: investigation of prenatal and postnatal exposure to persistent bioaccumulating toxicants using breast milk and placenta biomonitoring. *Chemosphere* **67**, S256-262.

Shin, K., Fogg, V. C., and Margolis, B. (2006). Tight junctions and cell polarity. *Annu Rev Cell Dev Biol* **22**, 207-235.

Shirai, T., Tsuda, H., Ogiso, T., Hirose, M., and Ito, N. (1987). Organ specific modifying potential of ethinyl estradiol on carcinogenesis initiated with different carcinogens. *Carcinogenesis* **8**, 115-119.

Silkworth, J. B., Brown, J.F. Jr. (1996). Evaluating the impact of exposure to environmental contaminants on human health. *Clinical Chemistry* **42**, 1345-1349.

Simpson JE, M. J., and Franklin CL (2002). Effect of bedding sterilization and type on the incidence of urogenital disease in the estrogenized nude mouse. *Contemp Topics (abstract)* **41**, 72.

Singal, R., Tu, Z. J., Vanwert, J. M., Ginder, G. D., and Kiang, D. T. (2000). Modulation of the connexin26 tumor suppressor gene expression through methylation in human mammary epithelial cell lines. *Anticancer Res* **20**, 59-64.

Sosinsky, G. E. (1996). Molecular organization of gap junction membrane channels. *J Bioenerg Biomembr* **28**, 297-309.

Steel, K. P. (1998). One connexin, two diseases. *Nat Genet* **20**, 319-320.

Stein, L. S., Boonstra, J., and Burghardt, R. C. (1992). Reduced cell-cell communication between mitotic and nonmitotic coupled cells. *Exp Cell Res* **198**, 1-7.

Steinberg, T. H., Civitelli, R., Geist, S. T., Robertson, A. J., Hick, E., Veenstra, R. D., Wang, H. Z., Warlow, P. M., Westphale, E. M., Laing, J. G., and et al. (1994). Connexin43 and connexin45 form gap junctions with different molecular permeabilities in osteoblastic cells. *EMBO J* **13**, 744-750.

Sugie, S., Mori, H., and Takahashi, M. (1987). Effect of in vivo exposure to the liver tumor promoters phenobarbital or DDT on the gap junctions of rat hepatocytes: a quantitative freeze-fracture analysis. *Carcinogenesis* **8**, 45-51.

Tan, L. W., Bianco, T., and Dobrovic, A. (2002). Variable promoter region CpG island methylation of the putative tumor suppressor gene Connexin 26 in breast cancer. *Carcinogenesis* **23**, 231-236.

Thurlimann, B., Keshaviah, A., Coates, A. S., Mouridsen, H., Mauriac, L., Forbes, J. F., Paridaens, R., Castiglione-Gertsch, M., Gelber, R. D., Rabaglio, M., Smith, I., Wardley, A., Price, K. N., and Goldhirsch, A. (2005). A comparison of letrozole and tamoxifen in postmenopausal women with early breast cancer. *N Engl J Med* **353**, 2747-2757.

Tormey, D. C., Rasmussen, P, and Jordan, V.C. (1987). Long-term adjuvant tamoxifen study: clinical update (Letter). *Breast Cancer Research Treatment* **9**, 157-158.

Tormey, D. C. a. J., V.C. (1984). Long-term tamoxifen adjuvant therapy in node-positive breast cancer: A metabolic and pilot clinical study. *Breast Cancer Research and Treatment* **4**, 297-302.

Trosko, J. E., and Ruch, R. J. (1998). Cell-cell communication in carcinogenesis. *Front Biosci* **3**, d208-236.

Trosko, J. E. a. C., C.C. (1983). Potential role of intercellular communication in the rate-limiting step in carcinogenesis. *Journal of American College of Toxicology* **2**, 5-22.

Van der Ven, K., van der Ven, H., Thibold, A., Bauer, O., Kaisi, M., Mbura, J., Mgaya, H. N., Weber, N., Diedrich, K., and Krebs, D. (1992). Chlorinated hydrocarbon content of fetal and maternal body tissues and fluids in full term pregnant women: a comparison of Germany versus Tanzania. *Hum Reprod* **7 Suppl 1**, 95-100.

Van Leeuwen, F. E., Benraadt, J., Coebergh, J. W., Kiemeney, L. A., Gimbrere, C. H., Otter, R., Schouten, L. J., Damhuis, R. A., Bontenbal, M., Diepenhorst, F. W., and et al. (1994). Risk of endometrial cancer after tamoxifen treatment of breast cancer. *Lancet* **343**, 448-452.

Van Rijen, H. V., R. Wilders, M.B. Rook, and H.J. Jongsma (2001). Dual Patch Clamp. In *Methods in Molecular Biology: Connexin Methods and Protocols* (R. Bruzzone, Giaume, Christian, Ed.), pp. 269. Humana Press, Totowa, New Jersey.

Vinken, M., Vanhaecke, T., Papeleu, P., Snykers, S., Henkens, T., and Rogiers, V. (2006). Connexins and their channels in cell growth and cell death. *Cell Signal* **18**, 592-600.

Vogel, V. G., Costantino, J. P., Wickerham, D. L., Cronin, W. M., Cecchini, R. S., Atkins, J. N., Bevers, T. B., Fehrenbacher, L., Pajon, E. R., Jr., Wade, J. L., 3rd, Robidoux, A., Margolese, R. G., James, J., Lippman, S. M., Runowicz, C. D., Ganz, P. A., Reis, S. E., McCaskill-Stevens, W., Ford, L. G., Jordan, V. C., and Wolmark, N. (2006). Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease

outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. *JAMA* **295**, 2727-2741.

Warshawsky, D. a. L. J. R. J. (2006). *Molecular carcinogenesis and the molecular biology of human cancer*. CRC Press, Boca Raton, Florida.

Werner, R. (1998a). In *Gap Junctions* (G. A. Perkins, Goodenough, D.A., Sosinsky, G.E., Ed.), pp. 13. IOS Press, Amsterdam, Netherlands.

Werner, R. (1998b). *Gap junctions*. IOS Press, Inc, Amsterdam, Netherlands.

Wilders, R., and Jongsma, H. J. (1992). Limitations of the dual voltage clamp method in assaying conductance and kinetics of gap junction channels. *Biophys J* **63**, 942-953.

Yamamoto, H., Ngan, C. Y., and Monden, M. (2008). Cancer cells survive with survivin. *Cancer Sci* **99**, 1709-1714.

Yano, T., Hernandez-Blazquez, F. J., Omori, Y., and Yamasaki, H. (2001). Reduction of malignant phenotype of HEPG2 cell is associated with the expression of connexin 26 but not connexin 32. *Carcinogenesis* **22**, 1593-1600.

Yano, T., Ito, F., Kobayashi, K., Yonezawa, Y., Suzuki, K., Asano, R., Hagiwara, K., Nakazawa, H., Toma, H., and Yamasaki, H. (2004). Hypermethylation of the CpG island of connexin 32, a candiate tumor suppressor gene in renal cell carcinomas from hemodialysis patients. *Cancer Lett* **208**, 137-142.

Yotti, L. P., Chang, C. C., and Trosko, J. E. (1979). Elimination of metabolic cooperation in Chinese hamster cells by a tumor promoter. *Science* **206**, 1089-1091.

Zhang A, W. Y., Lai HWL, Yew DT (2004). Apoptosis- A brief review. *Neuroembryology* **3**, 47-59.

Zhang, C. C., and Shapiro, D. J. (2000). Activation of the p38 mitogen-activated protein kinase pathway by estrogen or by 4-hydroxytamoxifen is coupled to estrogen receptor-induced apoptosis. *J Biol Chem* **275**, 479-486.

Zhang, G. J., Kimijima, I., Onda, M., Kanno, M., Sato, H., Watanabe, T., Tsuchiya, A., Abe, R., and Takenoshita, S. (1999). Tamoxifen-induced apoptosis in breast cancer cells relates to down-regulation of bcl-2, but not bax and bcl-X(L), without alteration of p53 protein levels. *Clin Cancer Res* **5**, 2971-2977.

Zhu, D., Caveney, S., Kidder, G. M., and Naus, C. C. (1991). Transfection of C6 glioma cells with connexin 43 cDNA: analysis of expression, intercellular coupling, and cell proliferation. *Proc Natl Acad Sci U S A* **88**, 1883-1887.

Ziambaras, K., Lecanda, F., Steinberg, T. H., and Civitelli, R. (1998). Cyclic stretch enhances gap junctional communication between osteoblastic cells. *J Bone Miner Res* **13**, 218-228.

Chapter 2 - Hypothesis and Objectives

2.1 Hypothesis

1. A decrease or loss in gap junctional intercellular communication can potentiate cancer formation
2. Restoration of gap junctional intercellular communication can decrease tumor growth

2.2 Objectives

1. To determine the effect of an environmental pollutant, TCDD, on gap junctional intercellular communication in human mammary epithelial cells (HMEC) and MCF-7 cells
2. To screen for gap junctional activators
3. To investigate the role of substituted quinolines, PQ1, on human breast cancer cell lines (*in vitro*) and nude mice (*in vivo*)
4. To evaluate the combinational effect of PQ1 and tamoxifen

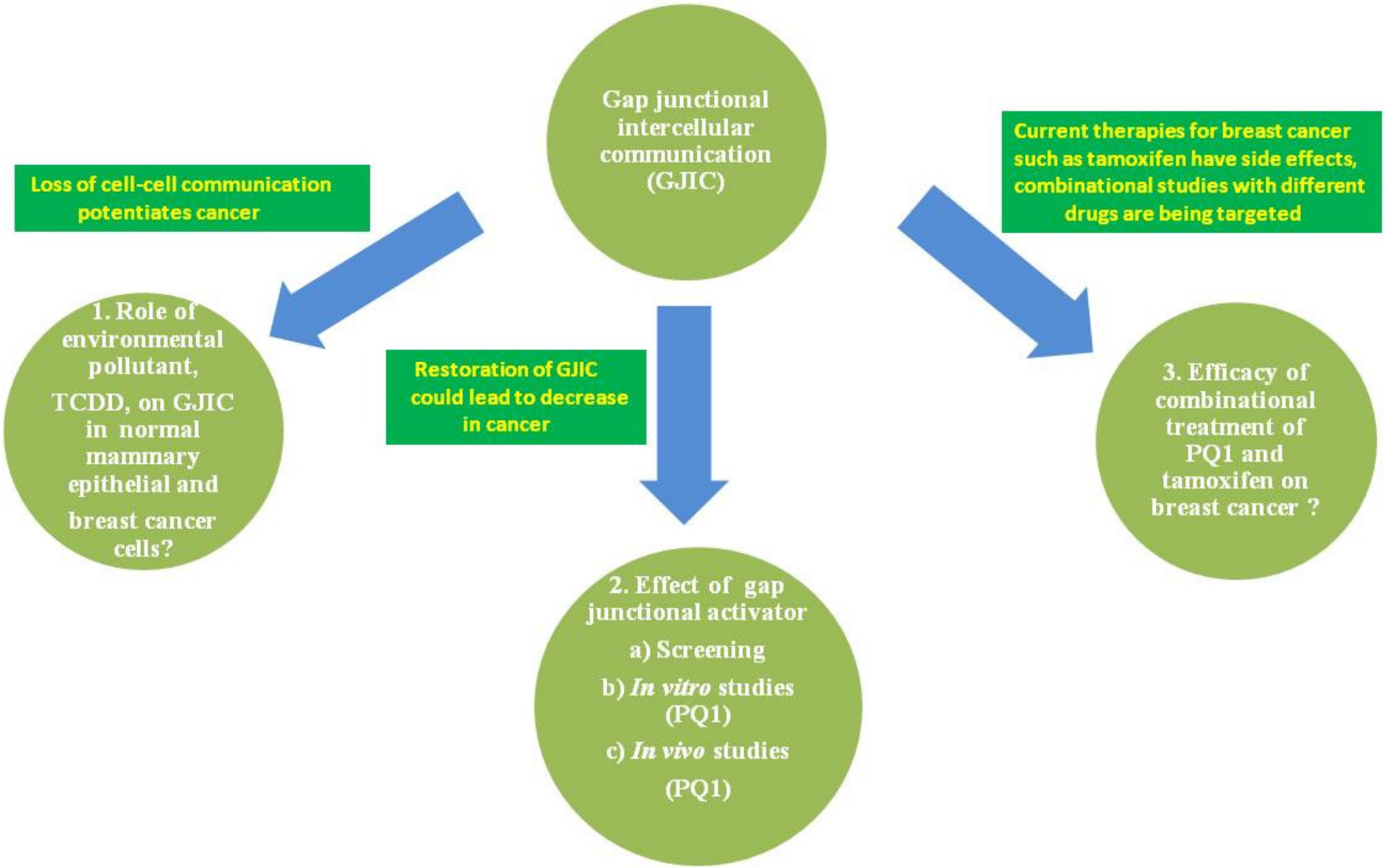


Figure 2.1 Schematic representation of the rationale and the questions being asked.

Dark green boxes represent rationale and the three circles represent objectives.

Chapter 3 - Regulation of gap junctional intercellular communication by TCDD in HMEC and MCF-7 breast cancer cells

3.1 Abstract

Previous studies suggest that many neoplastic tissues exhibit a decrease in gap junctional intercellular communication (GJIC). Many hydrocarbons and organochlorine compounds are environmental pollutants known to be carcinogenic. The effect of an organochlorine compound, TCDD, on GJIC in human breast cell lines has not been established. In the present study, we showed that TCDD causes an inhibition in the gap junctional activity in MCF-7 (breast cancer cells). In MCF-7 cells, an increase in the phosphorylated form of gap junctional protein, connexin 43 (Cx43), and PKC α was seen in the presence of TCDD. Gap junctional plaque formation was significantly decreased in MCF-7 cells in the presence of TCDD. Immunoprecipitation studies of PKC α showed that TCDD caused a significant 40% increase in the phosphorylated Cx43 in MCF-7 cells. TCDD also modulated the translocation of PKC α from the cytosol to the membrane and caused a 2-fold increase in the PKC α activity at 50 nM TCDD in MCF-7 cells. Calphostin C, an inhibitor of PKC α, showed a significant inhibition of PKC α activity in the presence of TCDD. Furthermore, TCDD also caused a decrease in the gap junctional activity and Cx43 protein in human mammary epithelial cells (HMEC). However, we observed a shift in the Cx43 plaques towards the perinuclear membrane in the presence of TCDD by confocal microscopy and Western blot. Overall, these results conclude that TCDD decreases GJIC by phosphorylating Cx43 via PKC α signaling pathway in MCF- 7 cells; however, TCDD decreases the GJIC by affecting the localization of Cx43 in HMEC. These new findings elucidate the differential mode of effect of TCDD in the downregulation of GJIC in HMEC and MCF-7 cells.

3.2 Introduction

Polycyclic aromatic hydrocarbons are ubiquitous environmental and food contaminants formed mainly during the incomplete combustion of organic materials (Blaha *et al*., 2002). Among these, 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD), is a highly persistent environmental pollutant and a known human carcinogen (Safe, 1990, 1995). More than 2-fold increase in the incidence of breast cancer was found in female workers working in TCDD-exposed chemical plant (Manz *et al*., 1991). TCDD was shown to alter mammary gland differentiation and increased susceptibility for mammary cancer in a carcinogen induced rat mammary cancer model (Jenkins *et al*., 2006). Many studies indicate the potential role of TCDD in regulating the activity of GJIC (Baker *et al*., 1995; Chipman *et al*., 2003; Sharovskaja *et al*. 2004). But the effect of TCDD on the GJIC in human breast cells has not been studied.

Gap junctional intercellular communication (GJIC) is formed by gap junctions (GJ), dynamic intercellular plasma membrane channels allowing the passage of small molecules (<1 kD) between adjacent cells (Mesnil, 2002; Carystinos *et al*., 2002; Vine and Bertram, 2002). Gap junctions are formed by the interaction of two hemichannels (connexon) in the adjacent cells, which, in turn are made of six subunits of connexin (Cx) proteins (Carystinos et al., 2002). The structure of connexins includes four hydrophobic membrane-spanning domains, two extracellular loops, one cytoplasmic loop, and an amino and carboxyl terminus in the cytoplasm (Goodenough *et al*., 1988; Hertzberg *et al*., 1988; Yeager and Gilula, 1992; Knudsen and Wheelock, 1992). In a cell, GJIC is mainly regulated by the phosphorylation of the C-terminus of connexin proteins (Carystinos *et al*., 2002). The ability of TPA to inhibit GJIC has been associated with the activation of PKC (Lampe, 1994; Rivedal and Opsahl, 2001; Zampighi *et al*., 2005). Evidence demonstrated that PKC may inhibit gap junction channels by inhibiting gap junction assembly and channel gating (Tomasetto *et al*., 1993). PKC α overexpression has been shown to cause tumor growth, and tumor resistance to cytotoxic chemotherapy. In a study conducted on breast cancer patients, PKC activity was found significantly higher in breast tumor tissue compared to normal tissue from the same patient (O'Brian *et al*., 1989). Studies have shown that many neoplastic cells show fewer or dysfunctional gap junctions and have reduced GJIC (Trosko and Ruch, 1998). Many studies also suggest that the alteration in connexin expression is important, as their forced expression, following cDNA transfection, can promote cell density inhibition and reverse the tumor phenotype (Mesnil, 2002). Tumor growth of communication-deficient human

tumor cells, SKHep1, was significantly higher than cells transfected with cDNA encoding Cx32 in athymic nude mice (Eghbali *et al*., 1991). Re-expression of hCx26 and hCx43 exhibited a classical tumor-suppressive behavior restoring growth-regulatory properties to metastatic mammary carcinoma cells and inducing differentiation (Hirschi *et al*., 1996). Consistently, these studies strongly suggest that the altered expression of connexins leads to loss of growth control and restoration of GJIC causes inhibition of cell proliferation thereby exhibiting tumor suppressive effect. Given the importance of GJIC in cancer, we used breast cancer as the model to test the effect of TCDD on GJIC. Cx43 is the most predominant form in breast epithelium and detected mostly in myoepithelial cells. Therefore, in our present study we observed the effect of TCDD on GJIC by measuring Cx43 expression. The strategy for the prevention and treatment of cancers is based on understanding the underlying mechanisms of carcinogenesis. Environmental pollutants have been shown to affect the GJIC but the mechanism still remains elusive. The aim of our study was to investigate how TCDD can affect the activity of GJIC in human breast cells. We directly examined the effect of TCDD on human breast cancer cells, MCF-7, and primary mammary epithelial cells, HMEC. Our study provides evidence showing that TCDD inhibits the GJIC through traditional and non-traditional pathways in cancerous and non-cancerous breast cells respectively.

3.3 Material and Methods

3.3.1 Cell lines and cell culture

MCF-7 human breast cancer cell line was purchased from American Type Culture Collection (Manassas, VA) and HMEC from Cambrex (Rockland, MA). MCF-7 cells were grown in Minimal Essential Media (MEM) supplemented with Earl's salts, glutamine, 1 mM sodium pyruvate, 0.01 mM nonessential amino acids, antibiotic/antimycotic, and 1.5 g/L sodium bicarbonate. MCF-7 cells were enriched with 10% fetal bovine serum (Atlanta biologicals, Lawrenceville, GA). HMEC were grown in mammary epithelial growth medium supplemented with bovine pituitary extract from Cambrex (Rockland, MA). Calphostin C, an inhibitor of PKC α, was purchased from Calbiochem (NJ, USA) and dissolved in dimethyl sulfoxide (DMSO).

3.3.2 Gap junctional activity - scrape load/dye transfer (SL/DT) assay

Cells were grown to 90% confluency on coverslips, dosed with 10, 50, 100 and 200 nM of TCDD and DMSO for an hour. Similarly, MCF-7 cells were dosed with calphostin C (100 nM) for 30 min and media were aspirated and cells were treated with varying concentrations of TCDD for an hour. TPA was used as a positive control for the phosphorylation of PKC α. Cells were washed three times with PBS. The 2.5 µl mixture of 1% (w/v) Lucifer yellow and 0.75% (w/v) of Rhodamine-dextran was added in the center of the coverslip. Two cuts crossing each other in the center of the coverslip were made. This cut allows the transfer of the Lucifer yellow dye as well as rhodamine-dextran in the cells at the line of cut. However, the passage of the Lucifer yellow dye from the injured cells to the membrane-intact cells is through gap junctions. After 3 min, cells were washed three times with PBS and incubated at 37°C in tissue culture media for 20 min. The cells were then washed with PBS and fixed in 2.5% paraformaldehyde for 10 min. Cells were mounted on a slide, sealed and visualized under a fluorescence microscope at 10X objective.

3.3.3 Western blot analysis

After reaching 90% confluency, MCF-7 cells and HMEC were harvested from a 75 cm^2 flask and 25 cm^2 flask respectively. Cells were grown in a phenol red-free DMEM with 5% charcoal dextran stripped serum, overnight. Cells were dosed with 0, 10, 50, 100, and 200 nM of TCDD and DMSO for an hour. DMSO was used as a solvent to dissolve TCDD. Cells were harvested in lysis buffer containing appropriate protease inhibitors. 30 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 mouse anti-PKC α antibody at 1:500 dilution (Santa Cruz Biotechnologies, Santa Cruz, CA), monoclonal mouse anti-Cx43 at 1:500 dilution (Fred Hutchinson, Seattle, WA), anti-phospho-Ser 368 Cx43 antibody at 1:500 dilution (Cell signaling technology, MA, USA) and rabbit anti-actin antibody at 1:1000 dilution (Sigma-Aldrich, Saint Louis, MO). The signal was detected by

enhanced chemiluminescence system (Pierce, Rockford, IL, USA). Lamin B1 antibody was used (ZL-5, Abcam, MA, USA) at 1:500 dilution to confirm the pure extraction of nuclear and cytoplasmic fraction.

3.3.4 Immunoprecipitation and translocation studies

MCF-7 cells were dosed with 10, 50, 100, and 200 nM TCDD and DMSO for an hour. Cells were harvested and 1 mg of whole cell extract was precleared with protein A/G-agarose beads (Santa Cruz Biotechnology, CA, USA) for 30 min. Then, samples were centrifuged at 2000 rpm for 5 min. Supernatant was collected and incubated with the anti-PKC α antibody at 1:2000 dilution overnight at 4°C. Samples were centrifuged at 2000 rpm for 5 min and pellet was washed three times with lysis buffer. Samples were run on 10% SDS-PAGE and immunoblotted with anti-Cx43 (Chemicon International Inc., Temecula, CA), anti-phospho-Ser 368 Cx43 and anti-PKC α antibodies. For translocation studies, MCF-7 cells were dosed with 10, 50, 100, and 200 nM TCDD. Cells were lysed with $MgCl_2$ and centrifuged at 35,000 rpm for 60 min at 4°C. Pellet was separated from the supernatant and resuspended in lysis buffer. Samples were sonicated and whole cell extracts were subjected to Western blot analysis. Nitrocellulose membranes were immunoblotted with anti-Cx43 and anti-actin antibodies. In HMEC, cytoplasmic and nuclear fractions were separated by using NE-PER kit (Cat # 78833, Pierce, Rockford, IL, USA).

3.3.5 Measuring kinase activity

The whole cell extract was obtained as described for Western blotting. PKC α activity was measured using Promega kinase activity assay kit (Promega, Madison, WI). The procedure was followed as recommended by the manufacturer. Results were visualized under UV light.

3.3.6 Immunofluorescent labeling and confocal microscopy

Cells were grown on coverslips in 6-well plates in phenol red-free DMEM media plus 5% charcoal dextran stripped serum overnight. Cells were treated with 10, 50, 100, 200 nM of TCDD and DMSO for 1 hr. Cells were first fixed with 2.5% paraformaldehyde for 10 min and

then neutralized with 50 mM glycine for 5 min. The cells were lysed with 0.15% Triton X-100 for additional 20 min. After washing with PBS, cells were blocked with 3% BSA in PBS for 2 hr and then incubated with primary antibodies, mouse anti-PKC α and rabbit anti-Cx43 antibodies (Chemicon International Inc, Temecula, CA), for 15 hr at 4°C. Following this step, cells were incubated with anti-mouse and anti-rabbit Alexa fluor 488 and 568 (Molecular probes, Eugene, OR, USA) for 2 hr at room temperature respectively. Samples were sealed and analyzed by a confocal microscope (Carl Zeiss LSM 510 META, Narashige, MN).

3.4 Results

3.4.1 Effect of TCDD on gap junctional activity

We examined the effect of TCDD on the GJIC in MCF-7 cells. By using scrape load/dye transfer (SL/DT) assay, the transfer of Lucifer yellow dye from one cell to another indicates GJIC and rhodamine-dextran is often used as a control as it cannot pass through intact plasma membrane. We observed a dose-dependent decrease in GJIC in presence of TCDD (Figure 3.1). 10 and 100 nM TCDD can inhibit Lucifer yellow dye transfer in MCF-7 cells (Figure 3.1A). MCF-7 cells treated with calphostin C and TCDD showed restoration of gap junctional activity at 10, 50, 100 nM of TCDD. At 200 nM TCDD, inhibition in GJIC was observed even in the presence of calphostin C (Figure 3.1B).

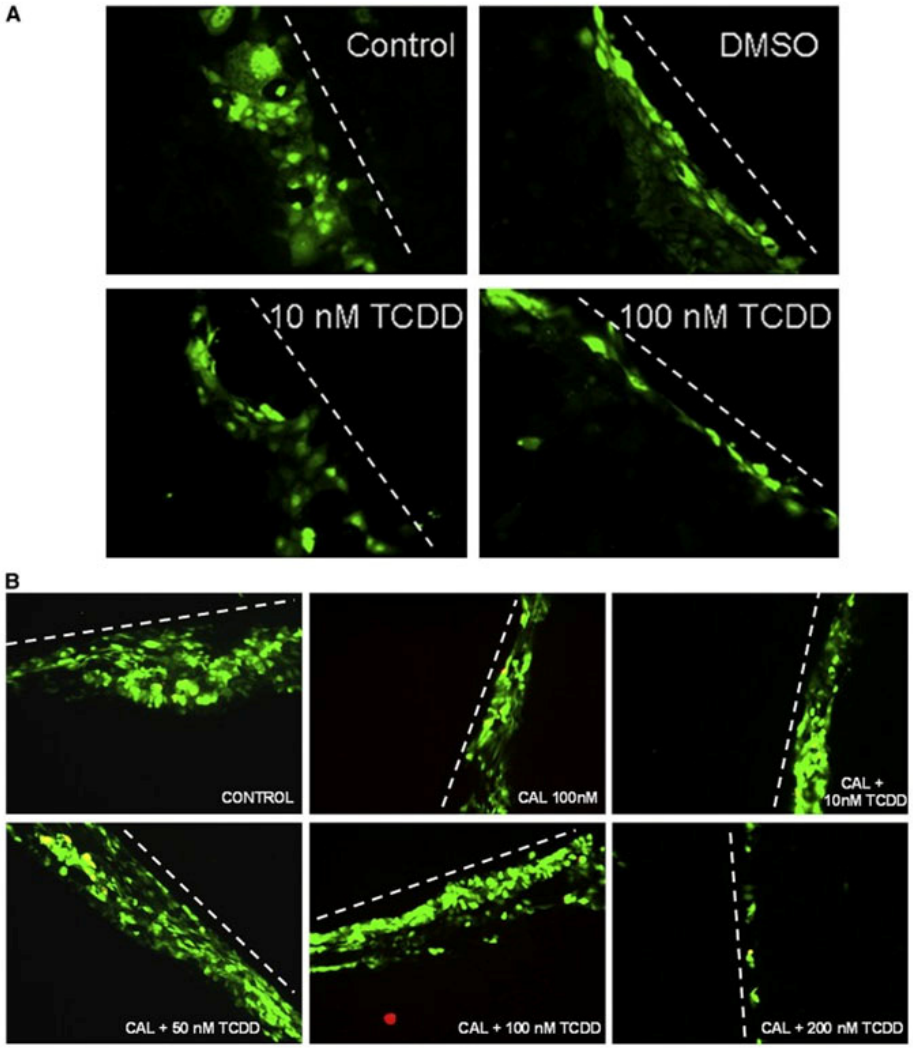


Figure 3.1 Scrape load/dye transfer assay (SL/DT)

A) MCF-7 breast cancer cells were seeded on coverslips in a 6-well plate until 90% confluency. Cells were then dosed with TCDD at various concentrations for an hour, loaded with dye, scraped, fixed in paraformaldehyde and viewed under fluorescence microscope. The cuts were made with a sharp blade in an X- fashion on the coverslip after the addition of the dye. Green fluorescence indicates Lucifer yellow, transferring from cell to cell only through gap junctions. Arrows indicate the line of cut. At 100 nM TCDD, there was a decrease in the transfer of Lucifer yellow dye away from the line of cut. Note: the difference in the Lucifer yellow dye transfer between the control and 100 nM TCDD. DMSO is a solvent used to dissolve TCDD. **B)** MCF-7 cells were treated with calphostin C, PKC α inhibitor, for 30 min and later with TCDD for 1 hr.

MCF-7 cells were prepared for the SL/DT assay as described above. Note: the increase in Lucifer dye transfer in 10, 50, and 100 nM TCDD pre-treated with calphostin C as compared to 10 and 100 nM TCDD alone without calphostin C. However, at 200 nM TCDD Lucifer yellow dye transfer is inhibited.

Furthermore, we examined the structural GJIC formation. Figure 3.2A shows the effect of TCDD on the Cx43 plaque formation in MCF-7 cells. 10-200 nM TCDD inhibited Cx43 plaque formation compared to control without TCDD or DMSO treatment in MCF-7 cells. On the other hand, in the presence of calphostin C, Cx43 plaque formation was seen at 10, 50, 100 nM TCDD but not at 200 nM TCDD (Figure 3.2B).

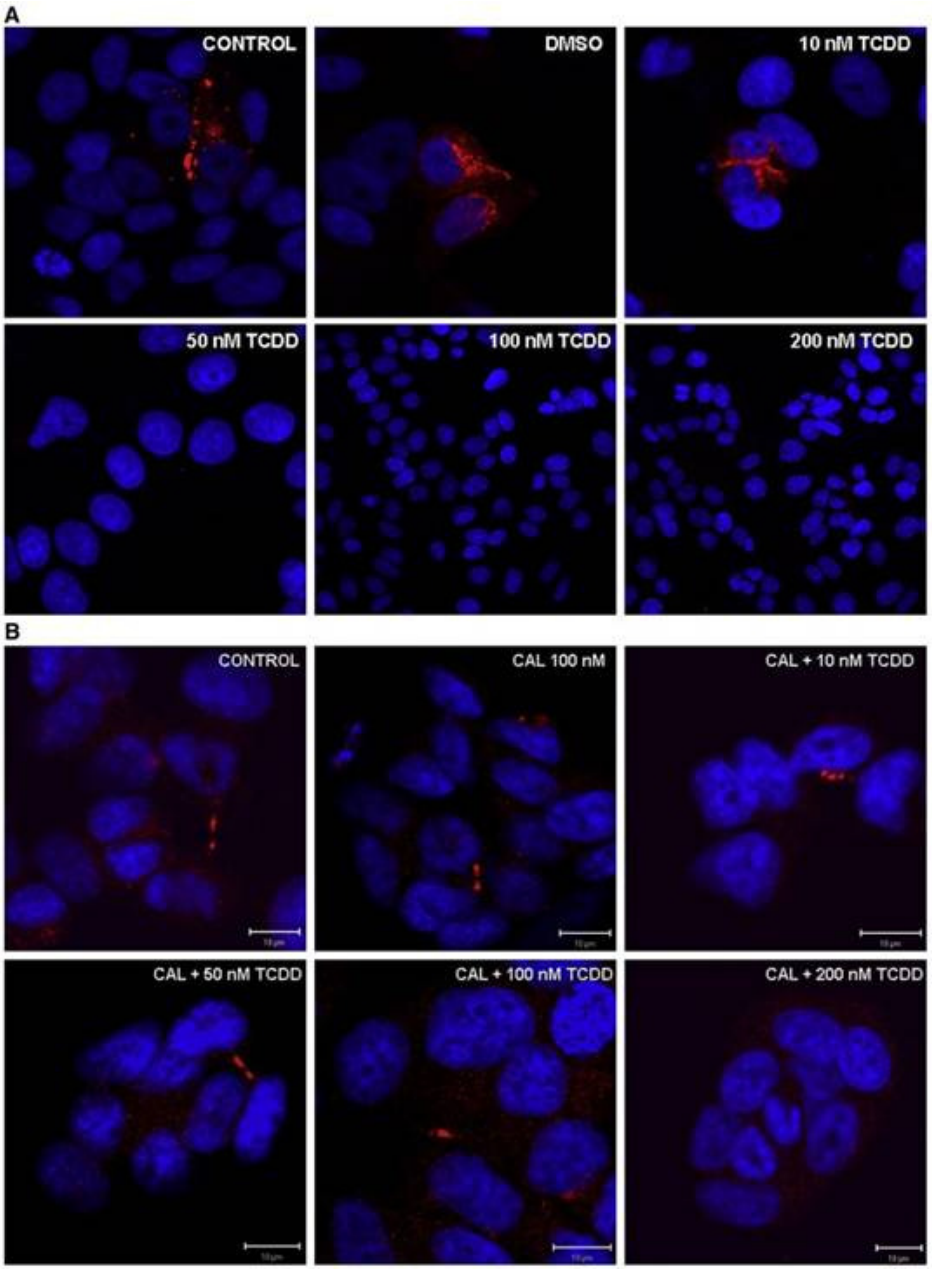


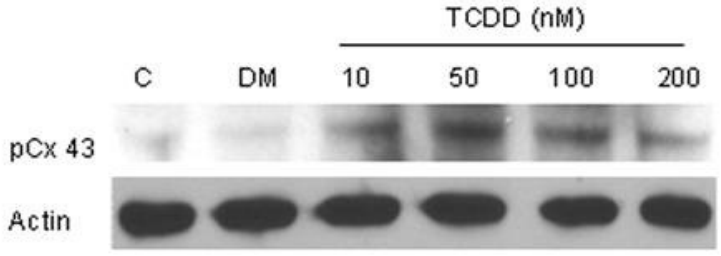
Figure 3.2 Confocal microscopy showing the effect of TCDD on the Cx43 plaque formation.

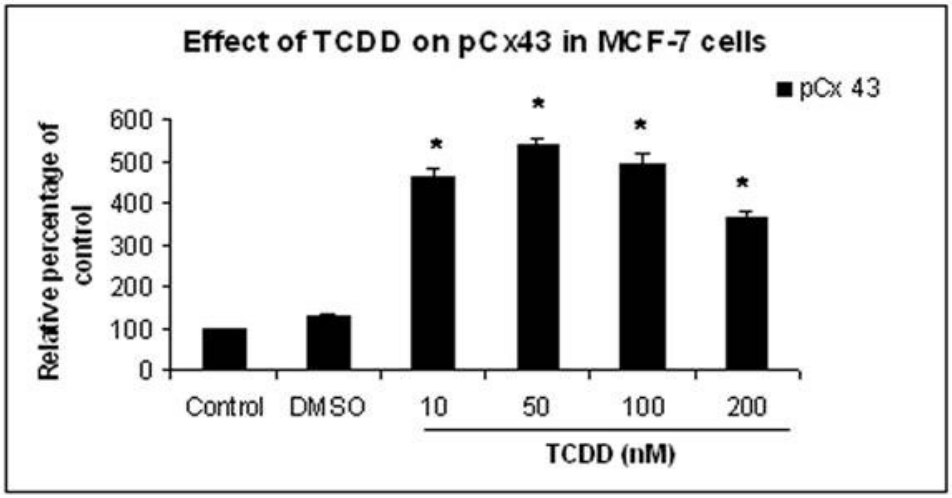
A) MCF-7 cells were dosed with 0, 10, 50, 100 and 200 nM TCDD for an hour. **B)** MCF-7 cells were treated with calphostin C (100 nM) for 30 min prior to the dosing of TCDD for an hour. Note: the presence of Cx43 plaques in 50 and 100 nM TCDD. However, at 200 nM TCDD Cx43 plaque formation was completely inhibited.

3.4.2 Effect of TCDD on the expression of Cx43 and PKC α

Cx43 is phosphorylated at multiple serine residues. One of the serine residues, serine 368, of Cx43 has been shown to be phosphorylated by PKC (Lampe and Lau, 2000). Therefore, the expression of both phospho-Ser 368 Cx43 and PKC proteins in the presence of TCDD was examined. We found that 10-200 nM TCDD caused an increase in phospho-Ser 368 Cx43 (Figure 3.3A). Similarly, a significant 40% increase in PKC α expression at 200 nM TCDD treatment compared to control or DMSO was seen (Figure 3.3B). However, 10 nM TCDD for 1 hr was not significant to cause an increase in PKC α expression.

A.





B.

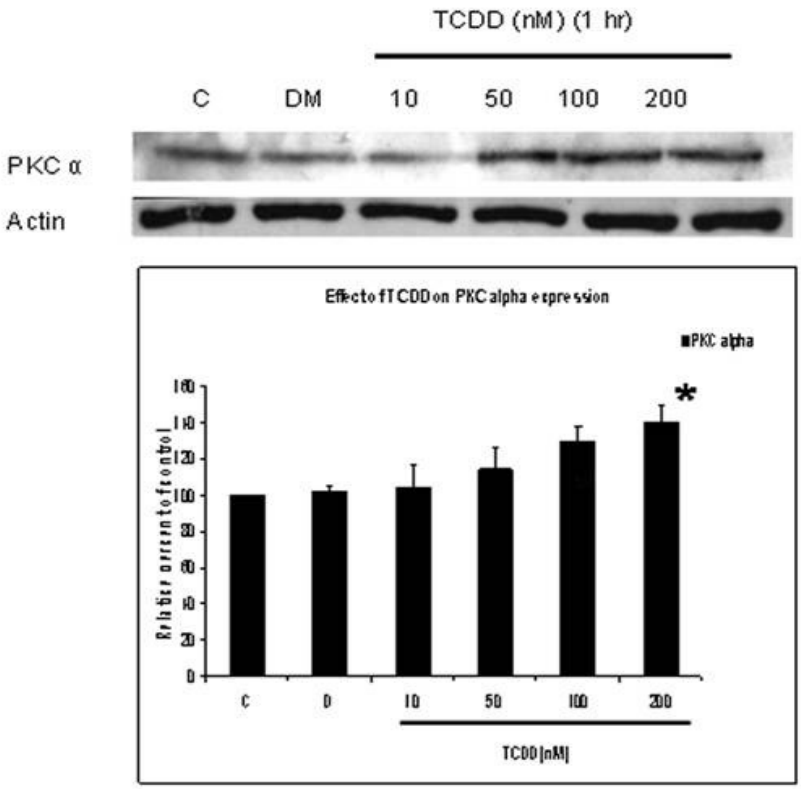


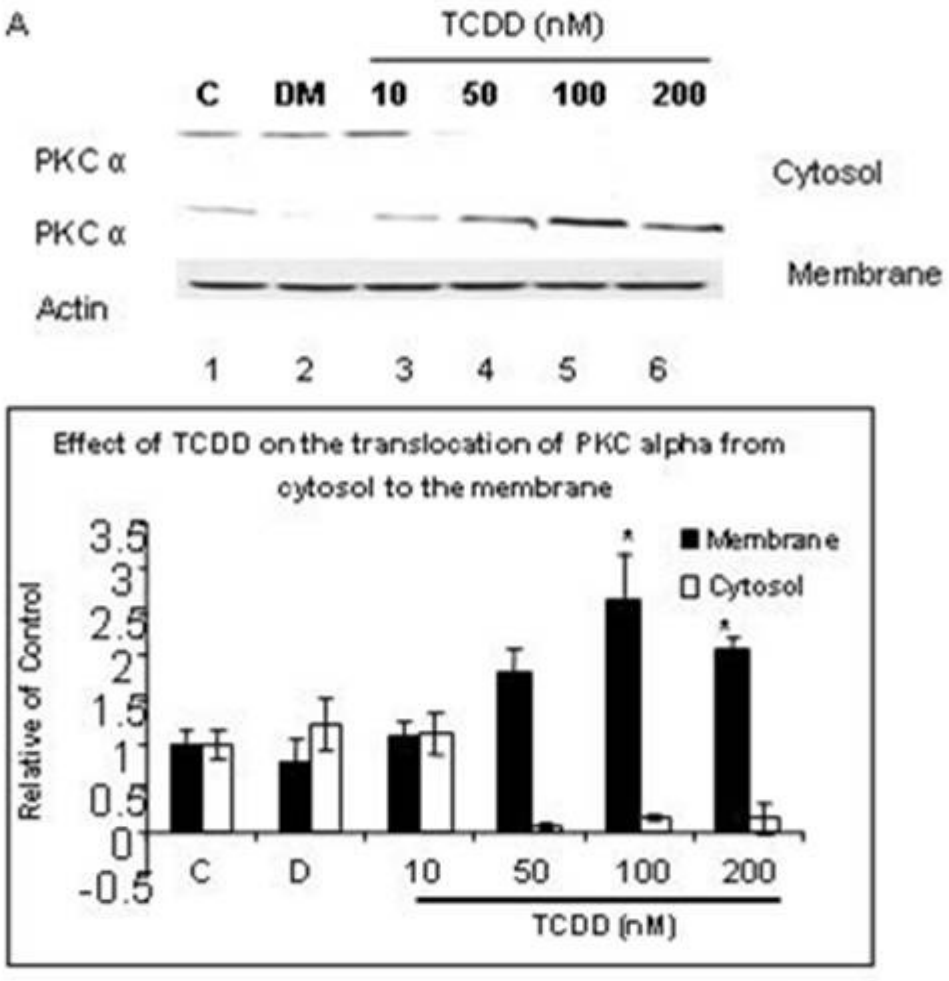
Figure 3.3 Expression profile of phospho-Ser 368 Cx43 and PKC α in presence of TCDD in MCF-7 cells.

A) Western analysis of phospho-Ser 368 Cx43 in MCF-7 cells dosed with different concentrations of TCDD. Actin is used as a loading control. A histogram representing three independent experiments is provided showing the increase in phospho-Ser 368 as a relative percentage of control, *p < 0.01. pCx43 is phosphorylated serine 368 Cx43. **B)** The effect of TCDD on the PKC α expression in MCF-7 cells. Graphical presentation of three independent experiments with the standard deviation is shown. DMSO is the vehicle used to dissolve TCDD. C = Control, DM = DMSO, *p < 0.05.

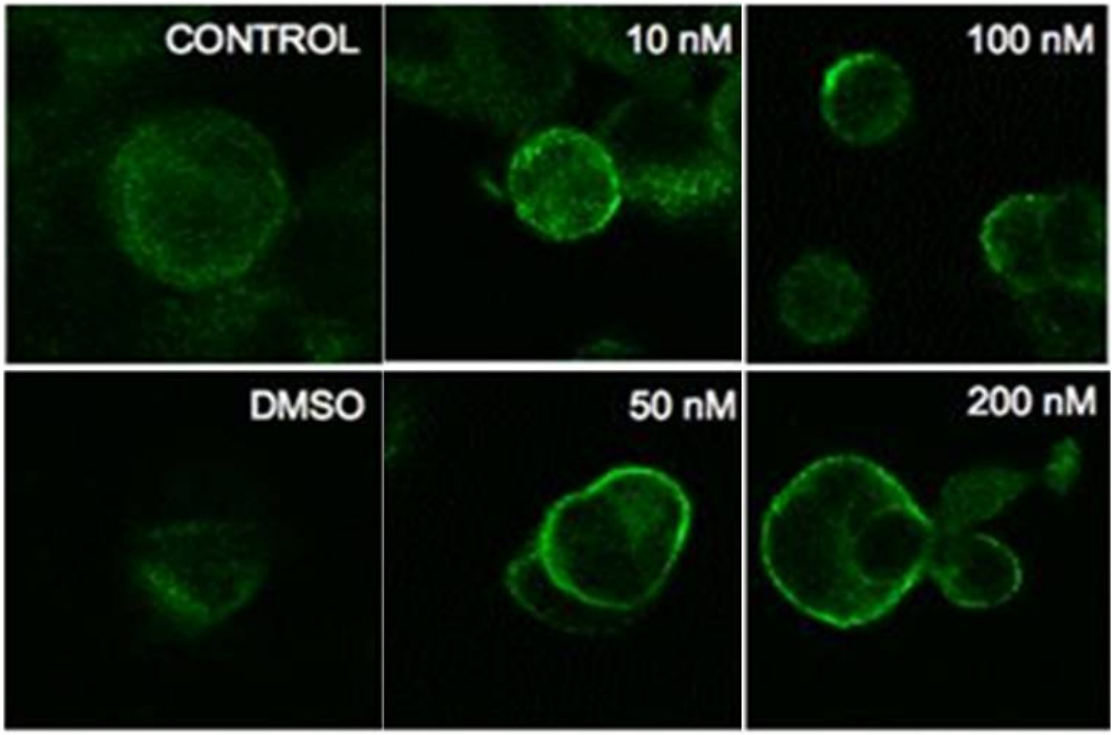
3.4.3 Translocation studies of PKC α in MCF-7 cells

Activation of PKC isoforms results in changes in their subcellular location. We found that TCDD increases the translocation of PKC α from the cytosol to the membrane. 10 nM TCDD was sufficient to translocate PKC α from the cytosol to the membrane (Figure 3.4A, lane

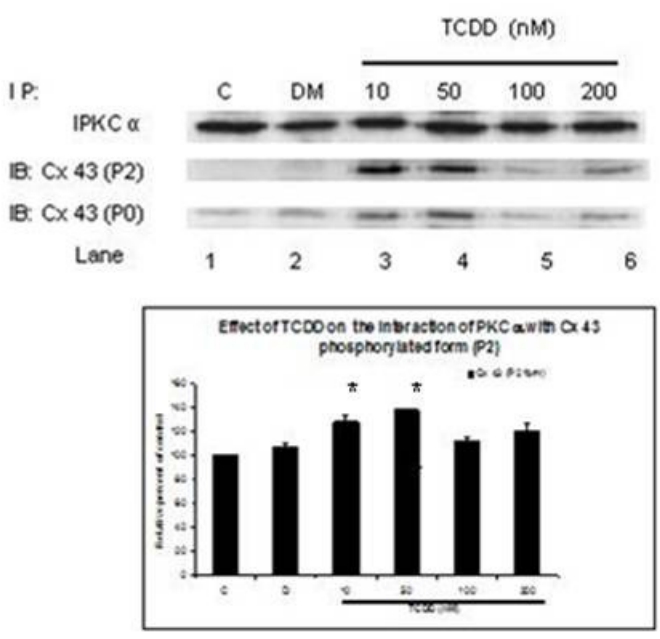
3), and 100 nM TCDD completely translocates PKC α to the membrane (Figure 3.4A, lane 5). This means that TCDD is involved in the activation of PKC α. The results were also confirmed by immunofluorescence studies (Figure 3.4B). Immunofluorescent PKC α was localized around the plasma membrane in TCDD treatment compared to control or DMSO. In HMEC, PKC α was not observed. Other classical PKCs (PKC β and PKC γ) were not affected by TCDD in both cell types (Appendix A, Figure S1).



B.


CONTROL
10 nM
100 nM
DMSO
50 nM
200 nM

C.


TCDD (nM)
I P:
C
DM
10
50
100
200
IPKC α
IB: Cx 43 (P2)
IB: Cx 43 (P0)
Lane
1
2
3
4
5
6
Effect of TCDD on the interaction of PKC α with Cx 43 phosphorylated form (P2)

D.

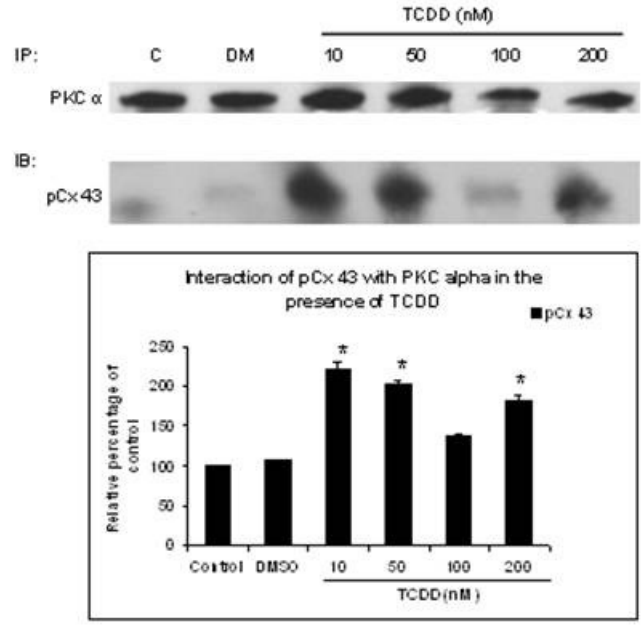

TCDD (nM)
IP:
C
DM
10
50
100
200
PKC α
IB:
pCx43
Interaction of pCx 43 with PKC alpha in the presence of TCDD
pCx 43
Relative percentage of control
Control
DMSO
10
50
100
200
TCDD (nM)

Figure 3.4 Localization of PKC α and the interaction of PKC α and Cx43.

A) Western blots showing the effect of TCDD on translocation of PKC α from the cytosol to the membrane in MCF-7 cells. Separation of cytosolic and membrane fractions was performed as described in Materials and Methods. 100 nM TCDD-treated cells caused a significant translocation of PKC α from the cytosolic fraction to the membrane fraction. Graphical representation of three independent experiments with ±SD, *$p < 0.05$. **B)** Immunofluorescence showing the localization of PKC α in the presence of TCDD in MCF-7 cells. Cells were treated with 10, 50, 100 and 200 nM TCDD for 1 hr. Immunofluorescent labeling was performed as described in Materials and Methods. PKC α was labeled with Alexa fluor 488 for 2 hr and then the cells were washed, sealed and observed under 40X oil objective confocal microscope, pin hole = 0.65 μm. Green fluorescence was seen around the cell membrane as the concentration of TCDD was increased. **C)** Interaction of PKC α and Cx43 in MCF-7 cells. Immunoprecipitated PKC α was subjected to Western blot analysis. The nitrocellulose membrane was immunoblotted against Cx43. P0 and P2 forms of Cx43 are shown separately. Graphical presentation of three experiments with standard deviation and statistical significance, *$p < 0.05$. **D)** Interaction of PKC α and pCx43 in MCF-7 cells. Immunoprecipitated PKC α was transferred to nitrocellulose membrane and immunoblotted with pCx43. A histogram representing three independent experiments, *$p < 0.01$.

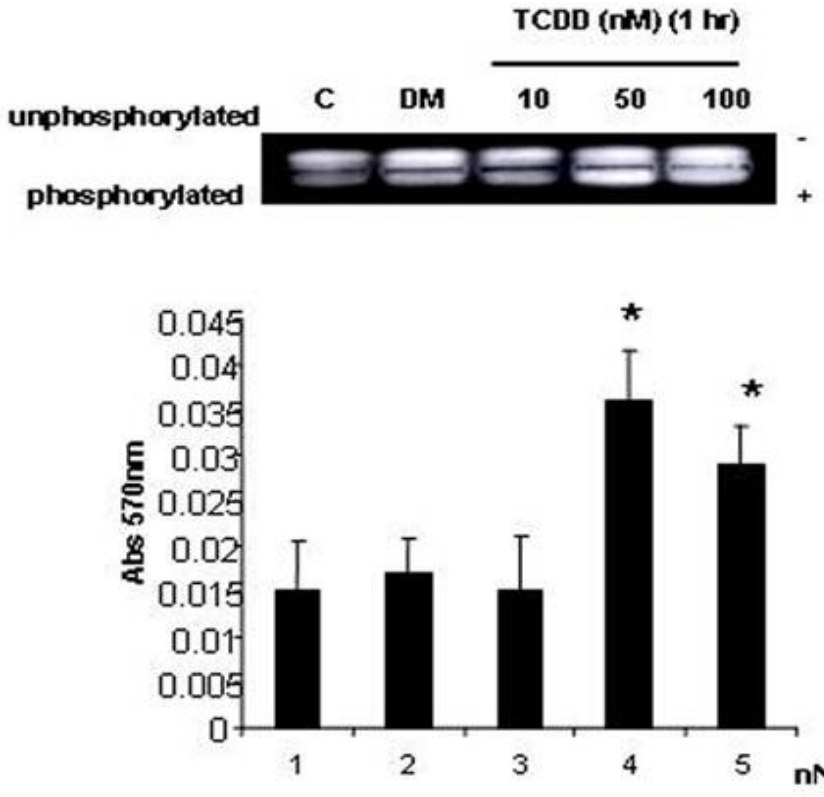
3.4.4 Immunoprecipitation studies showing interaction of phosphorylated Cx43 with PKC α

PKC α was immunoprecipitated from MCF-7 cells using Protein A/G-agarose beads and anti-PKC α antibodies (Figures 3.4C, D). The nitrocellulose membrane was probed with anti-Cx43 antibodies. The results showed a significant 40% increase in P2 form of Cx43 upon activation by 10 and 50 nM of TCDD in MCF-7 cells (Figure 3.4C). However, treatments of 100 and 200 nM TCDD only have a slight interaction of PKC α and Cx43 (Figure 3.4C lane 5, 6). This experiment was repeated using anti-phospho-Ser 368 Cx43 antibody (Figure 3.4D). Similarly, we found an increase in phospho-Ser 368 Cx43 at 10 and 50 nM TCDD. This suggests that in the presence of TCDD, PKC α phosphorylates Cx43 in MCF-7 cells.

3.4.5 Effect of TCDD on the PKC enzyme activity

PKC enzyme activity assay was performed with samples treated with calphostin C in the presence of TCDD and, TCDD alone. Promega PKC peptide substrate system was used to measure the PKC activity (Figure 3.5A). Fluorescence peptides separate according to charges. Phosphorylated peptides migrate towards anode. The results demonstrated that treatment of 50 nM TCDD significantly increased PKC enzyme activity by 2-fold in TCDD alone treated MCF-7 cells (Figure 3.5B). In the presence of calphostin C, PKC activity was significantly reduced at 10, 50, 100 nM TCDD compared to the control. However, at 200 nM TCDD, PKC enzyme activity was 20% more than the control.

A.



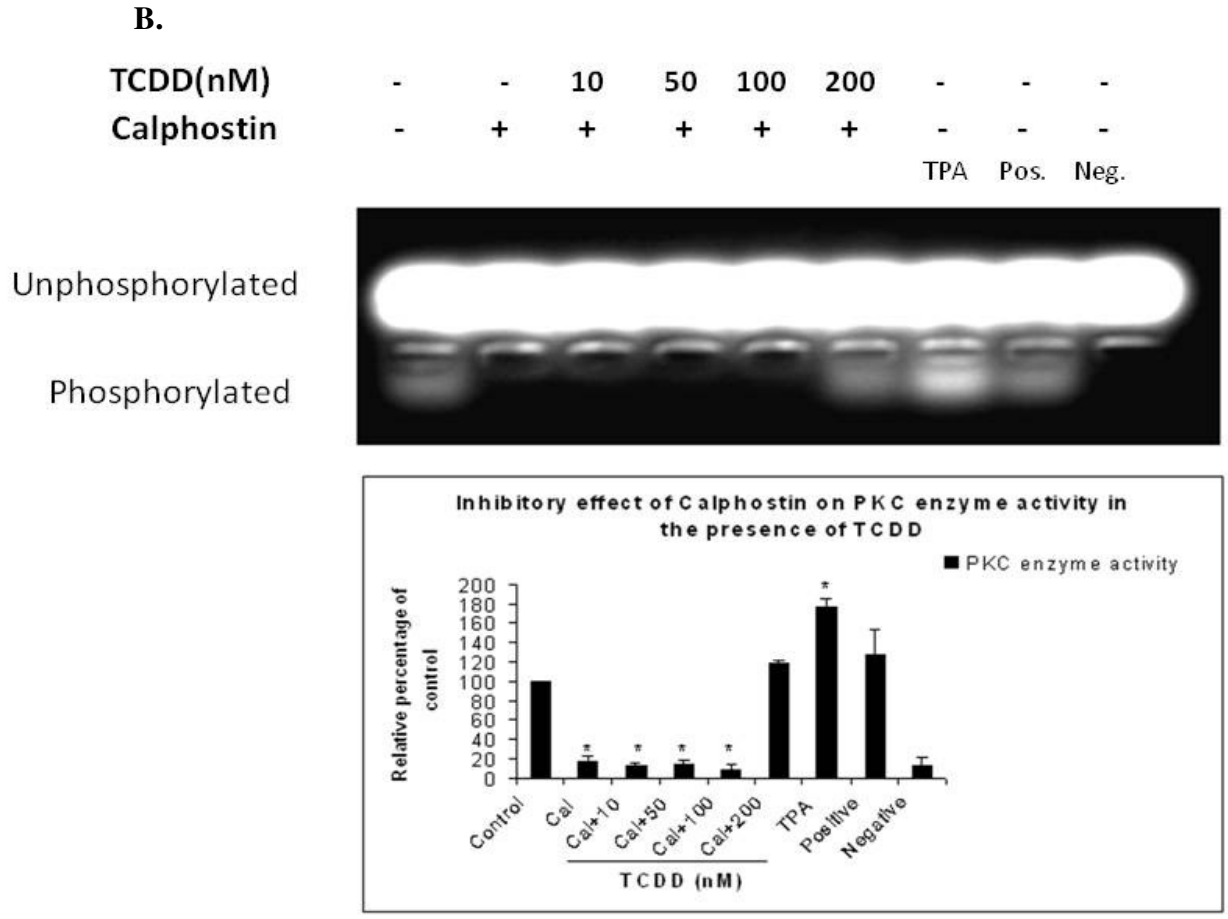


Figure 3.5 The effect of TCDD on PKC enzyme activity in MCF-7 cells.

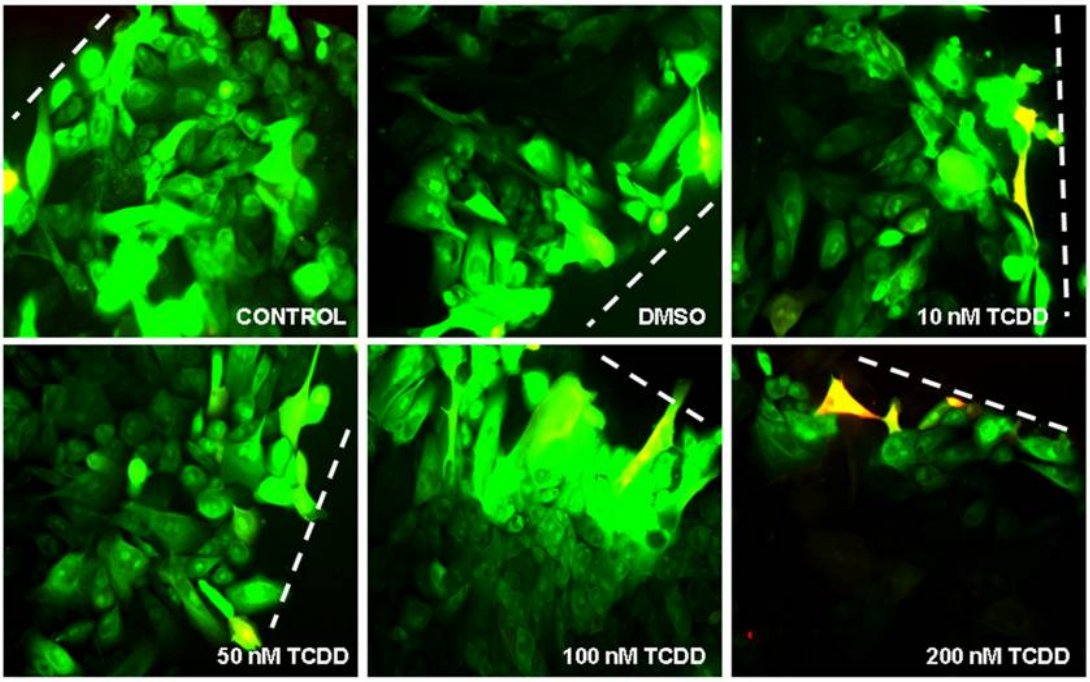
A) Cells were dosed with 10, 50, 100 and 200 nM of TCDD. Whole cell extract was used. PKC enzyme activity was performed according to the manufacturer's recommendation. Reaction mixtures were separated on agarose gel according to the charges of the substrate. Excision of the phosphorylated bands was measured using photospectrometer at 570 nm. Graphical representation of three experiments with ±SD and statistical significance, *$p < 0.05$. **B)** Cells were dosed with calphostin C for 30 min prior to 10, 50, 100 and 200 nM TCDD treatment for 1 hr. PKC enzyme activity was performed. Note: the significant decrease in the PKC activity in the presence of calphostin C treated with 10, 50 and 100 nM TCDD. However, at 200 nM TCDD, the PKC activity restored back indicating that the effect of 200 nM TCDD on PKC activity cannot be inhibited with 100 nM calphostin C. TPA was used as a positive control for PKC activity. Positive indicates the positive control provided by the manufacturer, which includes using purified PKC enzyme. Negative control includes the reaction mixture excluding any

sample/PKC enzyme. Graphical representation of three experiments with ±SD and statistical significance, *$p < 0.01$.

3.4.6 Effect of TCDD on primary mammary epithelial cells

Previous studies have indicated that the expression of Cx43 or gap junctional activity of normal cells is higher than neoplastic cells (Hirschi *et al.* 1996; Trosko and Ruch 1998). Since breast cancer cells have a lower level of Cx43 and gap junctional activity, we examined the effect of TCDD on normal breast cells, HMEC. Interestingly, higher concentration of TCDD is required to inhibit dye transfer. 200 nM TCDD caused a significant decrease in GJIC compared to control in HMEC (Figure 3.6A). Furthermore, 200 nM TCDD can cause a decrease in Cx43 (Figure 3.6B). Surprisingly, confocal microscopy showed the presence of Cx43 aggregates in the perinuclear area in the presence of 10-200 nM of TCDD (Figure 3.7A) compared to the Cx43 plaques on the membrane in the controls. Further evidence was provided by nuclear extraction of HMEC. 10 nM TCDD can cause an increase of Cx43 expression in the nuclear fraction compared to control (Figure 3.7B). However, at 200 nM TCDD, Cx43 expression was completely reduced in both cytoplasmic and nuclear fractions. Blots were stripped and Lamin B1 expression was observed only in the nuclear fraction and not in the cytoplasmic fraction (Figure 3.7B). Thus, these findings show for the first time that TCDD affects gap junctional activity in HMEC through different mechanism than MCF-7 cells.

A.


CONTROL
DMSO
10 nM TCDD
50 nM TCDD
100 nM TCDD
200 nM TCDD

B.

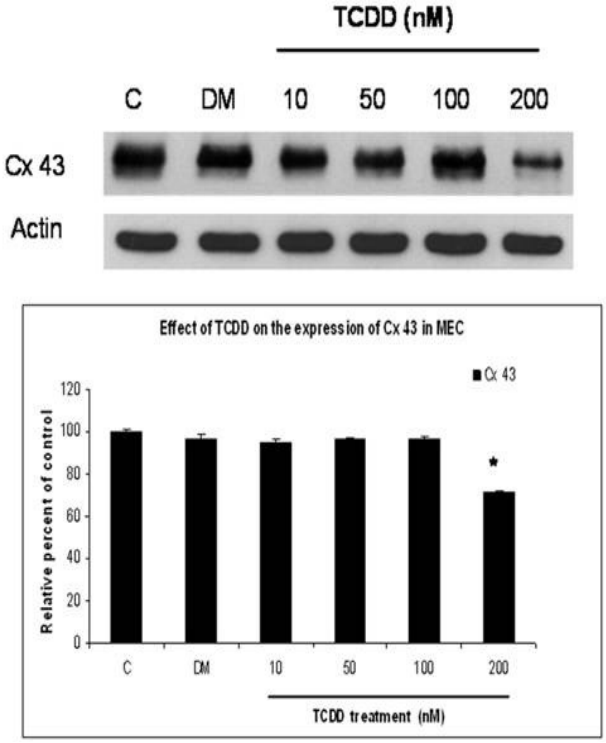
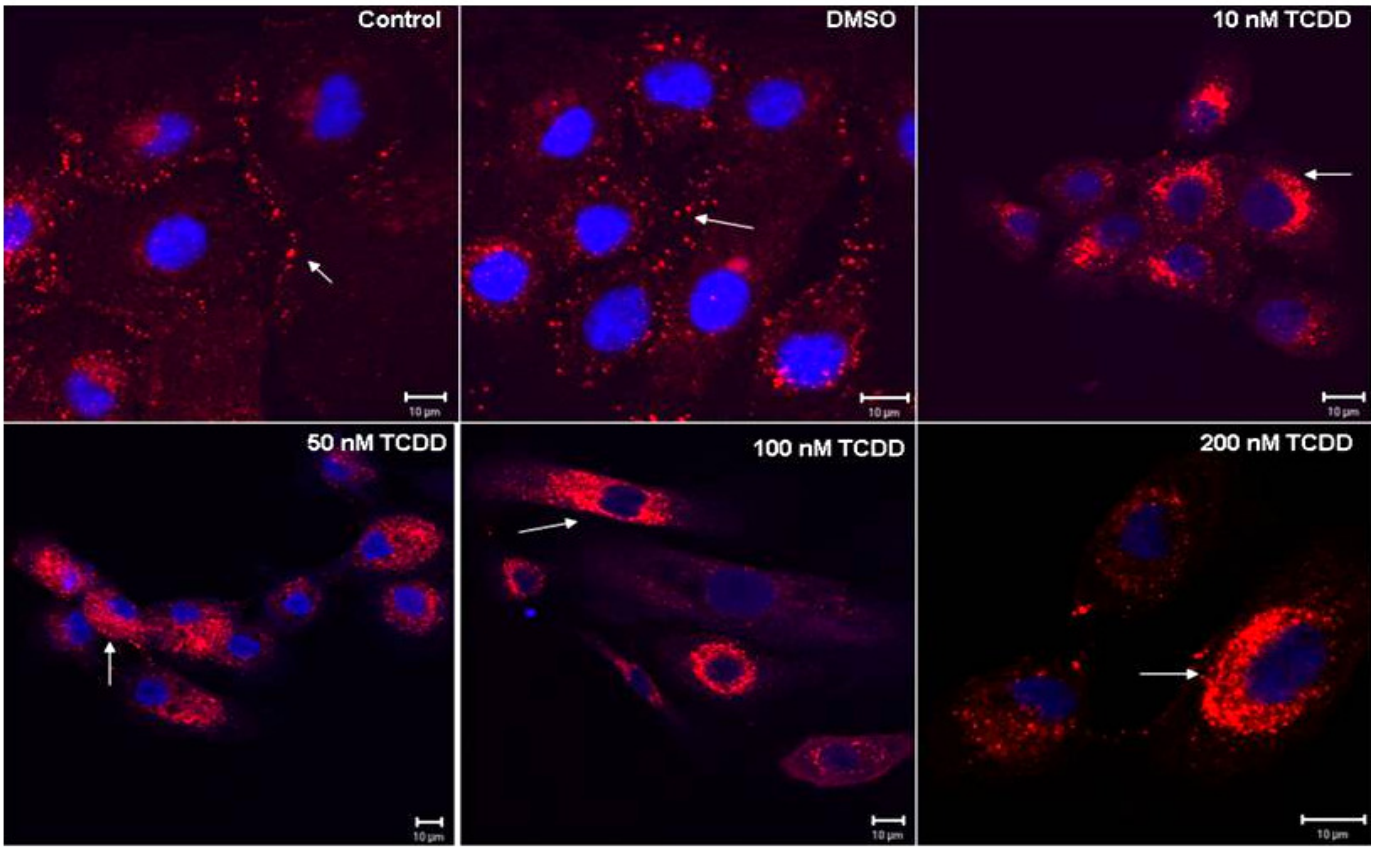
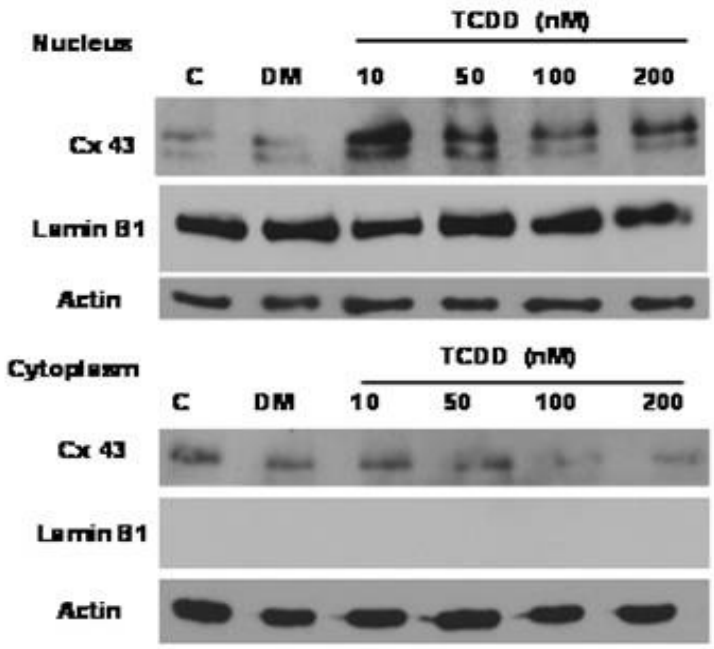

TCDD (nM)
C
DM
10
50
100
200
Cx 43
Actin
Effect of TCDD on the expression of Cx 43 in MEC
Cx 43
Relative percent of control
120
100
80
60
40
20
0
*
C
DM
10
50
100
200
TCDD treatment (nM)

Figure 3.6 Scrape load dye transfer assay (SL/DT) and expression of Cx43 in HMEC.
A) HMEC were seeded in a 6-well plate and dosed with different concentrations of TCDD for an hour. SL/DT assay was performed as described in Figure 3.1A. Pictures depict three independent experiments. **B)** Effect of TCDD on Cx43 expression. Cells were treated with DMSO and 10, 50, 100, 200 nM TCDD for 1 hr. A significant decrease in Cx43 at 200 nM TCDD compared to the control was observed, *$p < 0.05$.

A.



B.



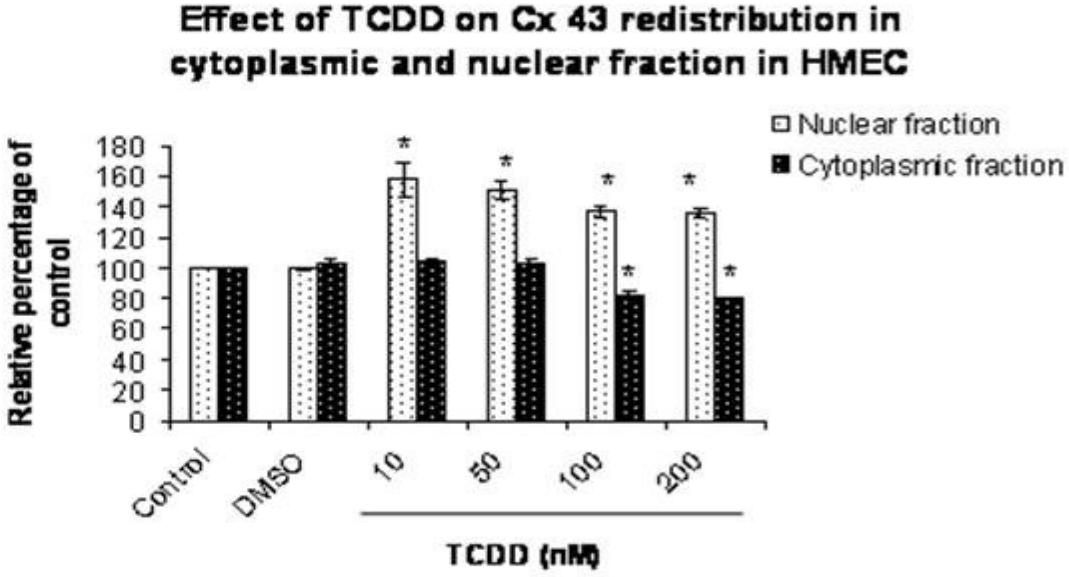


Figure 3.7 Confocal microscopy showing the effect of TCDD on Cx43 plaque formation and the localization of Cx43 in HMEC.

A) Cx43 plaque formation in the presence of 0, 10, 50, 100 and 200 nM TCDD. Cells were stained with DAPI (blue nuclear stain) and anti-Cx43 mouse antibody (red). Images were seen under 40X oil objective lens, pin hole = 0.76 μm and arrows indicate the plaque formation. Pictures represent three different experiments. **B)** Cx43 expression in nuclear and cytoplasmic fraction dosed with different concentrations of TCDD. All the Western blots are a representative of three individual experiments. Lamin B1 was used as a nuclear marker showing the separation of the nuclear and cytoplasmic fraction. Graphical representation of three experiments with ±SD and statistical significance, *$p < 0.01$ is shown.

3.5 Discussion

TCDD is a human carcinogen. It has been implicated as a reproductive toxin in humans, including its potential for breast cancer (Adami *et al*., 1995). Studies have been conducted to understand the risk of cancer in the presence of TCDD. TCDD is not a genotoxic, therefore, its exposure can affect many downstream pathways. We showed for the first time that TCDD has a differential effect on GJIC in primary mammary epithelial cells, HMEC and breast cancer cells, MCF-7. Baker *et al*. (1995) demonstrated that TCDD inhibits GJIC in the in vivo target of its tumor promoting effect and this effect may, in part, be directed through Ah receptor in primary culture of rat hepatocytes. In the present study, SL/DT was used to measure the activity of GJIC. Lucifer yellow dye (MW 457.2) passes from one cell to another through gap junctions whereas rhodamine-dextran (MW 10,000) passes only through injured plasma membrane. We found a decrease in the Lucifer dye transfer between the cells even in the presence of 10 and 100 nM of TCDD in MCF-7 cells (Figure 3.1A). This suggests that the gap junctional activity between the MCF-7 cells is decreased in the presence of TCDD. Plaques are the functional aggregations of many gap junctions on the plasma membrane. Clustering of gap junction channels into plaques is required for functional cell coupling. Since Cx43 is the most predominant form of connexin in breast epithelium, the effect of TCDD on Cx43 plaque formation was observed. Cx43 plaques were decreased in the presence of 10-200 nM TCDD in MCF-7 cells (Figure 3.2A). A complete loss in the Cx43 plaque formation was seen from 50-200 nM TCDD in MCF-7 cells. This suggests that TCDD affects the GJIC by affecting the formation or degradation of Cx43 plaques in MCF-7 cells.

Various tumor-promoting agents (Ruch *et al*., 2001) decrease GJIC, either by suppressing Cx expression or by inducing post-translational modifications such as phosphorylation, a process that is closely related to cellular processes such as trafficking, assembly/disassembly, gating of gap junction channels, and altered susceptibility to degradation (Lampe and Lau, 2000). The carboxy-terminus of Cx43 contains several serine and tyrosine phosphorylation sites, suggesting that this region of the molecule contains a complex array of potential regulatory sites (Lampe and Lau, 2000). Therefore, to understand the decrease in the gap junction activity by TCDD in MCF-7 cells, we investigated the phosphorylation of Cx43. Phospho-Ser 368 Cx43 antibody was specifically used to detect the phosphorylation of Cx43. PKC has been shown to phosphorylate Cx43 at serine 368 carboxyl terminal in vitro (Lampe *et al*., 2000; Saez *et al*., 1997; Shah *et al*., 2002). We found an increase in phospho-Ser 368 Cx43 in the presence of 10-200 nM TCDD in MCF-7 cells (Figure 3.3A). Since an increase was seen in the phosphorylated form of Cx43 in MCF-7 cells, we examined the expression of kinases in the presence of TCDD. PKC α was found to be significantly increased in the presence of TCDD in MCF-7 cells (Figure 3.3B). It could be due to the effect on the transcriptional, post-transcriptional, or translational level. Early observations that PKC isozymes are activated by tumor promoting phorbol esters (Castagna *et al*., 1982) suggested a key role for PKC in tumor promotion and progression (Mackay and Twelves, 2003). Increased levels of PKC have been associated with malignant transformation in a number of cell lines including breast (O'Brian *et al*., 1989), lung (Takenaga and Takahashi, 1996) and gastric carcinomas (Schwartz *et al*., 1993). In vitro studies suggest a positive correlation between elevated PKC levels and both the invasive and chemotactic potential of human breast cancer cell lines (Blobe *et al*., 1994).

To confirm the phosphorylation of Cx43 by PKC α, we examined the effect of TCDD on the activation and interaction of PKC α with Cx43. Among others, diacylglycerol and Ca^{2+} are known to activate PKC by translocating it from the cytoplasm to the membrane and this report showed that TCDD is an activator of PKC α in MCF-7 cells. We found an increase in the translocation of PKC α from the cytosol to the membrane under the effect of TCDD in MCF-7 cells (Figure 3.4A). The translocation of PKC α promotes the interaction of PKC α and Cx43 and subsequently the phosphorylation of Cx43. Translocation studies were further confirmed by confocal microscopy showing the localization of PKC α on the membrane in the presence of TCDD (Figure 3.4B). Immunoprecipitation studies showed an increase in the phosphorylated

form of Cx43 with an equal amount of PKC α being pulled out (Figures 3.4C, D). Interaction between PKC α and the phosphorylated form of Cx43 was increased significantly at 10 and 50 nM TCDD; however, interaction was decreased at 100 and 200 nM TCDD. Our study supports previous studies that other potential PKCs sites might be involved in regulating assembly or degradation of gap junctions and channel behavior (Lampe and Lau, 2004). PKC has received considerable attention because PKC activators, which promote tumorigenesis, both increase Cx43 phosphorylation and decrease GJIC in a number of different cell types (Berthoud *et al*., 1992; Brissette *et al*., 1991; Lampe, 1994; Reynhout *et al*., 1992).

To confirm the functionality of PKC α, an enzyme activity assay was performed. We observed an increase in the enzymatic activity of PKC α in the presence of TCDD (Figure 3.5A). Cells treated with 100 nM TCDD did not show further increased PKC enzyme activity. This suggests that TCDD may activate other GJIC-mediated pathways that are also involved in the inhibition of GJIC. Calphostin C, an inhibitor of PKC α, was used to provide further evidence that TCDD mediated inhibition of gap junctional activity involves PKC α pathway. SL/DT assay showed the restoration of Lucifer dye transfer in MCF-7 cells in the presence of calphostin (100 nM) followed by TCDD (Figure 3.1B). Confocal microscopy also showed the presence of Cx43 plaques in the presence of TCDD in MCF-7 cells pre-treated with 100 nM calphostin C (Figure 3.2B). A significant decrease in the PKC enzyme activity was observed in the presence of calphostin C (100 nM) in TCDD-treated MCF-7 cells (Figure 3.5B). All the experiments performed with calphostin C showed the restoration of the effect of TCDD at 200 nM in MCF-7 cells. It can be concluded that when TCDD is double the concentration of calphostin C, the inhibitory effect of calphostin C on TCDD-mediated PKC α pathway is lost. However, calphostin C (100 nM) was sufficient enough to block the effect of 10, 50 and 100 nM TCDD confirming that the TCDD-mediated inhibition of GJIC involves PKC α pathway.

To determine the effect of TCDD on non-cancerous human breast cells, primary human mammary epithelial cells were used. The dye transfer was tremendously reduced at 200 nM TCDD in HMEC (Figure 3.6A). Higher dose of TCDD caused a decrease in GJIC in HMEC compared to MCF-7 cells could be due to the fact that control HMEC exhibited more GJIC than control MCF-7 cells. This confirms the earlier studies suggesting a decrease in GJIC in cancerous cells compared to normal primary cells of the same tissue (Hirschi *et al*., 1996). Exposure of TCDD inhibits GJIC in both cell types, cancerous and non-cancerous cells. 200 nM

TCDD caused a decrease in the Cx43 expression (Figure 3.6B). This exhibits two different ways of regulation of GJIC by TCDD by phosphorylation of Cx43 in MCF-7 cells and by decrease in the expression of Cx43 in HMEC. Interestingly, PKCs expressions are not upregulated in control HMEC as in MCF-7 cells. In HMEC, a different effect of TCDD compared to MCF-7 cells was observed on the Cx43 plaque localization. In the presence of 10-200 nM TCDD, Cx43 plaques were redistributed from the plasma membrane to the perinuclear area in HMEC (Figure 3.7A). This effect could be due to the effect on the trafficking of Cx43 in HMEC. This suggests that TCDD causes the movement of Cx43 from the membrane to the perinuclear region or prevents the movement of Cx43 to the membrane. The pathways involved in the trafficking of Cx43 are not fully understood. Even though Cx43 plaques shifted to the perinuclear area supported by the appearance of Cx43 in the nuclear fraction at 10 nM TCDD, we still observed GJIC activity in HMEC by SL/DT. This could be explained by the presence of other connexin proteins, Cx26 and Cx32 in HMEC which can form homomeric or heteromeric GJs. To further provide evidence for the redistribution of Cx43 to the perinuclear area, we isolated nuclear and cytoplasmic fractions. Interestingly, Cx43 appears in the nuclear fraction and diminishes completely in the cytoplasmic fraction in the presence of 100 and 200 nM TCDD (Figure 3.7B). The expression of a nuclear protein, Lamin B1, was measured in the nuclear and the cytoplasmic fraction. Lamin B1 was detected only in the nuclear fraction confirming that there was no leakage of the nuclear proteins in the cytoplasmic fraction during the extraction procedure (Figure 3.7B). The differences in the level of Cx43 expression (Figure 3.6B) and nuclear and cytoplasmic fraction could be explained in part by HMEC are slow growing cells, being the primary cells, which can be grown to four passages without change in morphology. Therefore, minimal number of cells was used for nuclear extraction and the level of protein for both nuclear and cytoplasmic fractions was low, and another explanation is that the process of extraction might have led to the further loss of proteins.

In conclusion, TCDD is an environmental pollutant which affects GJIC in human breast cells. This report adds to the previous information that GJIC plays a vital role in breast cancer. The results provide evidence that TCDD inhibits GJIC through traditional and non-traditional pathways. The summary of the results observed in MCF-7 cells has been shown in Figure 3.8. In cancer cells, PKC α is upregulated and thus TCDD inhibits GJ through PKC α translocation and phosphorylation of Cx43. However, in non-cancerous cells such as primary mammary epithelial

cells, TCDD also inhibits GJ through a novel pathway, redistribution of Cx43 to the perinuclear fraction. In the absence of the changes in protein kinases, TCDD mediates the localization of Cx43 in normal mammary cells. This study provides the molecular mechanism showing how TCDD affects gap junctions. The findings reveal the potential risk of exposure to environmental pollutants like TCDD on the normal breast epithelium and cancerous breast tissue using breast cells.

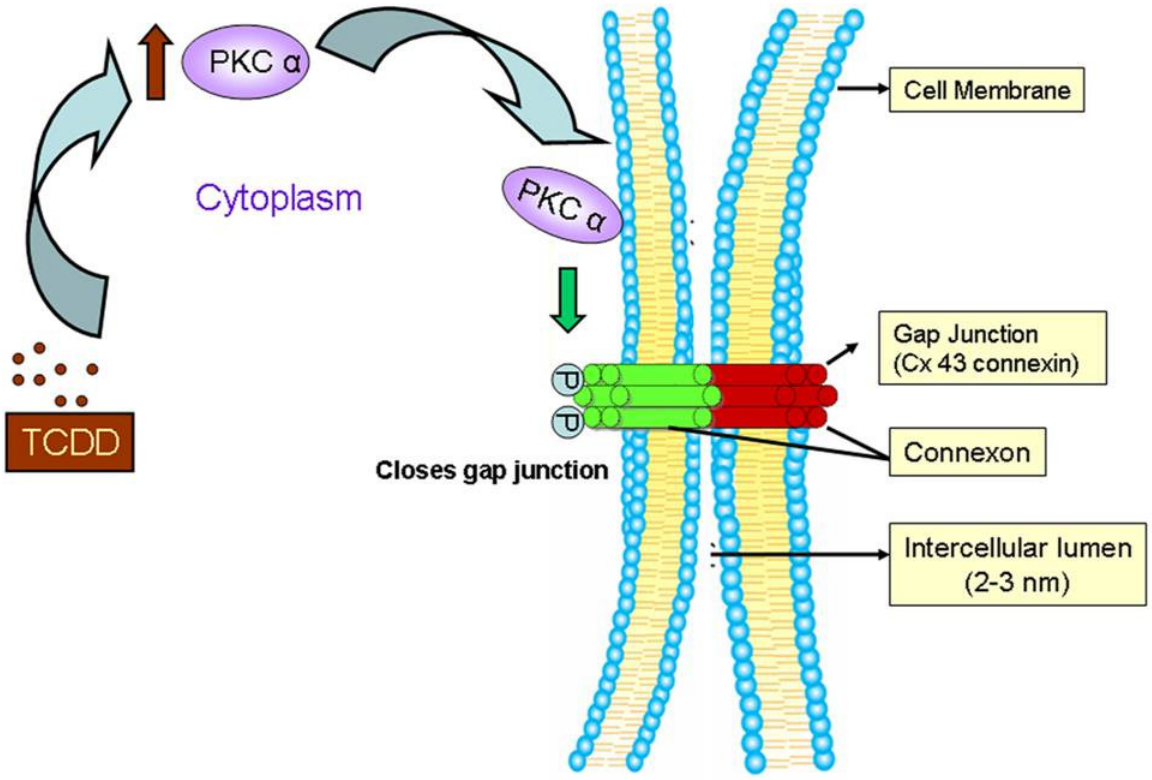


Figure 3.8 Proposed pathway showing the effect of TCDD on GJIC in MCF-7 cells.
TCDD increases the expression and activates PKC α, causing its translocation from cytosol to the membrane. Activation of PKC α causes phosphorylation of Cx43 on the plasma membrane, thereby decreasing the gap junctional activity.

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3.6 References

Adami, H.O., Lipworth, L., Titus-Ernstoff, L., Hsieh, C.C., Hanberg, A., Ahlborg, U., Baron, J.,Trichopoulos, D. (1995). Organochlorine compounds and estrogen-related cancers in women. *Cancer Causes Control* **6**, 551-566.

Baker, T.K., Kwiatkowski, A.P., Madhukar, B.V., Klaunig, J.E. (1995). Inhibition of gap junctional intercellular communication by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in rat hepatocytes. *Carcinogenesis* **16**, 2321-2326.

Berthoud, V.M., Ledbetter, M.L.S., Hertzberg, E.L., Saez, J.C. (1992). Connexin 43 in MDCK cells: regulation by a tumor-promoting phorbol ester and Ca^{2+}. *Eur. J. Cell Biol*. **57**, 40-50.

Blaha, L., Kapplova, P., Vondracek, J., Upham, B., Machala, M. (2002). Inhibition of gap-junctional intercellular communication by environmentally occurring polycyclic aromatic hydrocarbons. *Tox. Sci.* **65**, 43-51.

Blobe, G.C., Obeid, L.M., Hannun, Y.A. (1994). Regulation of protein kinase C and role in cancer biology. *Cancer Metastasis Rev*. **13**, 411-431.

Brissette, J.L., Kumar, N.M., Gilula, N.B., Dotto, G.P. (1991). The tumor promoter 12-O-tetradeconylphorbol-13-acetate and the *ras* oncogene modulate expression and phosphorylation of gap junction proteins. *Mol. Cell. Biol*. **11**, 5364-5371.

Carystinos, G.D., Bier, A., Batist, G. (2002). The role of connexin-mediated cell–cell communication in breast cancer metastasis. *J. Mammary Gland Biol. Neoplasia* **6**, 431-440.

Castagna, M., Takai, Y., Kaibuchi, K., Kikkawa, U., Nishizuka, Y. (1982). Direct activation of calcium-activated, phospholipids dependent protein kinase by tumor-promoting phorbol esters. *J. Biol. Chem*. **257**, 7847-7851.

Chipman, J.K., Mally, A., Edwards, G.O. (2003). Disruption of gap junctions in toxicity and carcinogenicity. *Tox. Sci*. **71**, 146-153.

Eghbali, B., Kessler, J.A., Reid, L.M., Roy, C., Spray, D.C. (1991). Involvement of gap junctions in tumorigenesis: transfection of tumor cells with connexin 32 cDNA retards growth *in vivo*. *Proc. Natl. Acad. Sci*. **88**, 10701-10705.

Goodenough, D.A., Paul, D.L., Jesaitis, L.A. (1988). Topological distribution of two connexin32 antigenic sites in intact and split rodent hepatocyte gap junctions. *J.Cell Biol.* **107**, 1817-1824.

Hertzberg, E.L., Disher, R.M., Tiller, A.A., Zhou, Y., Cook, R.G. (1988). Topology of the Mr 27,000 liver gap junction protein. Cytoplasmic localization of amino- and carboxyl termini and a hydrophilic domain which is protease-hypersensitive. *J. Biol. Chem*. **263**, 19105-19111.

Hirschi, K.K., Xu, C., Tsukamoto, T., Sager, R. (1996). Gap junction genes Cx26 and Cx43 individually suppress the cancer phenotype of human mammary carcinoma cells and restore differentiation potential. *Cell growth Differ.* **7**, 861-870.

Jenkins, S., Rowell, C., Wang, J., Lamartiniere, C.A. (2006). Prenatal TCDD exposure predisposes rat mammary cancer. *Reprod. Toxicol* **23** (3), 391-396.

Knudsen, K.A., Wheelock, M.J. (1992). Plakoglobin, or an 83-kD homologue distinctfrom beta-catenin, interacts with E-cadherin and N-cadherin. *J. Cell Biol*. **118**, 671-679.

Lampe, P.D. (1994). Analyzing phorbol ester effects on gap junction communication: a dramatic inhibition of assembly. *J. Cell Biol*. **127**, 1895-1905.

Lampe, P.D., Lau, A.F. (2000). Regulation of gap junctions by phosphorylation of connexins. *Arch. Biochem. Biophys*. **384**, 205-215.

Lampe, P.D., Lau, A.F. (2004). The effects of connexin phosphorylation on gap junctional communication. *Int. J. Biochem. Cell Biol*. **36** (7), 1171-1186.

Lampe, P.D., Tenbroek, E.M., Burt, J.M., Kurata, W.E., Johnson, R.G. (2000). Phosphorylation of connexin 43 on serine 368 by protein kinase C regulates gap junctional communication. *J. Cell Biol*. **149**, 1503-1512.

Mackay, H.J., Twelves, C.J. (2003). Protein kinase C: a target for anticancer drugs? *Endocr.-Relat. Cancer* **10**, 389-396.

Manz, A., Berger, J., Dwyer, J.H., Flesch-Janys, D., Nagel, S., Walsgott, H. (1991). Cancer mortality among workers in a chemical plant contaminated with dioxin. *Lancet* **338**, 959-964.

Mesnil, M. (2002). Connexins and cancer. *Biol. Cell* **94**, 493-500.

O'Brian, C.A., Vogel, V.G., Singletary, S.E., Ward, N.E. (1989). Elevated protein kinase C expression in human breast tumor biopsies relative to normal breast tissue. *Cancer Res*. **49**, 3215-3217.

Reynhout, J.K., Lampe, P.D., Johnson, R.G. (1992). An activator of protein kinase C inhibits gap junction communication between cultured bovine lens cells. *Exp. Cell Res*. **198**, 337-342.

Rivedal, E., Opsahl, H. (2001). Role of PKC and MAP kinase in EGF- and TPA-induced connexin43 phosphorylation and inhibition of gap junction intercellular communication in rat liver epithelial cells. *Carcinogenesis* **22**, 1543-1550.

Ruch, R.J., Trosko, J.E., Madhukar, B.V. (2001). Inhibition of connexin43 gap junctional intercellular communication by TPA requires ERK activation. *J. Cell. Biochem*. **83**, 163-169.

Saez, J.C., Naim, A.C., Czemik, A.J., Fishman, G.I., Spray, D.C., Hertzberg, E.L. (1997). Phosphorylation of connexin 43 and the regulation of neonatal rat cardiac myocyte gap junctions. *J. Mol. Cell. Cardiol*. **29**, 2131-2145.

Safe, S. (1990). Polychlorinated (PCBs), dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs) related compounds: environmental and mechanistic considerations which support the development of toxic equivalency factors (TEFs). *CRC Crit. Rev. Toxicol*. **21**, 51-88.

Safe, S. (1995). Modulation of gene expression and endocrine response pathways by 2,3,7,8-tetrachlorodibenzo-p-dioxin and related compounds. *Pharmacol. Ther.* **67**, 247-281.

Schwartz, G.K., Jiang, J., Kelsen, D., Albino, A.P. (1993). Protein kinase C: a novel target for inhibiting gastric cancer cell invasion. *J. Natl. Cancer Inst.* **85**, 402-407.

Shah, M.M., Martinez, A.M., Fletcher, W.H. (2002). The connexin 43 gap junction protein is phosphorylated by protein kinase A and protein kinase C: in vivo and in vitro studies. *Mol. Cell. Biochem.* **238**, 57-68.

Sharovskaja, J.J., Vaiman, A.V., Solomatina, N.A., Kobliakov, V.A. (2004). Inhibition of gap junction intercellular communications in cell culture by polycyclic aromatic hydrocarbons (PAH) in the absence of PAH metabolism. *Biochemistry* **69**, 413-419.

Takenaga, K., Takahashi, K. (1996). Effects of 12-otetradecanoylphorbol-13-acetate on adhesiveness and lung colonizing ability of Lewis lung carcinoma cells. *Cancer Res.* **46**, 375-380.

Tomasetto, C., Neveu, M.J., Daley, J., Horan, P.K., Sager, R. (1993). Specificity of gap junction communication among human mammary cells and connexin transfectants in culture. *J. Cell Biol.* **122**, 157-167.

Trosko, J.E., Ruch, R.J. (1998). Cell–cell communication in carcinogenesis. *Front. Biosci.* **3**, 208-236.

Vine, A.L., Bertram, J.S. (2002). Cancer chemoprevention by connexins. *Cancer Metastasis Rev.* **21**, 199-216.

Yeager, M., Gilula, N.B. (1992). Membrane topology and quaternary structure of cardiac gap junction ion channels. *J. Mol. Biol.* **223** (4), 929-948.

Zampighi, G.A., Planells, A.M., Lin, D., Takemoto, D. (2005). Regulation of lens cell-to-cell communication by activation of PKC γ and disassembly of Cx50 channels. *Investig. Ophthalmol. Vis. Sci.* **46**, 3247-3255.

Chapter 4 - Antitumor effect of substituted quinolines in breast cancer cells

4.1 Abstract

Cancer in part is caused by the disruption in cell homeostasis, affecting the ability to respond to extracellular signals, triggering some intracellular events which affect the gap junctional intercellular communication (GJIC). Cancer cells have reduced or altered GJIC capacity. One feasible approach to reduce growth of cancer cells is to enhance/alter GJIC. The capability of cells to communicate through gap junction is negatively related to their growth activity. A computational docking study showed that a new class of substituted quinolines (code name: PQs) has a relatively high binding to gap junction protein, connexin 43. Thus, PQs were used in this study to find their effect on human breast cancer cells. Scrape load/dye transfer and colony growth assay were performed to measure GJIC and determine the effect of the PQ compounds on the colony formation in human normal mammary epithelial cells (HMEC) and breast cancer cells, respectively. The results showed that PQs have a significant antitumor effect in human breast cancer cells compared to control without treatment or normal mammary epithelial cells. 200 nM PQ1 showed a 30% increase in the GJIC in T47D cells; however, there is no effect of PQ treatment on GJIC in normal mammary epithelial cells. In addition to an increase in GJIC, 80-95% growth attenuation was observed by PQ1 in colony growth assay. Moreover, an increase in caspase 3 with PQ-treated cells was observed, suggesting possible involvement in apoptosis. The results show that PQ1 compound have a promising role in exerting antitumor activity in human breast cancer cells. The treatment with PQ1 in T47D cells causes an increase in GJIC activity and active caspase 3, and a decrease in colony growth and cell viability. Substituted quinolines have demonstrated to activate gap junction activity and subsequently decrease colony and tumor growth.

4.2 Introduction

Breast cancer is the most common cancer in women worldwide and mortality from breast cancer is consistently due to tumor metastasis (Jemal *et al*. 2007). Progress in understanding the etiology of this disease and developing therapies has been slow due to multiple deregulations of various genes. The additive effects against mammary tumor cells might be achieved by combining anti-tumor agents directed against one or more altered mechanisms in cancer.

Cancer cells exhibit many defects in cell communication that contribute to the loss of tissue homeostasis (excess cell proliferation, invasion, and metastasis) (Loewenstein 1979; Loewenstein and Kanno 1966; Trosko *et al*. 1990; Wilgenbus *et al*. 1992; Yamasaki and Naus 1996). Intercellular communication in many organs is maintained via intercellular gap junction channels (GJIC). Gap junctions are the only communicating junctions found in animal tissues, in all species, which are responsible for the direct traffic of ions and molecules with molecular weights less than 1,200 Da (Martin and Evans 2004; Musil *et al*. 2000). These traffic ways are formed by the interaction between two hemichannels on the surface of opposing cells. Hemichannels are formed by the association of six proteins, the connexins. Because of the importance of intercellular junctions in the maintenance of the cellular homeostasis, the modulation of intercellular junctions and expression of connexin seems to be involved in carcinogenesis (Dubina *et al*. 2002; Holder *et al*. 1993; Ruch 1994).

Electrophysiological measurement and dye-transfer studies in vivo have shown that signal transmission through GJIC can be altered by a number of molecules (Bruzzone *et al*. 1996). Electron crystallography studies demonstrate that the dimension of the pore of gap junction channel varies from 40 Å in the cytoplasm to 15 Å at the membrane boundary with the extracellular gap (Foote *et al*. 1998). The reported low density structure (Makowski *et al*. 1977) only provides a general shape of the channel proteins. A recombinant gap junction was constructed, (Unger *et al*. 1999) which allows the computational docking studies of the channel proteins with small molecules.

Several small organic molecules, such as caffeic acid phenethyl ester (CAPE) (Na *et al*. 2000), sodium 4-phenylbutyrate (Ammerpohl *et al*. 2004), Liarozole (Acevedo and Bertram 1995), lycopene (Livny *et al*. 2002), and lovastatin (Ruch *et al*. 1993), have been reported for up-regulation or restoration of GJIC. CAPE is an active ingredient in honeybee propolis and has

antiviral, anti-inflammatory and anticancer properties (Bhimani *et al*. 1993). The effective concentration of CAPE is 17 μM in restoring GJIC and phosphorylation of Cx43 in WB-ras2 cells (Na *et al*. 2000). 4-Phenylbutyrate enhances GJIC in glioblastoma cells and consequently increases the efficiency of the bystander killing effect of HSV-tk suicide gene/ganciclovir technology (Ammerpohl *et al*. 2004). Phase I/II dose-escalating trial of lycopene in biochemical relapsed prostate cancer patients did not result in any discernible response in serum PSA (Clark *et al*. 2006). A phase II study of liarozole in advanced non-small cell lung cancer showed that it is ineffective as single agent therapy (O'Byrne *et al*. 1998). Lovastatin, an inhibitor of cellular cholesterol synthesis, has an IC_{50} value of ~8 μM over 72 hours against MCF-7 mammary cancer cells (Wei *et al*. 2007). Hence, new GJIC enhancers are needed, and the study of the mechanism of restoring GJIC would provide information for the design of selective GJIC enhancers. The restoration of GJIC mechanism may provide potential targets for anticancer therapy.

In search of new activators that enhance GJIC, potential interactions of a number of substituted quinolines (code name PQs) with the partial crystal structure of gap junction (Fleishman *et al*. 2006; Makowski *et al*. 1977; Veenstra 2003) using Autodock computational docking software (Goodsell and Olson 1990; Morris *et al*. 1996; Morris 1998), was examined. Dr. Duy H. Hua's lab observed bindings of PQs to the inert core of the hexameric hemichannel of gap junctions. In one of the minimum energy (-0.7 kcal/mol) bound structures, interactions (closed contact) between CF3 group of PQ1 and H-N of Leu144 of connexin (2.5 Å), and OCH3 group of PQ1 and CH2 of Phe81 of connexin (2.0 Å) are found. Consequently, Dr. Hua synthesized this class of quinolines and studied their GJIC and anticancer activities. PQ analogs were synthesized via a modification of the reported protocol (LaMontagne *et al*. 1982a; LaMontagne *et al*. 1982b; Lauer 1946) starting from 4-acetaminoanisole (compound 6), and the synthetic sequence is depicted in Scheme 1.

Tumor cells have reduced or altered GJIC capacity (Eghbali *et al*. 1991). It has been shown that the drug sensitivity was enhanced in Cx 43 overexpressing tumor cells (Huang *et al*. 2001). Cancer cells are characterized by the lack of growth control, by the inability to terminally differentiate and by resistance to apoptosis. Various oncogenes (e.g. ras, raf, neu, src, mos) down-regulate GJIC while several tumor suppressor genes can up-regulate GJIC (Na *et al*. 2000; Ruch *et al*. 1993; Zhang *et al*. 1994). Transfection of gap junction genes, connexins, into GJIC-deficient tumor cells can restore GJIC, growth control and reduce tumorigenicity (Na *et al*.

2000). Previous studies from Trosko and Chang clearly suggest that dietary factors can modulate GJIC by inducing various signal transducing systems (Trosko and Chang 2001). The modulation can either down-regulate GJIC leading to tumor promotion or it can up-regulate GJIC and lead to suppression of the initiated cells. Hence, increasing gap junction activity or enhancing GJIC in tumor cells provides the means to enhance anti-neoplastic therapies. The goal of our studies is to examine PQs that specifically activate GJIC activity and inhibit cancer cell growth. The results demonstrated that these small molecules may be the potential combinational therapeutic drugs for breast cancer.

4.3 Materials and Methods

4.3.1 Cell line and cell culture

T47D human breast cancer cell line was purchased from American Type Culture Collection (Manassas, VA). Cells were grown in RPMI medium supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 10% antibiotic-actinomycotic at 37°C with 5% CO_2 in 75cm^2 flasks.

4.3.2 Western Blot Analysis

Cells were grown in serum supplemented RPMI media until they were 90% confluent in 75 cm^2 flasks. Cells were kept in starving media containing phenol red-free DMEM with 5% charcoal dextran stripped serum, overnight. Cells were dosed with 0, 10, 100, 200 and 500 nM of PQ1 for 24 hr. Cells were washed three times with cold PBS and then were harvested using lysis buffer (20 mM Tris pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 0.5% Triton X-100) with 1:1000 dilution of protease inhibitors (Sigma-Aldrich, Saint Louis, MO). Cell lysate was sonicated and centrifuged at 13,000 rpm for 30 min at 4ºC. 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 1 hr at room temperature and then incubated with monoclonal mouse PKC α, 1:500 (Santa Cruz Biotechnologies, Santa Cruz, CA), mouse Cx43, 1:500 (Fred Hutchinson, Seattle,

Washington), rabbit actin, 1:1,000 (Sigma-Aldrich, Saint Louis, MO). Western blots were detected by enhanced chemiluminescence detection reagents (Pierce, Rockford, Illinois, USA).

4.3.3 Gap Junction Activity

For scrape load/ dye transfer (SL/DT) assay, cells were grown to 90% confluency on cover slips, dosed with 10, 100, 200 and 500 nM of PQ 1 for 40 min. After that cells were washed three times with PBS. The 2.5 µl of 1% (w/v) Lucifer yellow and 0.75% (w/v) of Rhodamine dextran was mixed and added in the center of the coverslip. Two cuts crossing each other in the center of the coverslip were made. After 3 min, cells were washed three times with PBS and incubated at 37℃ in tissue culture media for additional 20 min. The cells were then washed with PBS three times and fixed in 2.5% paraformaldehyde for 10 min. Cells were mounted on a slide, sealed and visualized under a fluorescence microscope at 10X objective.

4.3.4 Measurement of transepithelial electrical resistance

Cells were grown to 100% confluency on a 12-well transwell (BD Biosciences, San Jose, CA) and treated with 0, 100, 200, and 500 nM PQ1 for 48 hr. Transepithelial electrical resistance (TEER) of the T47D monolayers was measured by a high-precision. Resulting voltages were recorded with the aid of a differential amplifier with a high input resistance. Data were corrected for well area (given in $\Omega \bullet cm^2$).

4.3.5 Colony Growth Using Soft Agar

Cells were treated with 0, 10, 100, 200 and 500 nM PQ1 for 7 days. Base agar plates were prepared containing 0.8% agar and 0.4% agar in Ham's F12. Cells (5×10^4 cells/33 mm^2 well) were suspended in 100 µl of Ham's F12 with 0.4% agar and plated. These plates were maintained at 37℃ for 7 days and examined for the presence of colonies. Individual colonies of 50 µm or greater were examined.

4.3.6 MTT assay

MTT (3-(4, 5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was performed with adherent cell cultures in a 96-well plate dosed with 0, 10, 100, 200, and 500 nM PQ 1 for 24 hr. Solution containing MTT was metabolized by the cells (incubation period 1 ½ hr). MTT is a tetrazolium salt (yellowish) cleaved to formazan crystals by succinate dehydrogenase enzyme, active in viable cells. The more viable cells in a well, the more formazan dye will be produced. After solubilization of the MTT crystals with the 0.35 N HCl solubilization solution, the amount of dye will be measured spectrophotometrically at 540 nm.

4.3.7 General Methods for Organic Synthesis- Dr Hua's Lab

NMR spectra were obtained at 400 MHz for ^{1}H and 100 MHz for ^{13}C in $CDCl_3$, and reported in ppm. High-resolution mass spectra were obtained from ESI spectrometers. ESI spectra were acquired on a LCT Premier (Waters Corp., Milford, MA) time of flight mass spectrometer. Satisfactory ^{1}H and ^{13}C NMR spectra and high-resolution mass spectra of compounds PQ1, PQ2, PQ3, PQ4, PQ5, and 10 were obtained. Compounds PQ2–PQ5 have been reported in the literature (LaMontagne *et al*. 1982a; LaMontagne *et al*. 1982b; Lauer 1946).

Compound PQ3 (LaMontagne *et al*. 1982b) was treated with 1 equivalent of 3-iodopropylphthalimide (compound 9) and 1 equivalent of sodium bicarbonate in DMF at 80°C for 48 hr. After aqueous work-up and silica gel column chromatographic separation, a 72% yield (based on reacted PQ3) of compound 10 was obtained along with 77% recovery of PQ3. Compound 10 was heated under reflux (85°C) with an excess of hydrazine (65%) in ethanol for 3 hours. After aqueous work-up (dilution with 10% aqueous KOH solution and extraction with dichloromethane) and silica gel column chromatographic separation, an 80% yield of PQ1 was obtained.

4.3.8 Statistical analysis

The level of significance (see * in figure legends) was considered at $p < 0.05$ using Student's t-test analysis. All data are presented as mean ± S.D. of at least three independent experiments from different batches of cultures.

4.4 Results

4.4.1 Synthesis of PQ1

Intercellular communication in many organs is maintained via GJIC. Several GJIC enhancers have been reported; however, an effective clinical drug targeting gap junction is not available at this time. Our goal was to collaborate with Dr. Hua to synthesize small molecules that specifically activate GJIC activity and inhibit cancer cell growth. Dr. Hua's lab designed novel substituted quinolines using computational docking studies, and demonstrated their enhancement of GJs activities and growth inhibitory effect on human breast cancer cells. The structures of substituted quinolines are shown in Figure 4.1 and the syntheses of PQs are described in Materials and Methods. PQ1 is predicted to have a high binding affinity for connexin via computational docking.

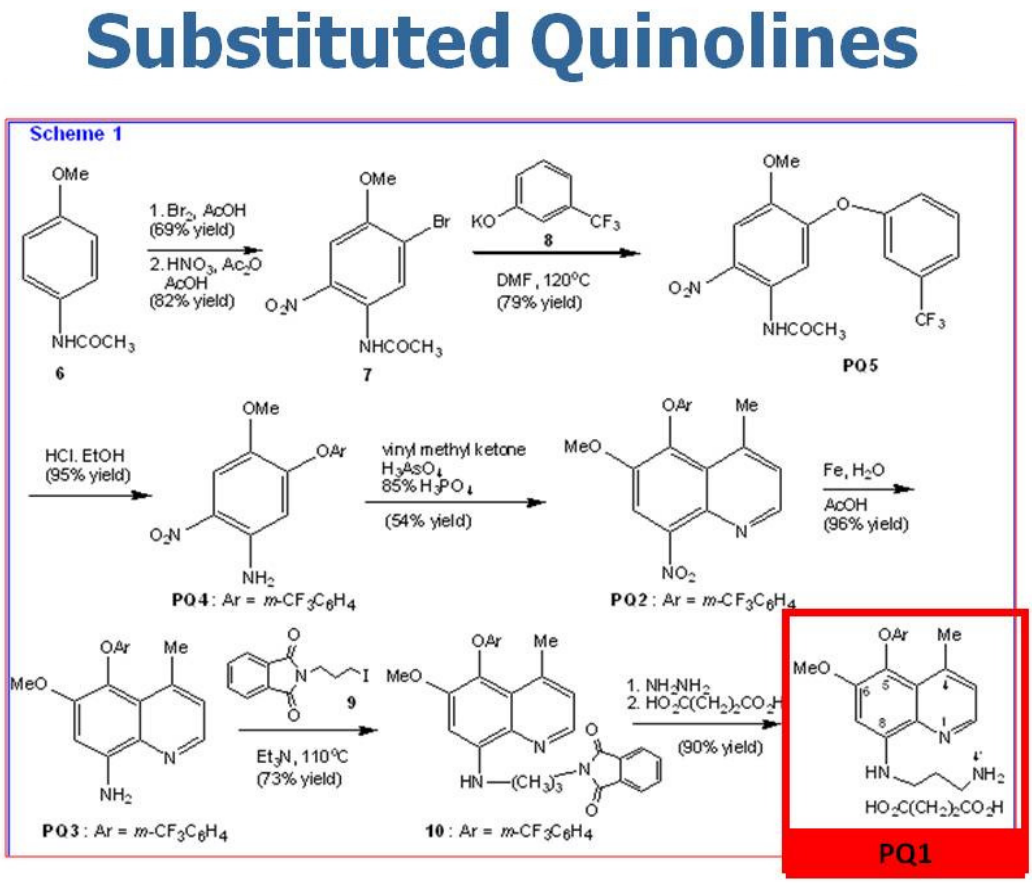
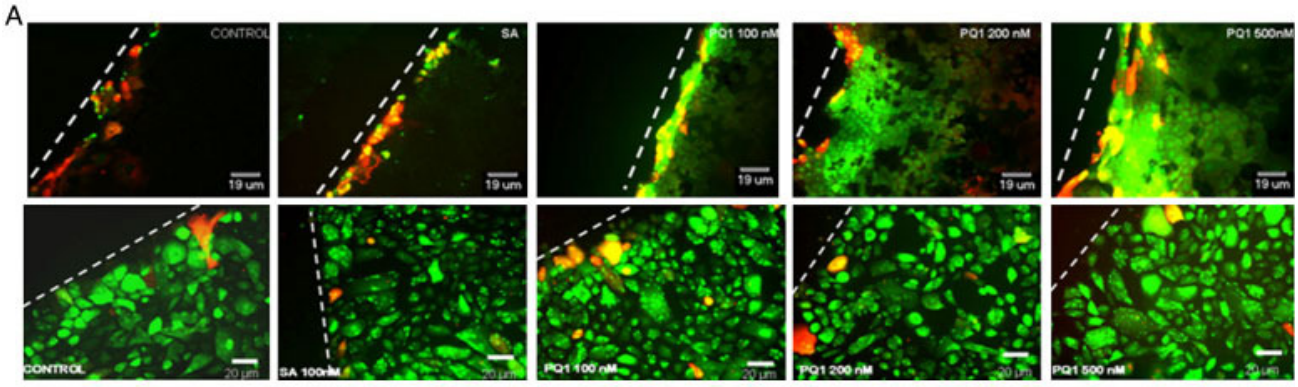


Figure 4.1 Syntheses of PQ analogs.

PQs were synthesized via a modified procedure from 4-acetaminoanisole (compound 6). Results of the isolation of PQ1 are described in Materials and Methods.

4.4.2 Effect of PQ1 on GJIC

We tested the efficacy of compounds PQ1-PQ5 on GJIC by parachute assay in different cell lines – MDA-453, MCF-7, ZR75, MDA-231, and T47D cells (Appendix A.2, Figures S2-S7). We found a significant increase in GJIC by PQ1 in T47D cells. Therefore, we chose to study the efficacy of PQ1 in T47D cells. We further tested the effect of PQ1 on the GJIC activity by another dye transfer method, SL/DT in T47D breast cancer cells. The results demonstrated that 100, 200 and 500 nM of PQ1 show a significant increase in gap junction activity in T47D cells compared to controls, without PQ1 treatment and succinic acid, using scrape load/dye transfer assay (Figure 4.2A, top panel). Conversely, PQ1 has no effect on GJIC activity of human primary epithelial cells (HMEC, normal cells) compared to its controls (Figure 4.2A, bottom panel). The distance of dye transfer from section cut to the farthest cells with dye was measured. A graphical representation of three experiments indicates that 200 nM PQ1 causes an 8.5-fold increase in distance of dye transfer compared to control (Figure 4.2B). HMECs have uniform uptake of Lucifer yellow. This is due to the existing high level of gap junction activity in these normal cells.



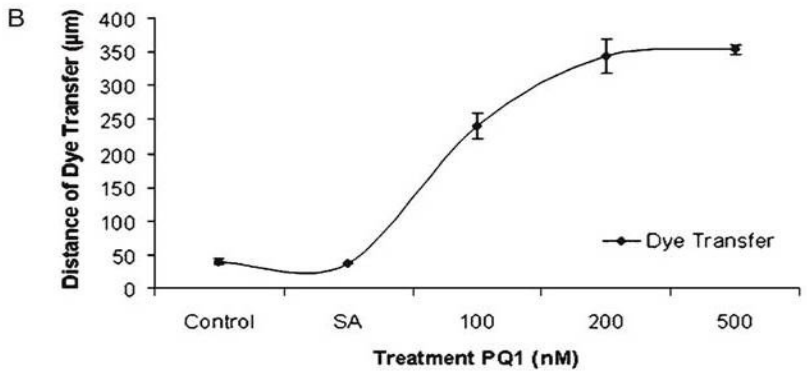


Figure 4.2 Gap Junction Activity.

A) T47D (Top panel), and **A)** HMEC cells (Bottom panel) were treated with 0, 100, 200, and 500 nM PQ1 for 1 hr. Controls are media alone and succinic acid. Scrape load/dye transfer assay was performed as described in Materials and Methods. White dash lines indicate a cross section cut of initial dye. Lucifer yellow was used as a gap junctional dye and rhodamine-dextran was used to mark the cutting site. Fluorescence green indicates the passages of dye from the cutting site, showing increase of GJIC. **B)** Graphical presentation of three experiments shows the distance of dye transfer of T47D cells.

4.4.3 Effect of PQ1 on tight junctions, colony growth assay and cell viability

Furthermore, 100, 200, or 500 nM of PQ1 has no significant effect on tight junction ion permeability by using transepithelial electric resistance (TEER) compared to control (Figure 4.3). This suggests that PQ1 enhances only affected gap junctional activity without having an effect on tight junction. These results demonstrated that PQ1 is sufficient to cause an increase in GJIC activity in SL/DT assay.

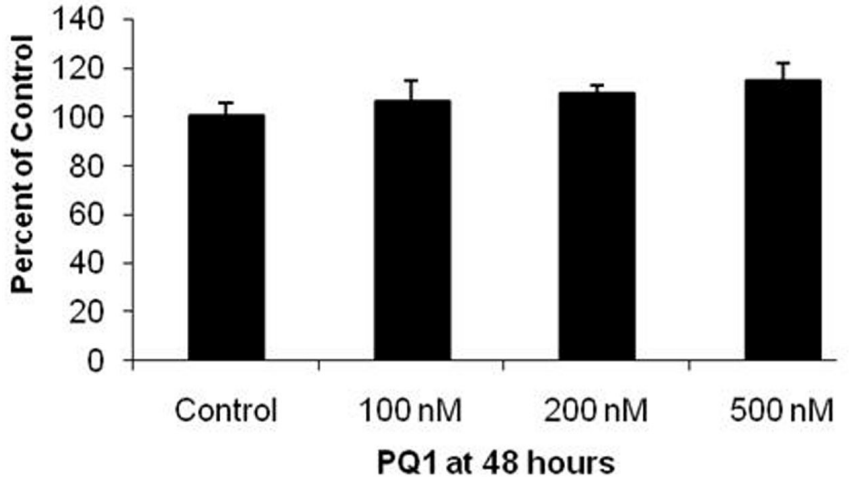


Figure 4.3 Effect of PQ1 on transepithelial electrical resistance (TEER).
T47D cells were treated with 0, 100, 200, and 500 nM PQ1 for 48 hr. TEER assay was performed as described in Materials and Methods. TEER values of each treatment in KΩ/cm^2 were converted to relative percent of control.

Various oncogenes (e.g. ras, raf, neu, src, mos) down-regulate GJIC while several tumor suppressor genes can up-regulate GJIC. Thus, we examined the effect of PQ1-upregulated gap junction activity in T47D colony growth formation. Cells were grown in soft agar to assess their capacity for anchorage-independent growth, which is a key feature of cell transformation. HMEC and T47D cells were treated with 10, 100, 1000, and 10,000 nM PQ1 for 7 days. A graphical presentation of three experiment results is presented in log scale of PQ1 concentration. The effect of PQ1 on T47D cells showed a significant inhibition of T47D cell colony growth compared to control (Figure 4.4). A 100 nM PQ1 inhibits 66% of colony growth compared to controls, without PQ1 treatment or 100 nM succinic acid. Interestingly, the same concentration (100 nM PQ1) has no effect on HMECs (data not shown). This suggests that 100 nM PQ1 can cause an increase in GJIC activity and subsequently can decrease colony growth of T47D cells.

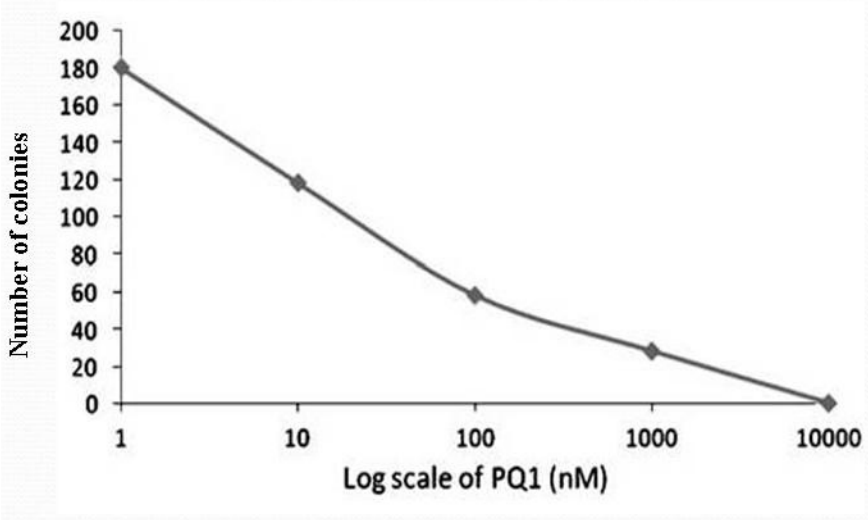


Figure 4.4 Effect of Substituted Quinolines on T47D Cells.

Base agar plates were prepared containing 0.8% agar and 0.4% agar in Ham's F12. Cells (5×10^4 cells/33 mm^2 well) were suspended in 100 µl of Ham's F12 with 0.4% agar and plated. These plates were maintained at 37℃ for 7 days and examined for the presence of colonies. Individual colonies of 50 µm or greater were examined. T47D cells were treated with 1, 10 and 100 nM PQ1 and SA (succinic acid) as a solvent control. Individual colonies of 50 µm or greater were examined. Statistical significance, *$p<0.05$, of at least three experiments.

We also determined the cytotoxicity of PQ1 in HMEC and T47D cells using MTT assay. Cells were treated with 10, 100, 200, 500, 1000 nM PQ1 for 24 hr. MTT assay was performed according to the manufacturer's recommendations. A 200 nM PQ1 has 67% cell viability compared to controls (Figure 4.5). 1 µM PQ1 can further decrease cell viability to 50% in T47D cells. However, treatments of 100 and 200 nM PQ1 have 95% and 103% HMEC cell viability compared to control (data not shown). Thus, PQ1 has no cytotoxic effect to HMECs.

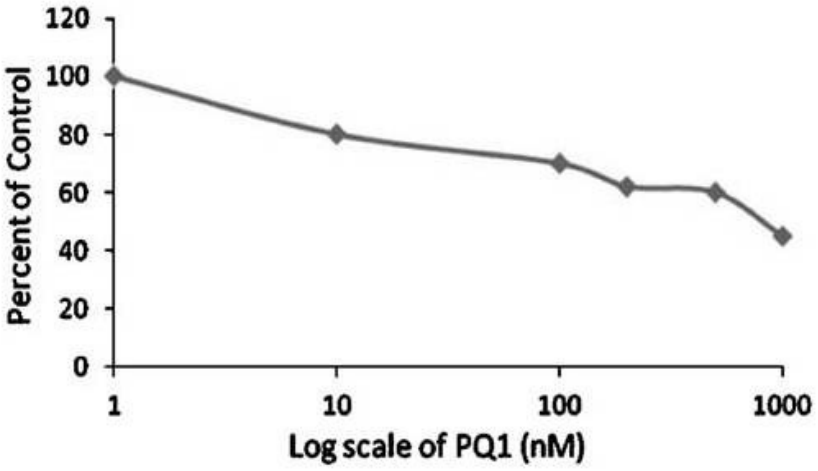


Figure 4.5 Effect of PQ1 on Cell Viability.

T47D breast cancer cells were treated with various concentrations of PQ1 for 24 hr. MTT assay was performed with adherent cell cultures using a culture medium free of phenol red and of serum. Solution containing MTT was metabolized by the cells (incubation period 3 hr). After solubilization of the MTT crystals with the solubilization solution MTT, the amount of dye was measured spectrophotometrically at 570 nm.

4.4.4 Effect of PQ1 on the expression of different connexins

Furthermore, whole cell extract of PQ1 treatment was analyzed for the changes in gap junctional proteins, connexins. Cells were treated with 10, 100, 200, and 500 nM PQ1 for 24 hr. Western blot analysis was performed against Cx26, Cx32, and Cx43 (Figure 4.6). The results show that PQ1 has no effect on Cx26, Cx32, and Cx43 expression. Interestingly, a decrease in phosphorylated Cx43 was observed in 500 nM PQ1 treatment of T47D cells. Anti-tubulin was used as a loading control. These results suggest that PQ1 does not affect the expression of connexins but directly causes a decrease in phosphorylation of connexin.

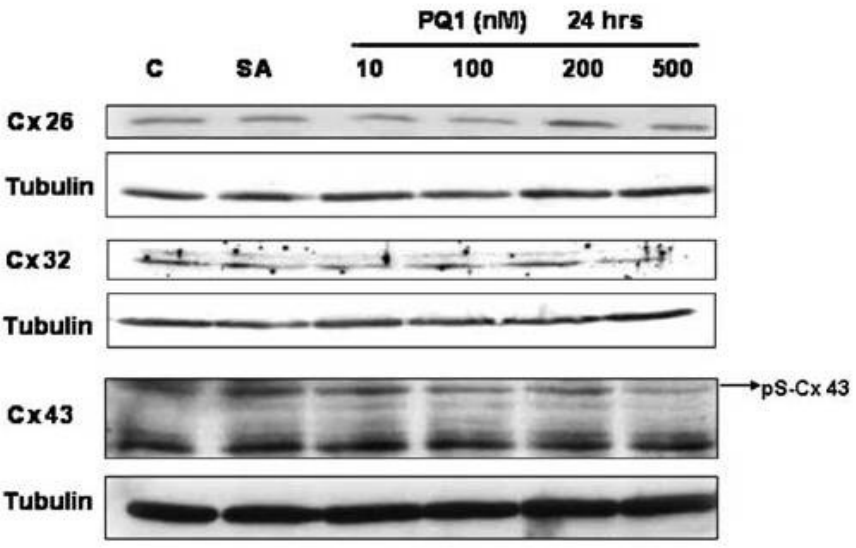


Figure 4.6 Effect of PQ1 on the expression of different connexins in T47D cells.
T47D cells were dosed with PQ1 for 24 hr. The Western blot analysis was performed as described in Materials and Methods. Whole cell extract was analysed for Cx43, Cx32, and Cx26. Experiment was performed at least three times.

4.4.5 PQ1 causing apoptosis

Mitochondrial damage in treatment of PQ1 was observed using electron microscopy (data not shown), suggesting that these cells are under stress and apoptotic conditions. We further examined the effect of PQ1 on apoptosis by detecting the active form of caspase 3. T47D cells were treated with 100, 500, and 1000 nM PQ1 for 24 hr. Western blot analysis was performed using active form of caspase 3 antibodies. A 200 nM PQ1 causes 1.5-fold increase of active caspase 3 compared to control (Figure 4.7). However, a decrease of caspase is observed at higher concentrations, a common effect in apoptotic protein expression. This is due to the cytotoxic response of the cells. A small pilot study was conducted to study the effect of PQ1 on T47D xenograft tumor growth in nude mice. However, statistical significance was not achieved as only three animals per group (control, PQ1-treated, and tamoxifen-treated) were included in the study. (A2, S 8-12)

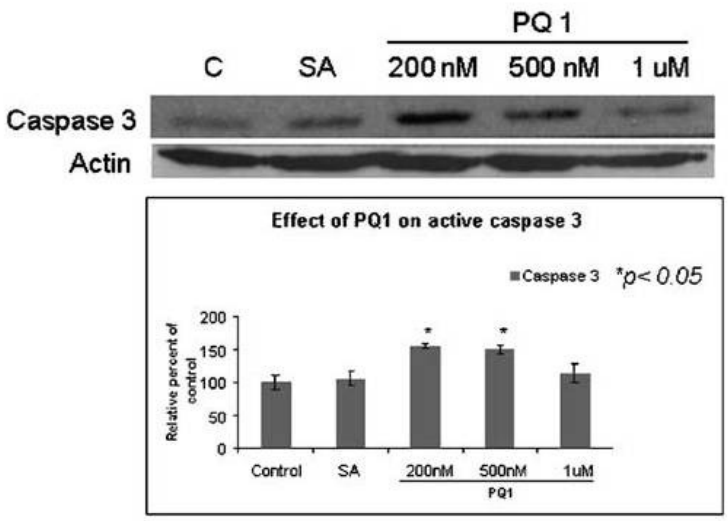


Figure 4.7 Effect of PQ1 on Active Caspase 3.

T47D cells were treated with 0, 200 nM, 500 nM and 1 μM PQ1 for 24 hr. Treatment with succinic acid was used as control. Western blot analysis was performed. Nitrocellulose membrane was blotted with the active form of caspase 3 antibodies (16 KDa). Actin acts as loading control. Graphical presentation of three experiments are presented with statistical significance, $p<0.05$.

4.5 Conclusions

Since an effective clinical drug targeting gap junction is not available presently, Dr. Hua used computational docking method to search for chemicals that bind to connexon. After screening several classes of molecules, substituted quinolines were focused on based on their relative binding constants and bioactivities. Quinolines are known for their anti-malarial [Wiesner 2003], antibacterial (Veyssier 2005) and anticancer (Boyle 2006; Cnubben 2005; Kawase 2003; Levitt 1999) activities. Recently, quinolines were examined in ATP-binding cassette drug transporter inhibition (Cnubben 2005), targeting tumor hypoxia (Boyle 2006), modulation of multidrug resistance (Kawase 2003), and tyrosine kinase inhibition (Levitt 1999). Beside activation of GJIC, quinolines may provide additional beneficial effects.

Over forty years, the loss of GJIC has been described in cancer cells and led to a hypothesis that defects in GJIC is involved in the carcinogenesis process (Loewenstein and

Kanno 1966). Numerous reports have been confirmed that gap junctions are frequently decreased or absent in cancer cells. Co-culture of tumor cells with normal cells, or with connexin-overexpressing cells, results in growth retardation of neoplastic component by the establishment of functional communication as observed with dye transfer (Zhu *et al*. 1992). This retardation of tumor growth could be prevented by co-culturing transformed cells with junctional competent normal cells transfected with a connexin-specific antisense reagent (Goldberg *et al*. 1994). Thus, these results indicate that signals from adjacent normal cells can reverse a malignant phenotype and that their failure to accomplish this is due to a lack of GJIC.

The relationship between cell communication and cell growth has been established that the capability of cells to communicate through gap junction is negatively related to their growth activity. We have demonstrated that increased GJIC activity in T47D cells can cause a decrease in cell growth (Figures 4.2 and 4.3). These results were also observed by Saez *et al*. (Saez *et al*. 2003) that an increase in GJIC is directly related to the anti-tumor effect in human mammary cancer cell line. Interestingly, PQ1 only affects T47D breast cancer cells and not normal mammary epithelial cells. In normal tissues, gap junctions are active and well-regulated between the cytoplasm of contacting cells (Loewenstein 1979; Loewenstein 1981; Yamasaki and Naus 1996). Through the passage of signaling molecules, GJIC contributes to the regulation of cell proliferation, differentiation, cell death, and homeostatic maintenance. Numerous studies clearly show that altered GJIC is involved in cell cycle progression. In most cell types, GJIC is reduced in the late G1, S and M phases (Ruch 1994). The specific cell cycle state in which GJIC and/or connexin expression are modified, however, depends on both the cell type and the nature of the connexin species being investigated. We found that PQ1 has no effect on connexin expression; however, it causes a decrease in phosphorylation of connexin (Figure 4.6). Upregulation of GJIC activity is dependent on the unphosphorylated connexins. Thus the observation is consistent with the increase of GJIC activity (Figure 4.2) and a decrease of phosphorylation of Cx43 (Figure 4.6).

In summary, PQ1 specifically enhances GJIC activity and does not affect the transepithelial electrical resistance of T47D cells. An increase of PQ1-induced GJIC activity causes a significant decrease of colony cell growth; however, PQ1 has no effect on primary mammary epithelial cells. Since normal epithelial cells have well-regulated gap junction channels, the change of GJIC activity in these cells was not observed. The decrease of cell

viability and colony cell growth is subsequently of PQ1-induced apoptosis as the result of upregulation of active caspase 3. Thus, PQ1 is the first known compound to enhance GJIC activity in T47D breast cancer cells.

Acknowledgements

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4.6 References

Acevedo P, Bertram JS. (1995). Liarozole potentiates the cancer chemopreventive activity of and the up-regulation of gap junctional communication and connexin43 expression by retinoic acid and beta-carotene in 10T1/2 cells. *Carcinogenesis* **16** (9), 2215-22.

Ammerpohl O, Thormeyer D, Khan Z, Appelskog IB, Gojkovic Z, Almqvist PM, Ekstrom TJ. (2004). HDACi phenylbutyrate increases bystander killing of HSV-tk transfected glioma cells. *Biochem Biophys Res Commun* **324** (1), 8-14.

Bhimani RS, Troll W, Grunberger D, Frenkel K. (1993). Inhibition of oxidative stress in HeLa cells by chemopreventive agents. *Cancer Res* **53** (19), 4528-33.

Boyle RG, Travers, S. (2006). Hypoxia: targeting the tumor. *Anti-cancer Agents in Med. Chem.* **6**, 281-286.

Bruzzone R, White TW, Paul DL. (1996). Connections with connexins: the molecular basis of direct intercellular signaling. *Eur J Biochem* **238** (1), 1-27.

Clark PE, Hall MC, Borden LS, Jr., Miller AA, Hu JJ, Lee WR, Stindt D, D'Agostino R, Jr., Lovato J, Harmon M and others. (2006). Phase I-II prospective dose-escalating trial of lycopene in patients with biochemical relapse of prostate cancer after definitive local therapy. *Urology* **67**(6),1257-61.

Cnubben NHP, Wortelboer, H. M., van Zanden, J. J., Rietjens, V. M. C. M., van Bladeren, P. J. (2005). Metabolism of ATP-binding cassette drug transporter inhibitors: complicating factor for multidrug resistance. *Expert Opinion on Drug Metabol. & Toxicol.* **1**, 219-232.

Dubina MV, Iatckii NA, Popov DE, Vasil'ev SV, Krutovskikh VA. (2002). Connexin 43, but not connexin 32, is mutated at advanced stages of human sporadic colon cancer. *Oncogene* **21**(32), 4992-6.

Eghbali B, Kessler JA, Reid LM, Roy C, Spray DC. (1991). Involvement of gap junctions in tumorigenesis: transfection of tumor cells with connexin 32 cDNA retards growth in vivo. *Proc Natl Acad Sci* **88**(23), 10701-5.

Fleishman SJ, Unger VM, Ben-Tal N. (2006). Transmembrane protein structures without X-rays. *Trends Biochem Sci* **31**(2), 106-13.

Foote CI, Zhou L, Zhu X, Nicholson BJ. (1998). The pattern of disulfide linkages in the extracellular loop regions of connexin 32 suggests a model for the docking interface of gap junctions. *J Cell Biol* **140** (5), 1187-97.

Goldberg GS, Martyn KD, Lau AF. (1994). A connexin 43 antisense vector reduces the ability of normal cells to inhibit the foci formation of transformed cells. *Mol Carcinog* **11** (2), 106-14.

Goodsell DS, Olson AJ. (1990). Automated docking of substrates to proteins by simulated annealing. *Proteins* **8** (3), 195-202.

Holder JW, Elmore E, Barrett JC. (1993). Gap junction function and cancer. *Cancer Res* **53** (15), 3475-85.

Huang RP, Hossain MZ, Huang R, Gano J, Fan Y, Boynton AL. (2001). Connexin 43 (cx43) enhances chemotherapy-induced apoptosis in human glioblastoma cells. *Int J Cancer* **92** (1), 130-8.

Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ. (2007). Cancer statistics, 2007. CA *Cancer J Clin* **57** (1), 43-66.

Kawase M, Motohashi, N. (2003). New multidrug resistance reversal agents. *Curr. Drug Targets* **4,** 31-43.

LaMontagne MP, Blumbergs P, Strube RE. (1982a). Antimalarials. 14. 5-(aryloxy)-4-methylprimaquine analogues. A highly effective series of blood and tissue schizonticidal agents. *J Med Chem* **25** (9), 1094-7.

LaMontagne MP, Markovac A, Khan MS. (1982b). Antimalarials. 13. 5-Alkoxy analogues of 4-methylprimaquine. *J Med Chem* **25** (8), 964-8.

Lauer WMR, C.; Arnold, R. T.; Drake, N. L.; Hook, J. V.; Tinker, J. .(1946). Some derivatives of 8-aminoquinoline. *J. Am. Chem. Soc*. **68,** 1546-1548.

Levitt ML, Koty, P. P. (1999). Tyrosine kinase inhibitors in preclinical development. . *Invest. New Drugs* **17**, 213-226.

Livny O, Kaplan I, Reifen R, Polak-Charcon S, Madar Z, Schwartz B. (2002). Lycopene inhibits proliferation and enhances gap-junction communication of KB-1 human oral tumor cells. *J Nutr* **132** (12), 3754-9.

Loewenstein WR. (1979). Junctional intercellular communication and the control of growth. *Biochim Biophys Acta* **560** (1), 1-65.

Loewenstein WR. (1981). Junctional intercellular communication: the cell-to-cell membrane channel. *Physiol Rev* **61** (4), 829-913.

Loewenstein WR, Kanno Y. (1966). Intercellular communication and the control of tissue growth: lack of communication between cancer cells. *Nature* **209** (5029), 1248-9.

Makowski L, Caspar DL, Phillips WC, Goodenough DA. (1977). Gap junction structures. II. Analysis of the x-ray diffraction data. *J Cell Biol* **74** (2), 629-45.

Martin PE, Evans WH. (2004). Incorporation of connexins into plasma membranes and gap junctions. *Cardiovasc Res* **62** (2), 378-87.

Morris GM, Goodsell DS, Huey R, Olson AJ. (1996). Distributed automated docking of flexible ligands to proteins: parallel applications of AutoDock 2.4. *J Comput Aided Mol Des* **10** (4), 293-304.

Morris GM, Goodsell, D.S., Halliday, R.S., Huey, R., Hart, W.E., Belew, R.K., Olson, A.J. (1998). Automated docking using Lamarckian genetic algorithm and an empirical binding free energy function. *J. Comp. Chem*. **19,** 1639-1662.

Musil LS, Le AC, VanSlyke JK, Roberts LM. (2000). Regulation of connexin degradation as a mechanism to increase gap junction assembly and function. *J Biol Chem* **275** (33), 25207-15.

Na HK, Wilson MR, Kang KS, Chang CC, Grunberger D, Trosko JE. (2000). Restoration of gap junctional intercellular communication by caffeic acid phenethyl ester (CAPE) in a ras-transformed rat liver epithelial cell line. *Cancer Lett* **157** (1), 31-8.

O'Byrne KJ, Han C, Mitchell K, Lane D, Carmichael J, Harris AL, Talbot DC. (1998). Phase II study of liarozole in advanced non-small cell lung cancer. *Eur J Cancer* **34** (9), 1463-6.

Ruch RJ. (1994). The role of gap junctional intercellular communication in neoplasia. *Ann Clin Lab Sci* **24** (3), 216-31.

Ruch RJ, Madhukar BV, Trosko JE, Klaunig JE. (1993). Reversal of ras-induced inhibition of gap-junctional intercellular communication, transformation, and tumorigenesis by lovastatin. *Mol Carcinog* **7** (1), 50-9.

Saez CG, Velasquez L, Montoya M, Eugenin E, Alvarez MG. (2003). Increased gap junctional intercellular communication is directly related to the anti-tumor effect of all-trans-retinoic acid plus tamoxifen in a human mammary cancer cell line. *J Cell Biochem* **89** (3), 450-61.

Trosko JE, Chang CC. (2001). Mechanism of up-regulated gap junctional intercellular communication during chemoprevention and chemotherapy of cancer. *Mutat Res* **480-481**:219-29.

Trosko JE, Chang CC, Madhukar BV, Klaunig JE. (1990). Chemical, oncogene and growth factor inhibition gap junctional intercellular communication: an integrative hypothesis of carcinogenesis. *Pathobiology* **58** (5), 265-78.

Unger VM, Kumar NM, Gilula NB, Yeager M. (1999). Three-dimensional structure of a recombinant gap junction membrane channel. *Science* **283** (5405), 1176-80.

Veenstra RD. (2003). Gap junction channel structure. *Recent Res. Devel. Biophys.* **2**, 65-94.

Veyssier P, Bryskier, A. (2005). Dihydrofolate reductase inhibitors, nitroheterocycles (furans) and 8-hydroxyquinolines. Internal Medicine, Hospital de Compiegne, Bryskier, A. Ed.,Compiegne, Fr. P .941-963.

Wei N, Mi MT, Zhou Y. (2007). Influences of lovastatin on membrane ion flow and intracellular signaling in breast cancer cells. *Cell Mol Biol Lett* **12** (1), 1-15.

Wiesner J, Ortmann, R., Jomaa, H., Schlitzer, M. (2003). New antimalarial drugs. . Angew. Chem. Int. Ed. (**42**), 5274-5293.

Wilgenbus KK, Kirkpatrick CJ, Knuechel R, Willecke K, Traub O. (1992). Expression of Cx26, Cx32 and Cx43 gap junction proteins in normal and neoplastic human tissues. *Int J Cancer* **51**(4), 522-9.

Yamasaki H, Naus CC. (1996). Role of connexin genes in growth control. *Carcinogenesis* **17**(6), 1199-213.

Zhang ZQ, Lin ZX, Lu YY. (1994). Studies on the reduction of malignant phenotypes in a highly metastatic human lung carcinoma-correlated changes of intercellular communication, cytoskeletons, oncogenes and antioncogene. *Cancer Res* **16**(2), 88-92.

Zhu D, Kidder GM, Caveney S, Naus CC. (1992). Growth retardation in glioma cells cocultured with cells overexpressing a gap junction protein. *Proc Natl Acad Sci* **89** (21), 10218-21.

Chapter 5 - Combinational treatment of PQ1 and tamoxifen induces increased apoptosis in T47D breast cancer cells

5.1 Abstract

Tamoxifen is a drug of choice for endocrine-responsive breast tumor patients. However, tamoxifen resistance has become a major concern for the treatment of breast cancer. Combinational therapies of tamoxifen and different drugs are being frequently studied. In the current study, we tested the efficacy of PQ1 (code name) in combination with tamoxifen in T47D cells. Colony growth assay was performed using soft agar to measure the colony growth while cell proliferation was measured by MTT assay in T47D cells. Confocal microscopy was used to measure Ki67, survivin and BAX expression along with western blot. APO-BrdU labeling indicated the apoptosis induced by the treatment in T47D cells. We observed a 55% decrease in the colony growth in the presence of combination of PQ1 and tamoxifen; while tamoxifen alone has little effects. Combination of 10 μM tamoxifen and PQ1 200 nM or 500 nM resulted in only 16% cell viability compared to controls at 48 hr in T47D cells by MTT assay. We found a significant increase in BAX protein at 1 hr in the presence of 500 nM PQ1 alone, 10 μM tamoxifen alone and combination of PQ1 and tamoxifen. A 2-fold increase was observed in active caspase 3 in the presence of combinational treatment of 10 μM tamoxifen and 200 or 500 nM PQ1. Also, flow cytometric analysis showed a 50% increase in the number of apoptotic cells in the presence of combination of tamoxifen and PQ1 compared to the control. Furthermore, the results show that the expression of survivin is not affected by PQ1 (200 or 500 nM) whereas tamoxifen alone or in combination with PQ1 significantly reduces survival of T47D cells. We observed a significant increase in BAX expression, caspase 3 activation and DNA fragmentation in combinational treatment of tamoxifen and PQ1 as compared to their individual treatments. Tamoxifen alone and combination with PQ1 showed a decrease in the survivin expression while PQ1 alone shows to be independent of survivin-mediated pathway. The combinational treatment of tamoxifen and PQ1 showed a significant decrease in cell viability compared to tamoxifen treatment alone. The present study demonstrates for the first time that combinational treatment of

tamoxifen and PQ1 (gap junctional activator) can be used to potentiate apoptosis of T47D human breast cancer cells. This could alter either the length or dose of tamoxifen clinically used for breast cancer patients.

5.2 Introduction

Tamoxifen is one of the most commonly and successfully used chemotherapeutic agent for the treatment of endocrine responsive breast cancers (Clarke *et al.*, 2001). Tamoxifen is better known as a selective estrogen receptor modulator (SERM) because of its multiple activities (Osborne, 1998). Clinical experience with this drug likely now exceeds 10 million patient years. Unfortunately, in most patients, cancers that initially respond to tamoxifen gradually acquire resistance to the treatment and require alternative systemic therapies (Berstein *et al.*, 2003). Despite extensive experience with this drug, the precise mechanisms that confer resistance remain unknown. A number of mechanisms have been proposed to control antiestrogen resistance in ER^+ breast cancer (Riggins *et al.*, 2007), but many details of these mechanisms continue to be unclear (Riggins *et al.*, 2008). These include changes in the host immunity, host endocrinology, or antiestrogen pharmacokinetics (Clarke *et al.*, 2001). Under host endocrinology, some tumors spontaneously become hormone-independent despite the presence of estrogen receptors; in others, tumors that are initially ER^+ become ER^- over time (Hull *et al.*, 1983; Encarnacion *et al.*, 1993). Numerous trials have been conducted using the combinational treatment of chemotherapy plus tamoxifen but the results have been controversial (Mouridsen *et al.*, 1988; Goldhirsch A, 1989; Fisher *et al.*, 1990; Rivkin *et al.*, 1994; group, 1997; Pritchard *et al.*, 1997).

Gap junctions are the intercellular plasma membrane channels which allow the passage of small molecules from one cell to other. The flux of molecules through the channels is called the gap junctional intercellular communication (GJIC) (Vinken *et al.*, 2006). GJIC exists in most of the mammalian cells and is involved in cell growth, differentiation, and homeostasis. Since decades it has been shown that the mitotic cells in the cell cycle show decreased GJIC (PO Lague, 1970; Goodall and Maro, 1986; Stein *et al.*, 1992). Therefore, it leads to a state that the cell-cell communication is negatively related to the capability of the cell to grow. Due to its effect on the cell growth, many studies were conducted to find a co-relation between the GJIC

and cancer. Many *in vitro* studies showed that tumor-promoting agents lead to a decrease in GJIC (Murray and Fitzgerald, 1979; Yotti *et al.*, 1979; Enomoto *et al.*, 1981; Trosko, 1983) while *in vivo* studies supported the *in vitro* data (Sugie *et al.*, 1987; Mesnil *et al.*, 1988). Protein and mRNA analysis showed a decrease in connexin expression in preneoplastic lesions as well as hepatocellular carcinomas (Fitzgerald *et al.*, 1989; Neveu MJ, 1989).

In our previous study we found a gap junctional activator, substituted quinolines (PQ1). We showed that PQ1 (200 nM) showed a 30% increase in the GJIC in T47D cells; however, there was no effect of PQ1 treatment on GJIC in normal mammary epithelial cells. In addition to an increase in GJIC, 80-95% growth attenuation was observed by PQ1 in colony growth assay. Moreover, an increase in caspase 3 with PQ-treated cells was observed, suggesting a possible involvement in apoptosis (Gakhar *et al.*, 2008).

The antitumor effects of tamoxifen are thought to be due to its antiestrogenic activity, mediated by competitive inhibition of estrogen binding to estrogen receptors (Osborne, 1996). The inhibition of expression of estrogen-regulated genes causes decrease in cell growth and proliferation (Langan Fahey SM, 1994). Tamoxifen may also directly affect the programmed-cell death (Ellis *et al.*, 1997). Our previous studies conducted on PQ1 have also shown an increase in caspase-3 and a decrease in breast tumor growth. Therefore, we examined the effect of combinational treatment of tamoxifen and the gap junctional activator, PQ1 on breast cancer cells. In the present study, we found an increase in T47D human breast cancer cell death observed by different mechanisms in the presence of combinational therapy of PQ1 and tamoxifen. A significant increase in BAX and caspase 3 followed by an increased apoptosis by APO-BrdU incorporation at different dosing time in the presence of both tamoxifen and PQ1 was observed. Furthermore, a decrease in the colony growth, MTT assay and an increased DNA fragmentation were observed in the combinational treatment of PQ1 and tamoxifen.

5.3 Materials and Methods

5.3.1 Cell Lines and 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), 10% antibiotic-antimycotic at 37°C with 5% CO_2 in 75 cm^2 flasks. Tamoxifen citrate was purchased from Sigma (Saint Louis, MO).

5.3.2 Cell Morphology

Cells (5,000 cells/ml) were seeded in a 6-well plate and dosed with 200 nM PQ1 alone, 10 μM tamoxifen alone, combination of tamoxifen and PQ1, and combination of tamoxifen and estrogen (10 nM) for 24, 48 and 72 hr. Cells were observed under a microscope at 40X objective.

5.3.3 Colony Growth Using Soft Agar Assay

Cells were treated with ethanol, 200 nM PQ1, 10 μM tamoxifen, and combination of 200 nM PQ1 and 10 μM tamoxifen for 7 days. Base agar plates were prepared containing 0.8% agar and 0.4% agar in RPMI. Cells (5×10^4 cells/33 mm^2 well) were suspended in 100 μl of RPMI with 0.4% agar and plated. These plates were maintained at 37°C for 7 days and examined for the presence of colonies. Individual colonies of 50 μm or greater were examined.

5.3.4 MTT Assay

The MTT [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was performed with cell cultures in a 96-well plate incubated with 200 and 500 nM PQ1, 10 μM tamoxifen alone, and combination of 10 μM tamoxifen and 200 or 500 nM PQ1 for 1, 48 and 72 hr. The MTT solution was metabolized by the cells (incubation period 1 hr) at 37°C. MTT is a tetrazolium salt (yellowish) cleaved to formazan crystals by succinate dehydrogenase. In viable cells, more formazan dye will be produced. After solubilization of MTT crystals with the 0.35 N HCl solubilization solution, dye was measured spectrophotometrically at 570 nm with the background subtraction at 650 nm.

5.3.5 Western Blot Analysis

Cells were grown in serum-supplemented RPMI media until they were 90% confluent in 25-cm^2 flasks. Cells were incubated with PQ1 alone, 10 μM tamoxifen alone, and combination of tamoxifen and PQ1 for 1, 48 and 72 hr. Cells were washed 3 times with cold PBS and harvested using cell 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, St. Louis, MO) for 10 min followed by rotation on a shaker for 30 min at 4°C. Cells were vortexed and centrifuged at 13,000 rpm for 30 min at 4°C. Forty μg of whole cell extract was resolved by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membrane (Midwest Scientific, St. Louis, MO). Nitrocellulose membrane was blocked in 5% milk for 1 hr at room temperature and incubated with polyclonal rabbit Bcl2, (1:200), mouse BAX (1:200, Santa Cruz Biotechnologies, Santa Cruz, CA), polyclonal rabbit caspase 3 (1:500, BD Pharmingen, San Diego, CA), polyclonal rabbit survivin (1:1,000, Novus Biologicals, CO, USA) and polyclonal rabbit actin (1:1,000, Sigma-Aldrich, Saint Louis, MO). Western blots were detected by enhanced chemiluminescence (ECL) detection (Amersham, Pittsburg, PA).

5.3.6 Immunofluorescence and Confocal Microscopy

Cells were grown on coverslips in 6-well plates in RPMI media. Cells were treated with PQ1 alone, tamoxifen alone, and combination of tamoxifen and PQ1 for 1, 48 and 72 hr. Cells were fixed with 2% paraformaldehyde for 20 min and then neutralized with 50 mM glycine for 5 min. The cells were lysed with 0.1% Triton X-100 for additional 10 min. After washing with PBS, cells were blocked with 2.5% BSA in PBS for 2 hr and then incubated with primary antibodies, rabbit Ki67 (1:250, Santa Cruz Biotechnologies, Santa Cruz, CA) and mouse BAX (1: 50, Santa Cruz, CA), rabbit survivin (1:250, Novus Biologicals, CO, USA) for 15 hr at 4°C. Following this step, cells were incubated in DAPI for a minute and then incubated with anti-mouse and anti-rabbit Alexa fluor 488 and 568 (Molecular probes, Eugene, OR, USA) for 4 hr at 4°C, respectively. Cells were analyzed for nuclear morphology by staining with DAPI. Samples were sealed and analyzed by a confocal microscope (Carl Zeiss LSM 510 META, Narashige, MN).

5.3.7 Apoptosis Assay by Flow Cytometry

Cells were grown in a 35 mm^2 dish and then dosed with PQ1 alone, tamoxifen alone, and combination of tamoxifen and PQ1 for 1, 48 and 72 hr. Cells were trypsinized and stained with APO-BrdU TUNEL Assay kit (Molecular Probes # A23210, Carlsbad, CA) according to the manufacturer's protocol. APO-BrdU binding was analyzed by flow cytometry using a BD FACSCalibur system and the data obtained was analyzed using the CellQuest software.

5.4 Results and Discussion

5.4.1 Current treatment with tamoxifen

Breast cancer is the second leading cause of cancer deaths in North American women. Tamoxifen has been the single agent of choice in treating hormone responsive breast cancer cases since 1971. Even though tamoxifen has been shown to be 50% more effective than placebo in preventing the occurrence of breast cancer in high-risk population, the risk of developing uterine cancer has increased by more than 40% (Mandlekar and Kong, 2001). The line of treatment with tamoxifen includes the usage of the drug for at least 5 years in most of the patients. Long-term treatment with tamoxifen induces tamoxifen resistance; however, the mechanism of which is still being elucidated (Osborne, 1998). Therefore, it is necessary to develop effective modalities to enhance the efficacy of tamoxifen. In our present study we propose to observe the combinational effect of PQ1 and tamoxifen. PQ1 has been shown to be a gap junctional activator and induced cell death in both *in vitro* and *in vivo* treatments (Gakhar *et al.*, 2008). Tamoxifen has also shown to induce apoptotic cell death both *in vitro* and *in vivo* (Perry *et al.*, 1995; Gelmann, 1996; Martin *et al.*, 1996). This effect was concentration-dependent. At nanomolar (nM) concentrations of tamoxifen, only growth arrest occurs whereas at micromolar (μM) concentrations induction of cell death was observed in cell cultures. We observed the effect of both PQ1 and 10 µM tamoxifen on T47D human breast cancer cell line. Our previous study suggested a tremendous increase in GJIC in the presence of PQ1 in T47D cells compared to other breast cancer cell lines (MCF7, MDA231, MDA453 and ZR75) (Gakhar *et al.*, 2008).

5.4.2 PQ1 and tamoxifen affects cell morphology, proliferation and colony growth in T47D cells

Cell morphology was tremendously affected in the presence of 200 nM PQ1 for 24, 48 and 72 hr in which cells were being detached from the plate. In the presence of 10 μM tamoxifen for 24, 48, and 72 hr, cells showed a marked change in the morphology, including shrinkage, irregular shape and some cells were floating in the media (Figure 5.1 A, B, C). The combination of PQ1 and tamoxifen showed the complete loss of structure of cells as well. Cells partially regained back their structure in the presence of 10 μM tamoxifen and 10 nM 17β-estradiol. These results suggest that the combinational treatment of tamoxifen and PQ1 can potentiate the effect of tamoxifen.

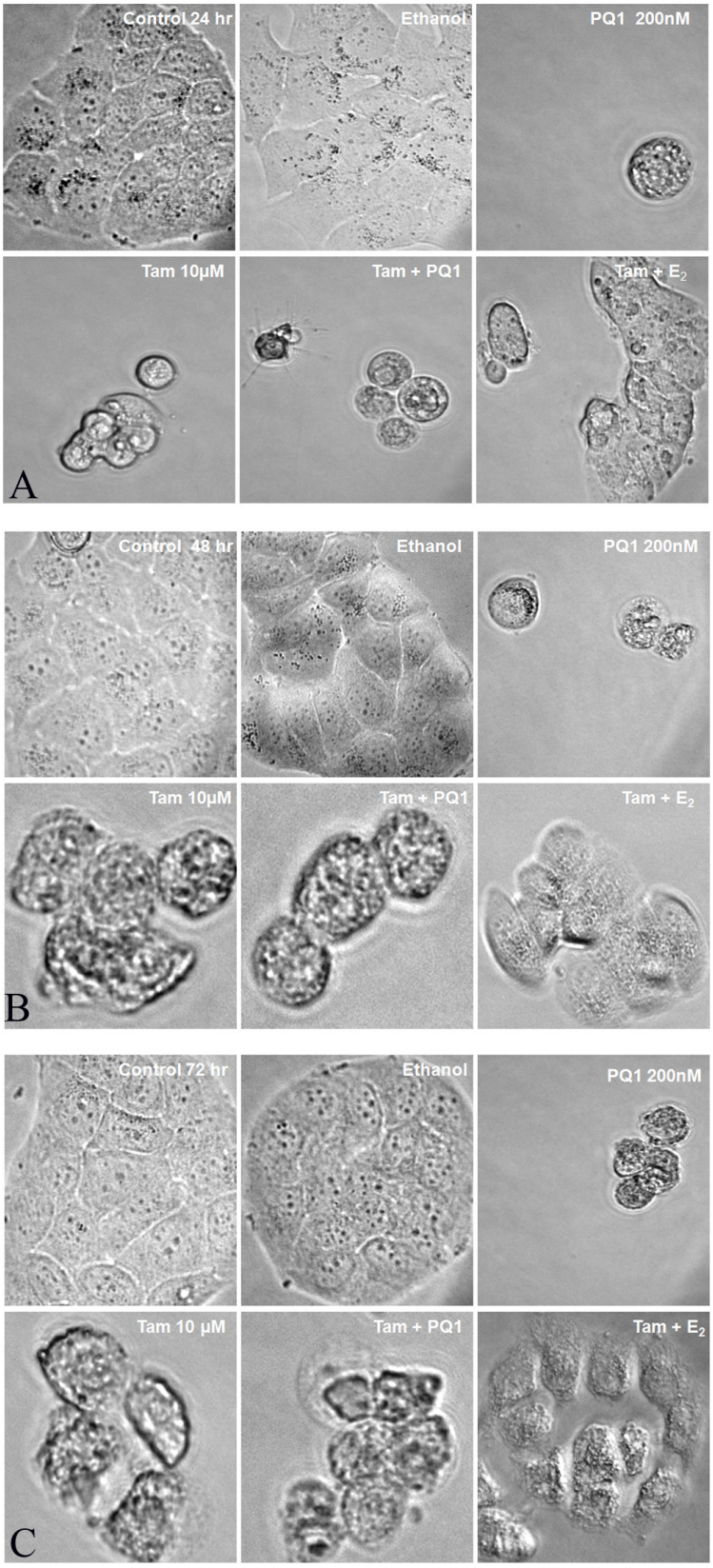

Figure 5.1 Effect of PQ1 and tamoxifen on cell morphology in T47D cells.

A) T47D cells were seeded in a 6-well plate for 24 hr in the presence of 200 nM PQ1, 10 µM tamoxifen, combination of tamoxifen and PQ1, and the combination of tamoxifen and 10 nM 17β-estradiol (an antagonist for tamoxifen action). **B)** Cells were dosed for 48 hrs with the same treatment as above. **C)** T47D cells were treated with the same treatment as in **(A)** for 72 hr. Ethanol was used as a solvent control for tamoxifen at different time intervals. In the presence of PQ1 and tamoxifen alone and in combination, the cell structure was seen to be lost significantly at 48 and 72 hrs. At 10 nM 17β-estradiol and tamoxifen, a partial restoration in the cell structure was observed at 48 and 72 hr compared to full restoration at 24 hr. Tam=tamoxifen

Colony growth assay measures the colony formation in soft agar. Soft agar is used to measure the anchorage-independence (feature of cancerous cells) of the cells. We observed a 55% decrease in the colony growth in the presence of combination of PQ1 and tamoxifen (Figure 5.2). This suggests that the combinational treatment of tamoxifen and PQ1 is sufficient to cause a significant decrease of colony growth at 7-day incubation compared to tamoxifen or PQ1 treatment alone. Ethanol treatment, a solvent control, shows no effect in colony growth of T47D cells.

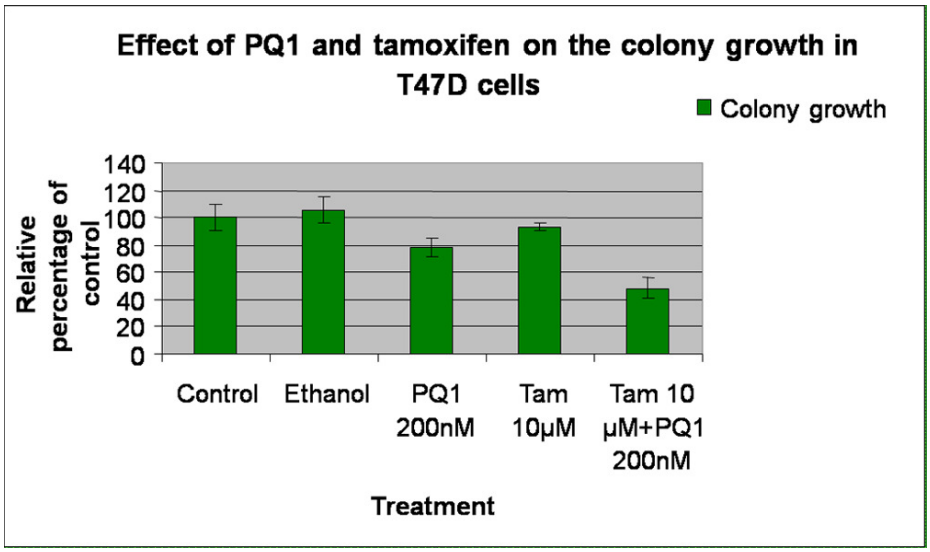

Figure 5.2 Combination of 200 nM PQ1 and tamoxifen decreases the colony growth in T47D cells.

Cells were dosed with 200 nM PQ1, 10 µM tamoxifen and combination of both for 7 days in a soft agar. After 7 days, colonies > 50 µm were counted. Combination of PQ1 and tamoxifen

resulted in a 55% decrease in the colony growth compared to 20% and 10% decrease in the presence of 200 nM PQ1 and tamoxifen alone, respectively. Graphical representation of three experiments with ± SD and statistical significance *p < 0.05. Note that the significance *p< 0.05 was found in all the treatments compared to control at 48 and 72 hr.

In MTT assay, 200 and 500 nM PQ1 showed a 60% and 50% cell viability at 48 hr, respectively (Figure 5.3). Tamoxifen (10 µM) showed 30% cell viability whereas combination of tamoxifen and PQ1 resulted in only 16% cell viability at 48 hr compared to controls at 48 hr in T47D cells. Both tamoxifen (10 µM) and PQ1 (200 and 500 nM) resulted in a decrease in cell growth by 50% compared to tamoxifen treatment alone at 48 hr. At 72 hr, combinational treatment of tamoxifen and PQ1 (200 and 500 nM) resulted in 20% and 13% cell viability, respectively. Thus, 48 hr is sufficient to cause a significant decrease with combinational treatment of tamoxifen and PQ1. Interestingly, cell viability was not affected by either 200 or 500 nM PQ1 at 1 hr; however, tamoxifen treatment alone resulted in 28% decrease in cell growth. The cell viability was greatly affected by MTT assay at 48 hr compared to 72 hr in the presence of PQ1 alone or tamoxifen alone or their combinations.

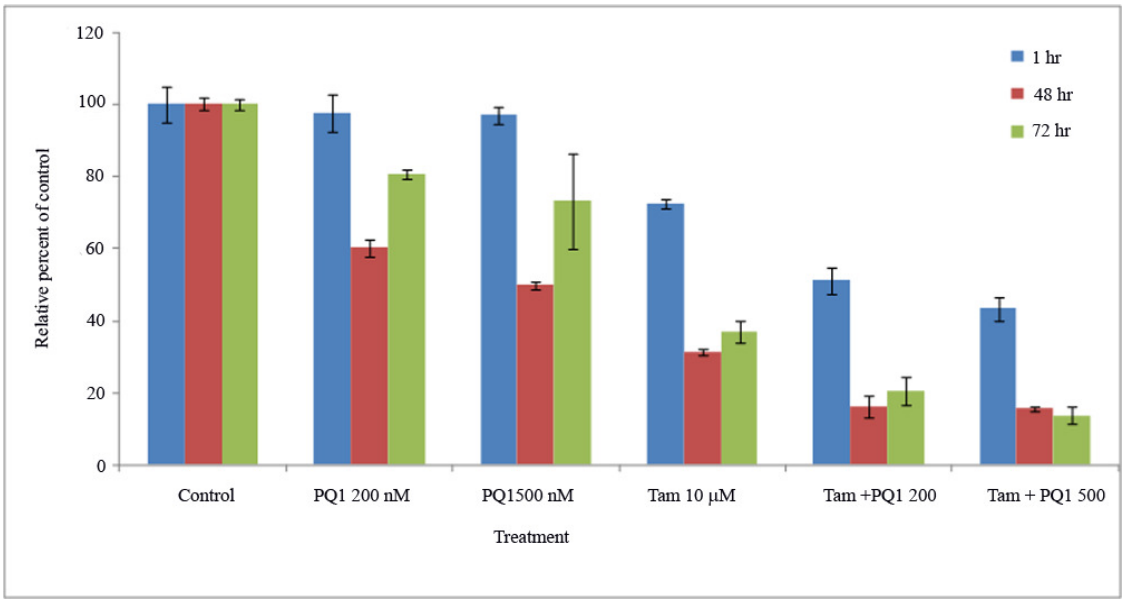

Figure 5.3 MTT assay to measure the T47D cell proliferation in the presence of tamoxifen and PQ1.

Cells were treated with 200 and 500 nM PQ1 and 10 µM tamoxifen and combination of tamoxifen and either 200 or 500 nM PQ1 for 1, 48 and 72 hr. At 1 hr, 50 % decrease in cell growth was observed in the presence of tamoxifen and 500 nM PQ1. At 48 hr, PQ1 200 and 500 nM alone, tamoxifen alone, combination of tamoxifen and 200 nM PQ1, and combination of tamoxifen and 500 nM PQ1 showed a cell viability of 60, 50, 30, 16, and 16 %, respectively. At 72 hr the combination of both tamoxifen and either PQ1 200 or 500 nM resulted in 20% and 13% cell viability, showing no further decrease compared to 48 hr. Graphical representation of three experiments with ± SD and statistical significance *$p < 0.005$ for 48 hr and **$p < 0.05$ for 72 hr . Note the *p values indicate the significance between the tamoxifen treatment alone or with PQ1 combination.

5.4.3 Tamoxifen and PQ1 affecting Ki67 expression

The expression of proteins in some instances is measured in symptomatic breast cancer to identify the prognostic factors which are associated with the biological behavior of individual tumors. Ki67 is a nuclear protein widely used as a marker for cell proliferation. Tamoxifen has been shown to decrease the expression of Ki67 in breast cancer patients and in breast cancer cell lines (Clarke *et al.* 1993). Therefore, the effect of combinational treatment of tamoxifen and PQ1 on Ki67 staining in T47D cells was measured. Ki67 staining was decreased in the presence of 200 nM PQ1 at 24, 48 and 72 hr (Figure 5.4). In the presence of tamoxifen alone, no Ki67 staining was observed at 24, 48, and 72 hr. Combinational treatment of tamoxifen and PQ1 also showed no Ki67 staining whereas combinational treatment of estrogen and tamoxifen showed an expression of Ki67 suggesting that estrogen can partially antagonize the effect of tamoxifen. These results suggest that PQ1 has an effect on Ki67 staining and it does not antagonize the effect of tamoxifen when used in combination while estrogen can reverse the effect of tamoxifen. Furthermore, PQ1 clearly has an effect on proliferation of T47D cells.

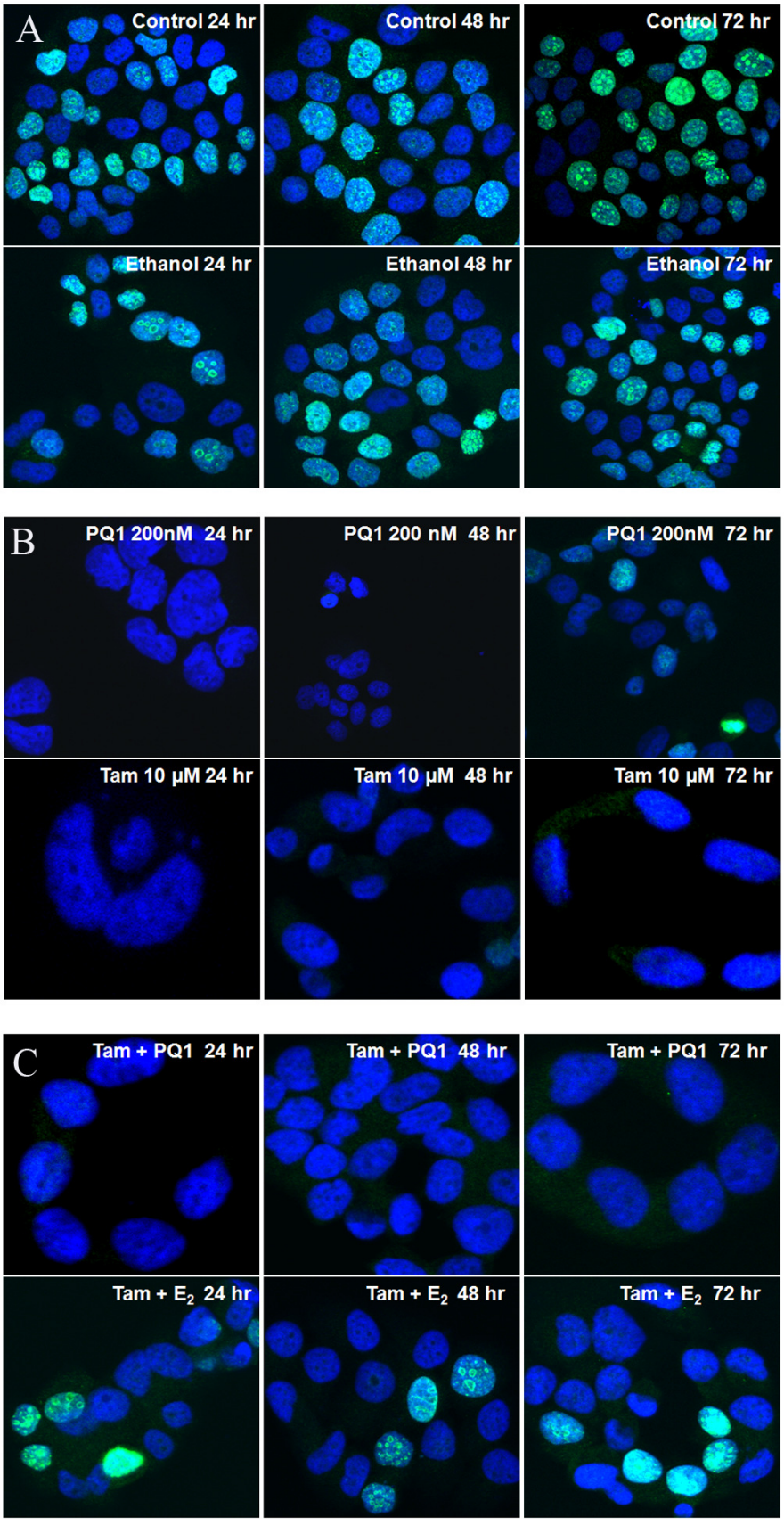
A
Control 24 hr
Control 48 hr
Control 72 hr
Ethanol 24 hr
Ethanol 48 hr
Ethanol 72 hr
B
PQ1 200nM 24 hr
PQ1 200 nM 48 hr
PQ1 200nM 72 hr
Tam 10 µM 24 hr
Tam 10 µM 48 hr
Tam 10 µM 72 hr
C
Tam + PQ1 24 hr
Tam + PQ1 48 hr
Tam + PQ1 72 hr
Tam + E2 24 hr
Tam + E2 48 hr
Tam + E2 72 hr

Figure 5.4 Confocal Microscopy showing the effect of PQ1 and tamoxifen on Ki67 expression.

Cells were treated with 200 nM PQ1, 10 μM tamoxifen, combination of 200nM PQ1 and tamoxifen, and combination of 10 nM 17β-estradiol for 24, 48 and 72 hr. Ethanol was used as a solvent control for tamoxifen. Cells treated with 200 nM PQ1, tamoxifen or combination of tamoxifen and PQ1 showed a tremendous decrease in Ki67 expression. 17β-estradiol and tamoxifen partially restored the expression of Ki67.

5.4.4 Effect of PQ1 and tamoxifen on apoptotic proteins

Programmed cell death, or apoptosis occurs either by activation of the death receptors or by a breach in the mitochondrial membrane integrity. Cytochrome C, a key player in induction of apoptosis cascade, is released from the inside of the mitochondria into the cytosol of the cell by the interaction of two important proteins involved in apoptosis, BAX and Bcl2. Upon apoptotic signals, proapoptotic protein, BAX gets activated while antiapoptotic proteins like Bcl2 prevent apoptosis by heterodimerization with BAX (Otter *et al.*, 1998; Broker *et al.*, 2005). Overall, the ratio of BAX and Bcl2 determines the integrity of the mitochondrial membrane. In the present study, we conducted a time-dependent study by treating cells with PQ1 and tamoxifen in combination and alone for 1, 48 and 72 hr. We found a significant increase in BAX protein at 1 hr in the presence of 500 nM PQ1 alone, tamoxifen alone and combination of PQ1 and tamoxifen (Figure 5.5A). We also observed a decrease in Bcl2 in the presence of tamoxifen alone and in combination of tamoxifen and PQ1 at 1 hr. At 48 hr, a significant decrease was seen in Bcl2 expression at 500 nM PQ1 and combination of tamoxifen and PQ1. Also, there was a significant increase in BAX in combinational treatment at 48 hr. Zhang *et al.* showed a decrease in Bcl2 but no effect on BAX in MCF-7 cells in the presence of 10 μM tamoxifen at 72 hr (Zhang *et al.*, 1999). We found that at 72 hr, BAX and Bcl2 were significantly increased and decreased in the presence of tamoxifen and combination of tamoxifen and either PQ1 200 or 500 nM, respectively. These results implicate that the combinational treatment not only significantly increases BAX but also decreases Bcl2. The ratio of Bcl2 and BAX is decreased significantly at 1 hr in the presence of PQ1 alone and tamoxifen alone or in combination. Therefore, it indicates

that PQ1 has a rapid action on BAX activation. The effect on reduction of Bcl2 expression is relatively slow, staying constant at 48 hr before increasing again at 72 hr.

A. **B.**

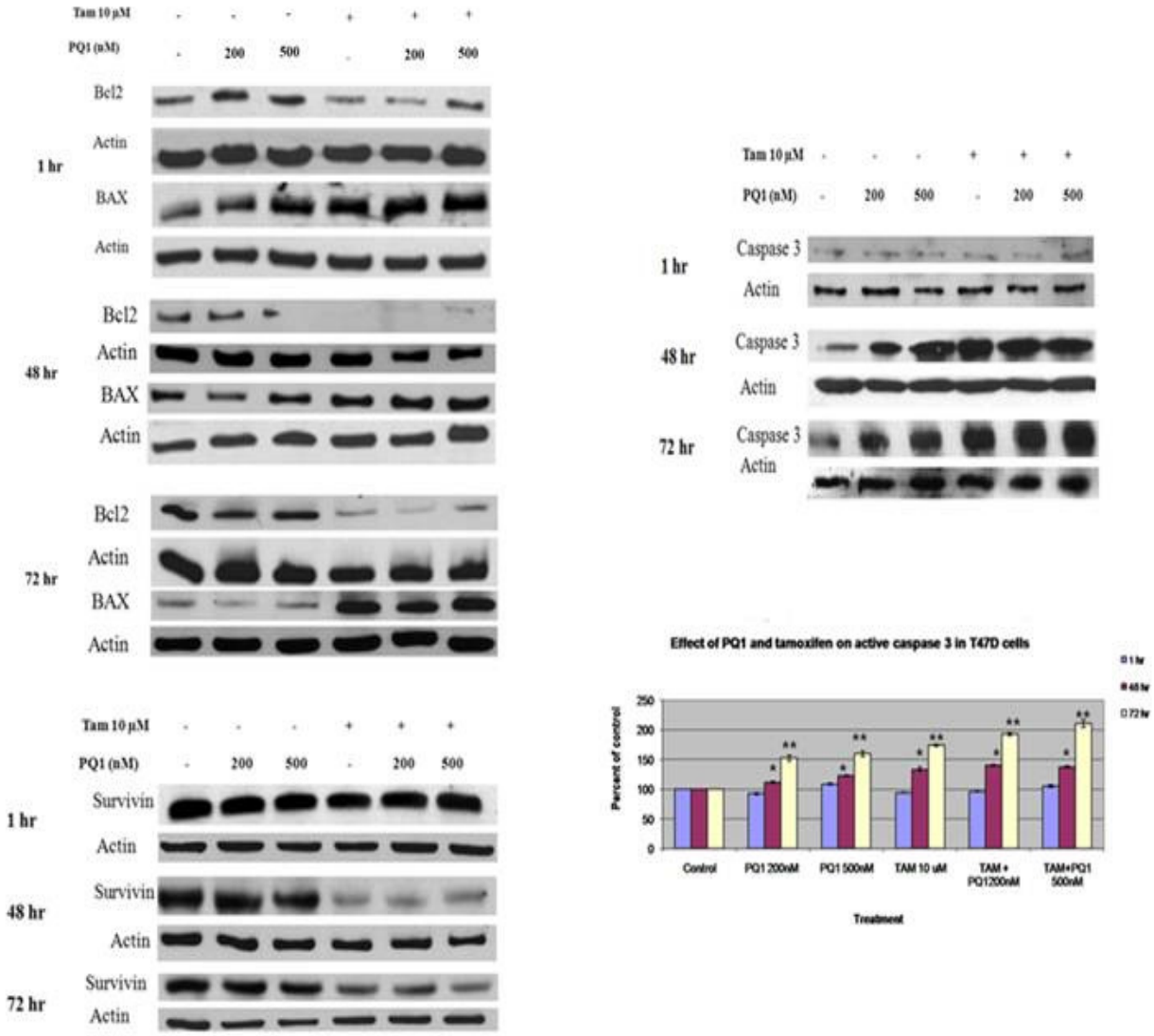

Figure 5.5 Expression of proteins involved in apoptosis pathway- BAX, caspase 3, Bcl2 and survivin.

A) Bcl2 and BAX levels were measured in cells were treated with 200 and 500 nM PQ1 and 10µM tamoxifen and combination of tamoxifen with 200 or 500 nM PQ1 for 1, 48 and 72 hr. A significant increase in BAX was observed at all the treatments compared to control. Survivin measured for the same treatments at 1, 48 and 72 hr. A significant decrease in survivin was

observed in the presence of tamoxifen alone and in combination with both 200 and 500 nM PQ1 at 48 hr. However, no effect on survivin was seen in the presence of 200 and 500 nM PQ1 alone. **B)** Caspase 3 expression was measured for the same treatments at 1, 48 and 72 hr. Histogram showing the changes observed in active caspase 3 at 1, 48 and 72 hr with different treatments. A 2-fold increase was seen at 48 and 72 hr in the presence of combination of tamoxifen with 200 and 500 nM PQ1. A histogram of three experiments with ± SD and statistical significance *p< 0.05 for 48 hr and **p< 0.05 for 72 hr. Actin was used as a loading control for all the proteins.

After BAX activation, a cascade of events driven primarily by the activation of proteolytic caspases results in the processing of intracellular structural proteins and regulatory enzymes that culminates in apoptotic cell death (Zhang A, 2004). Caspase 3 activation, an executioner caspase, is considered to be one of the last steps involved in the apoptosis cascade pathway (Mandlekar *et al.*, 2000). Therefore, we examined the effect of combination of tamoxifen and PQ1 on caspase 3 activation. Interestingly, we did not find activation of caspase 3 at 1 hr (Figure 5.5B). However, there was a significant increase in caspase 3 at 48 and 72 hr in the presence of PQ1 alone, tamoxifen alone and in combination of tamoxifen and PQ1 indicating caspase 3 activation takes time and stays at least through 72 hr. A 50% increase in active caspase 3 was observed at 200 and 500 nM PQ1 at 72 hr. A 2-fold increase was observed in active caspase 3 both in the presence of combinational treatment of tamoxifen and 200 nM PQ1 and tamoxifen and 500 nM PQ1. This suggests that combination of both PQ1 and tamoxifen results in increased T47D cell death.

In the present study, we also observed the effect of PQ1 and tamoxifen on the inhibition of apoptosis (IAP) protein, survivin (a group of proteins involved in inhibition of caspase 3, 7, and 9) (Altieri, 2003; Gazzaniga *et al.*, 2003). Increase in expression of surviving is believed to protect cells against a possible default induction of apoptosis in the case of aberrant mitosis (Li *et al.*, 1998). Many studies on clinical specimen have shown that survivin expression is invariably upregulated in human cancers and is associated with resistance to chemotherapy linked to poor prognosis, suggesting that surviving modulates the survival of cancer cells (Yamamoto *et al.*, 2008). Gazzaniga *et al.* found that survivin cannot be used as a prognostic factor for the relapse of superficial bladder cancer (Gazzaniga *et al.*, 2003). However, in pre-clinical bladder tumor models, inhibition of survivin expression and/or function has been shown to impede tumor cell

proliferation, and markedly induce spontaneous or chemotherapy induced apoptosis (Margulis *et al.*, 2008). We found no effect on survivin at 1 hr (Figure 5.5A); however, at 48 and 72 hr we found a tremendous decrease in survivin expression in the treatment of tamoxifen alone and combinational treatment of tamoxifen and PQ1 (200 or 500 nM). This suggests that tamoxifen decreases the survivin expression whereas PQ1 alone has no effect on survivin expression in T47D cells up to 72 hrs.

We also observed survivin and BAX in T47D cells at 1, 48 and 72 hr by confocal microscopy. We found a nuclear localization of survivin and cytoplasmic as well as nuclear localization of BAX (Figure 5.6A, B). The confocal microscopy results showed the absence of survivin in tamoxifen treated cells at 48 and 72 hr.

5.4.5 Measurement of Apoptosis by Nuclear Morphology and Flow Cytometry

The effect of tamoxifen and PQ1 on the nuclear staining by using DAPI was measured. Apoptosis is characterized by morphologic changes such as shrinkage of the cell, condensation of chromatin, and disintegration of the cell into small fragments, apoptotic bodies (Kerr *et al.*, 1972). In the present study, we found more cells undergoing the process of apoptosis exhibited by blebbing, and fragmentation in the presence of PQ1 and tamoxifen alone and in combination (data not shown). APO-BrdU TUNEL assay kit was used to detect the DNA fragmentation of apoptotic cells. In apoptosis, DNA fragmentation exposes 3'-OH groups at which deoxynucleotidyl transferase (TdT) can add deoxyribonucleotides. 5-bromo-2'-deoxyuridine 5'-triphosphate (BrdUTP) is an analog of deoxythymidine which gets incorporated at the 3'-OH group. We found an increase in the BrdUTP incorporation in the presence of PQ1 and tamoxifen alone and in combination at 48 and 72 hr (Figure 5.7). A 50% increase in apoptotic cells was observed at 48 and 72 hr in the presence of combinational treatment of tamoxifen and PQ1. There was no effect on the apoptosis in the presence of PQ1 or tamoxifen at 1 hr. Even though the apoptosis cascade starts in an hour shown by an increase in BAX and a decrease in Bcl2, it requires > 1 hr for the activation of caspases and DNA damage.

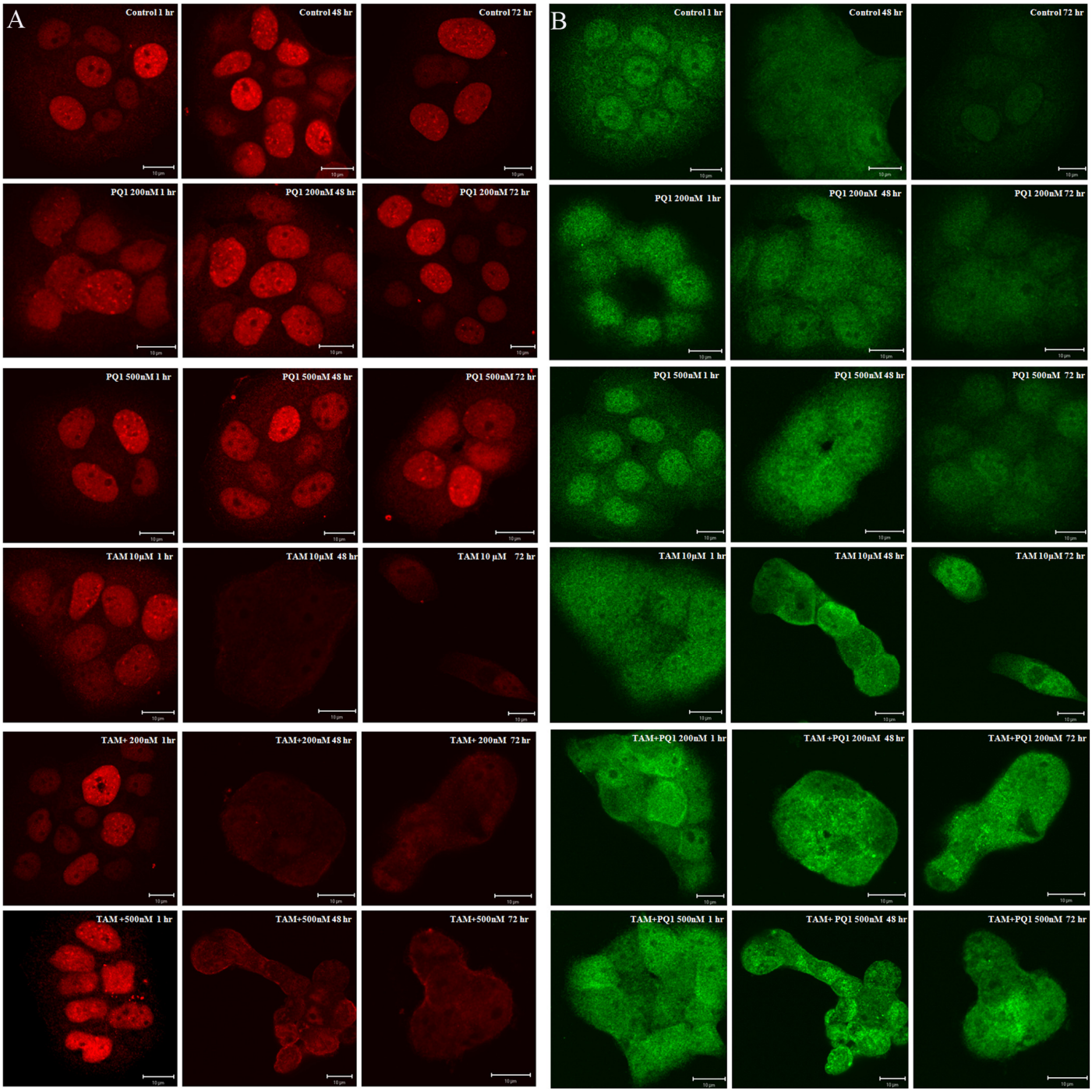

Figure 5.6 Confocal microscopy showing the expression of survivin and BAX proteins.

A) Cells were treated with 200 and 500 nM PQ1 and 10 µM tamoxifen and combination of tamoxifen and either 200 or 500 nM PQ1 for 1, 48 and 72 hr. Tamoxifen treatment resulted in a decrease in survivin expression. Cells were stained with antirabbit Alexa fluor 568. **B)** Cells

were treated the same way as in **(A)**; however; expression of BAX protein was observed using mouse secondary Alexa fluor 488 antibody. Cells were visualized by a confocal microscope (Carl Zeiss LSM 510 META). An increase in BAX was observed in the presence of tamoxifen.

The results imply that the combination of PQ1 (200 and 500 nM) and tamoxifen (10 µM) results in an increase in apoptosis compared to the individual treatments.

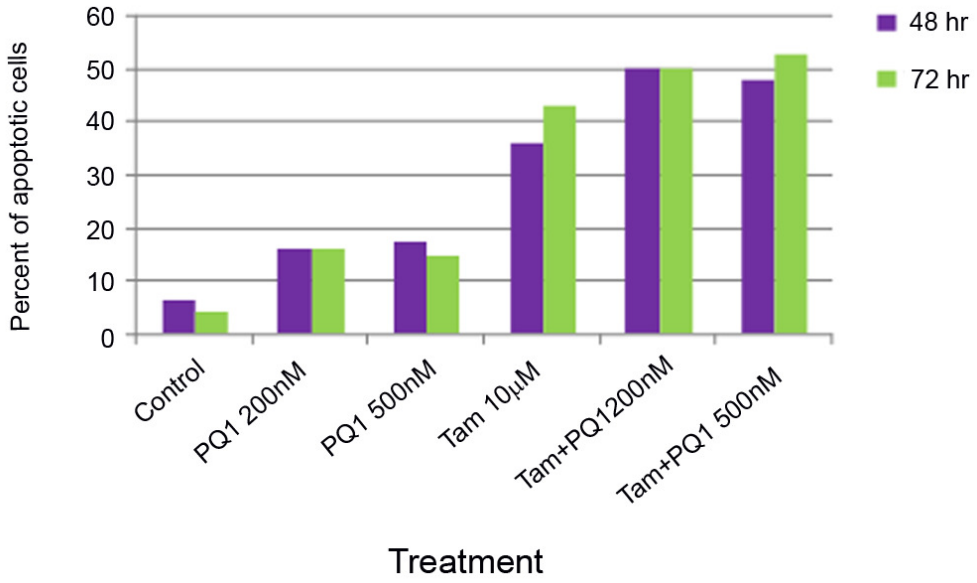

Figure 5.7 Flow cytometric analysis of apoptotic cells by APO-BrdU labeling.
Cells were treated with 200 and 500 nM PQ1 and 10µM tamoxifen and combination of tamoxifen and 200 or 500 nM PQ1 for 48 and 72 hr. A 50% increase in apoptotic cell number was seen at both 48 and 72 hr in the presence of both tamoxifen and 200 nM or 500 nM PQ1.

5.5 Conclusion

Tamoxifen has been the drug of choice for the treatment of endocrine responsive breast tumors, but tamoxifen resistance has been an issue for very long time. We demonstrate for the first time that the combinational effect of PQ1, a gap junction activator, with tamoxifen has a potential use for treatment against breast cancer. We found a decrease in cell proliferation by MTT assay and significant decrease in the colony growth assay. We observed a significant increase in BAX, caspase 3 activation and DNA fragmentation in the presence of combinational treatment of PQ1 and tamoxifen as compared to their individual treatment. We also found that

survivin is significantly decreased in the presence of tamoxifen alone and combination of tamoxifen with either 200 or 500 nM PQ1. However, PQ1 alone does not affect survivin expression in T47D cells as observed at 1, 48 and 72 hr. The possibility of PQ1 affecting other caspases in inducing apoptosis has not been covered by the current study; therefore, we cannot rule out the involvement of other caspases. We propose that PQ1 might allow tamoxifen (MW= 371.51) to pass through gap junctions between the cells, causing a rapid action of tamoxifen. In support of our data, Jensen and Glazer showed that forced expression of Cx43 in MCF-7 cells resulted in increased cell sensitivity to cisplatin at high density (Jensen and Glazer, 2004). Our work is based on the same concept as Jensen and Glazer demonstrating that the combinational therapy of tamoxifen and PQ1 shows more promising role in inducing apoptosis by caspase 3 activation. But in our studies we did not overexpress the connexins, we used a gap junctional activator which can be easily administered with tamoxifen. However, more studies needs to be done to observe the combinational effect of PQ1 and tamoxifen *in vivo*.

5.6 Significance

Our study suggests that the combinational treatment may allow a decrease in tamoxifen concentration (lower than 10μM) in clinical use. This will have a strong implication that combinational treatment with gap junctional activators can lower the concentration of chemotherapeutic agent and thus may reduce side effect of these drugs. In future we would be conducting combinational studies of PQ1 and tamoxifen (dose reduced from μM to nM) and observe its effect on cell-based systems. Also, much of the work presented has focused in breast cancer cells; however, the role of gap junctional activators (PQ1) in drug sensitivity need not be limited to breast cancer. A variety of other cancers may take advantage of very similar mechanism and as a result other diseases may benefit from gap junction modulating pathway.

Abbreviations: Substituted quinolines (code name PQ), Tamoxifen (TAM), MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

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5.7 References

Altieri, D. C. (2003). Survivin in apoptosis control and cell cycle regulation in cancer. *Prog Cell Cycle Res* **5**, 447-452.

Berstein, L. M., Zheng, H., Yue, W., Wang, J. P., Lykkesfeldt, A. E., Naftolin, F., Harada, H., Shanabrough, M., and Santen, R. J. (2003). New approaches to the understanding of tamoxifen action and resistance. *Endocr Relat Cancer* **10**, 267-277.

Broker, L. E., Kruyt, F. A., and Giaccone, G. (2005). Cell death independent of caspases: a review. *Clin Cancer Res* **11**, 3155-3162.

Clarke, R., Leonessa, F., Welch, J. N., and Skaar, T. C. (2001). Cellular and molecular pharmacology of antiestrogen action and resistance. *Pharmacol Rev* **53**, 25-71.

Clarke, R. B., Laidlaw, I. J., Jones, L. J., Howell, A., and Anderson, E. (1993). Effect of tamoxifen on Ki67 labelling index in human breast tumours and its relationship to oestrogen and progesterone receptor status. *Br J Cancer* **67**, 606-611.

Ellis, P. A., Saccani-Jotti, G., Clarke, R., Johnston, S. R., Anderson, E., Howell, A., A'Hern, R., Salter, J., Detre, S., Nicholson, R., Robertson, J., Smith, I. E., and Dowsett, M. (1997). Induction of apoptosis by tamoxifen and ICI 182780 in primary breast cancer. *Int J Cancer* **72**, 608-613.

Encarnacion, C. A., Ciocca, D. R., McGuire, W. L., Clark, G. M., Fuqua, S. A., and Osborne, C. K. (1993). Measurement of steroid hormone receptors in breast cancer patients on tamoxifen. *Breast Cancer Res Treat* **26**, 237-246.

Enomoto, T., Sasaki, Y., Shiba, Y., Kanno, Y., and Yamasaki, H. (1981). Tumor promoters cause a rapid and reversible inhibition of the formation and maintenance of electrical cell coupling in culture. *Proceedings of the National Academy of Sciences of the United States of America* **78**, 5628-5632.

Fisher, B., Redmond, C., Legault-Poisson, S., Dimitrov, N. V., Brown, A. M., Wickerham, D. L., Wolmark, N., Margolese, R. G., Bowman, D., Glass, A. G., and et al. (1990). Postoperative chemotherapy and tamoxifen compared with tamoxifen alone in the treatment of positive-node breast cancer patients aged 50 years and older with tumors responsive to tamoxifen: results from the National Surgical Adjuvant Breast and Bowel Project B-16. *J Clin Oncol* **8**, 1005-1018.

Fitzgerald, D. J., Mesnil, M., Oyamada, M., Tsuda, H., Ito, N., and Yamasaki, H. (1989). Changes in gap junction protein (connexin 32) gene expression during rat liver carcinogenesis. *J Cell Biochem* **41**, 97-102.

Gazzaniga, P., Gradilone, A., Giuliani, L., Gandini, O., Silvestri, I., Nofroni, I., Saccani, G., Frati, L., and Agliano, A. M. (2003). Expression and prognostic significance of LIVIN, SURVIVIN and other apoptosis-related genes in the progression of superficial bladder cancer. *Ann Oncol* **14**, 85-90.

Gelmann, E. P. (1996). Tamoxifen induction of apoptosis in estrogen receptor-negative cancers: new tricks for an old dog? *J Natl Cancer Inst* **88**, 224-226.

Goldhirsch A, G. R. (1989). In *Adjuvant therapy of primary breast cancer* (G. A. Senn H-J, Gelber RD, Osterwalder B, Ed.), pp. 153-162. Springer-Verlag.

Goodall, H., and Maro, B. (1986). Major loss of junctional coupling during mitosis in early mouse embryos. *J Cell Biol* **102**, 568-575.

group, T. I. B. C. S. (1997). Effectiveness of adjuvant chemotherapy in combination with tamoxifen for node-positive postmenopausal breast cancer patients. *Journal of Clinical Oncology* **15**, 1385-1394.

Gakhar, G, Ohira, T., Shi, A., Duy, H Hua, Nguyen, T.A. (2008) Antitumor effect of substituted quinolines in breast cancer cells. *Drug Development Research* **69**, 526-534.

Hull, D. F., 3rd, Clark, G. M., Osborne, C. K., Chamness, G. C., Knight, W. A., 3rd, and McGuire, W. L. (1983). Multiple estrogen receptor assays in human breast cancer. *Cancer Res* **43**, 413-416.

Jensen, R., and Glazer, P.M. (2004). Cell-interdependent cisplatin killing by Ku/DNA-dependent protein kinase signaling transduced through gap junctions. *Proc. Natl. Acad. Sci*. **101**, 6134-6139.

Kerr, J. F., Wyllie, A. H., and Currie, A. R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* **26**, 239-257.

Langan Fahey SM, J. V., Fritz NF, Robinson SP, Waters D, Tormey DC (1994). In *Long term tamoxifen treatment for breast cancer* (V. C. Jordan, Ed.), pp. 27-56. University of Wisconsin Press, Madison.

Li, F., Ambrosini, G., Chu, E. Y., Plescia, J., Tognin, S., Marchisio, P. C., and Altieri, D. C. (1998). Control of apoptosis and mitotic spindle checkpoint by survivin. *Nature* **396**, 580-584.

Mandlekar, S., and Kong, A. N. (2001). Mechanisms of tamoxifen-induced apoptosis. *Apoptosis* **6**, 469-477.

Mandlekar, S., Yu, R., Tan, T. H., and Kong, A. N. (2000). Activation of caspase-3 and c-Jun NH2-terminal kinase-1 signaling pathways in tamoxifen-induced apoptosis of human breast cancer cells. *Cancer Res* **60**, 5995-6000.

Margulis, V., Lotan, Y., and Shariat, S. F. (2008). Survivin: a promising biomarker for detection and prognosis of bladder cancer. *World J Urol* **26**, 59-65.

Martin, G., Melito, G., Rivera, E., Levin, E., Davio, C., Cricco, G., Andrade, N., Caro, R., and Bergoc, R. (1996). Effect of tamoxifen on intraperitoneal N-nitroso-N-methylurea induced tumors. *Cancer Lett* **100**, 227-234.

Mesnil, M., Fitzgerald, D. J., and Yamasaki, H. (1988). Phenobarbital specifically reduces gap junction protein mRNA level in rat liver. *Mol Carcinog* **1**, 79-81.

Mouridsen, H. T., Rose, C., Overgaard, M., Dombernowsky, P., Panduro, J., Thorpe, S., Rasmussen, B. B., Blichert-Toft, M., and Andersen, K. W. (1988). Adjuvant treatment of

postmenopausal patients with high risk primary breast cancer. Results from the Danish adjuvant trials DBCG 77 C and DBCG 82 C. *Acta Oncol* **27**, 699-705.

Murray, A. W., and Fitzgerald, D. J. (1979). Tumor promoters inhibit metabolic cooperation in cocultures of epidermal and 3T3 cells. *Biochem Biophys Res Commun* **91**, 395-401.

Neveu MJ, H. J., Paul DL and Pitot HC (1989). Regulation of the expression of proto-oncogenes and the gene of the 27-KD gap junction protein during multistage hepatocarcinogenesis in the rat. *Proceedings of American Association Cancer Research* **30**, 207.

Osborne, C., Elledge RM, Fuqua SAW (1996). Estrogen receptors in breast cancer therapy. *Science Medicine* **3**, 32-41.

Osborne, C. K. (1998). Tamoxifen in the treatment of breast cancer. *N Engl J Med* **339**, 1609-1618.

Otter, I., Conus, S., Ravn, U., Rager, M., Olivier, R., Monney, L., Fabbro, D., and Borner, C. (1998). The binding properties and biological activities of Bcl-2 and Bax in cells exposed to apoptotic stimuli. *J Biol Chem* **273**, 6110-6120.

Perry, R. R., Kang, Y., and Greaves, B. (1995). Effects of tamoxifen on growth and apoptosis of estrogen-dependent and -independent human breast cancer cells. *Ann Surg Oncol* **2**, 238-245.

PO Lague, H. D., H Rubin, C Tobias (1970). Electrical coupling: Low resistance Junctions between mitotic and interphase fibroblasts in tissue culture. *Science* **170**, 464-466.

Pritchard, K. I., Paterson, A. H., Fine, S., Paul, N. A., Zee, B., Shepherd, L. E., Abu-Zahra, H., Ragaz, J., Knowling, M., Levine, M. N., Verma, S., Perrault, D., Walde, P. L., Bramwell, V. H., Poljicak, M., Boyd, N., Warr, D., Norris, B. D., Bowman, D., Armitage, G. R., Weizel, H., and Buckman, R. A. (1997). Randomized trial of cyclophosphamide, methotrexate, and fluorouracil chemotherapy added to tamoxifen as adjuvant therapy in postmenopausal women with node-positive estrogen and/or progesterone receptor-positive breast cancer: a report of the National Cancer Institute of Canada Clinical Trials Group. Breast Cancer Site Group. *J Clin Oncol* **15**, 2302-2311.

Riggins, R. B., Lan, J. P., Zhu, Y., Klimach, U., Zwart, A., Cavalli, L. R., Haddad, B. R., Chen, L., Gong, T., Xuan, J., Ethier, S. P., and Clarke, R. (2008). ERRgamma mediates tamoxifen resistance in novel models of invasive lobular breast cancer. *Cancer Res* **68**, 8908-8917.

Riggins, R. B., Schrecengost, R. S., Guerrero, M. S., and Bouton, A. H. (2007). Pathways to tamoxifen resistance. *Cancer Lett* **256**, 1-24.

Rivkin, S. E., Green, S., Metch, B., Cruz, A. B., Abeloff, M. D., Jewell, W. R., Costanzi, J. J., Farrar, W. B., Minton, J. P., and Osborne, C. K. (1994). Adjuvant CMFVP versus tamoxifen versus concurrent CMFVP and tamoxifen for postmenopausal, node-positive, and estrogen receptor-positive breast cancer patients: a Southwest Oncology Group study. *J Clin Oncol* **12**, 2078-2085.

Stein, L. S., Boonstra, J., and Burghardt, R. C. (1992). Reduced cell-cell communication between mitotic and nonmitotic coupled cells. *Exp Cell Res* **198**, 1-7.

Sugie, S., Mori, H., and Takahashi, M. (1987). Effect of in vivo exposure to the liver tumor promoters phenobarbital or DDT on the gap junctions of rat hepatocytes: a quantitative freeze-fracture analysis. *Carcinogenesis* **8**, 45-51.

Trosko, J. E. a. C., C.C. (1983). Potential role of intercellular communication in the rate-limiting step in carcinogenesis. *Journal of American College of Toxicology* **2**, 5-22.

Vinken, M., Vanhaecke, T., Papeleu, P., Snykers, S., Henkens, T., and Rogiers, V. (2006). Connexins and their channels in cell growth and cell death. *Cell Signal* **18**, 592-600.

Yamamoto, H., Ngan, C. Y., and Monden, M. (2008). Cancer cells survive with survivin. *Cancer Sci* **99**, 1709-1714.

Yotti, L. P., Chang, C. C., and Trosko, J. E. (1979). Elimination of metabolic cooperation in Chinese hamster cells by a tumor promoter. *Science* **206**, 1089-1091.

Zhang A, W. Y., Lai HWL, Yew DT (2004). Apoptosis- A brief review. *Neuroembryology* **3**, 47-59.

Chapter 6 - Hydronephrosis and Urine retention in Estrogen-Implanted Athymic Nude Mice

6.1 Abstract

Subcutaneous estrogen pellet implantation is commonly practiced to induce tumor promotion in nude (Nu-FOXn1^{nu}) mice for xenograft tumor modeling. Twelve out of twenty-three, 18-week old nude mice, developed dysuria after estrogen pellet implantation. Physical examination revealed variably distended abdomen with palpable distended bladder. All mice were euthanized and necropsied. Blood samples of the mice were collected and serum estrogen concentration was measured. Necropsy revealed variable dilation of the urinary bladder and unilateral or bilateral dilations of ureters and kidneys. Microscopically, 12/23 mice and 13/23 mice showed dilated urinary bladder and hydronephrosis, respectively. In conclusion, hydronephrosis and urine retention were observed in estrogen-implanted nude mice.

6.2 Background

Estrogens have been associated with tumor development in many experimental animals and humans. Estrogen acts as a tumor promoter in rat liver carcinogenesis (Shirai *et al.*, 1987), spontaneous human endometrial and mammary carcinomas (Nenci *et al.*, 1988). Therefore, estrogen pellets are frequently used as a tumor promoter in animal models of human neoplasia. Besides being a tumor promoter, estrogen has been shown to increase urethral tone in mice (Game *et al.*, 2008).

Urogenital infections have been shown to flare up in elderly women. In post-menopausal women, estrogen therapy reduces vaginal atrophy (Rozenberg *et al.*, 2004), which normalizes vaginal flora thereby effective in reducing urinary tract infections (Robinson and Cardozo, 2003). On the contrary, hydronephrotic effect of high dose estrogen was observed in DDD (deutsche Maus at Denken) strain male mice (Mannen *et al.*, 1993). In another study conducted by Buhl *et al.*, estrogen-treated adult male CF1 (Carworth Farms 1) mice showed an increase in

urine volume. In both male and female gonadectomized DS (mutation disorganization) strain mice exposed to 17β-estradiol, significant urine retention and 100% hydronephrosis were observed (Kuroda *et al.*, 1985). An additional study found that 17β-estradiol treatment administered via subcutaneous pellets caused hydronephrosis and urine retention in female C57BL/6J mice (Levin-Allerhand *et al.*, 2003). The results observed in lab animals seem to be contradictory to the studies conducted in humans. It could be due to different dose regimen of estrogen in lab animals and also post-menopausal women are deprived of estrogen for a very long time which could trigger many pathways.

Estrogen implantation is commonly practiced to promote tumor growth in nude (Nu-FOXn1^{nu}) mice. After 1 week of estrogen implantation, $1x10^7$ T47D human breast cancer cells were injected at the right inguinal region. Multiple mice developed dermatitis and/or bloated abdomens after four weeks of estrogen implantation. The tumor growth study was terminated and mice received supportive care prior to euthanasia.

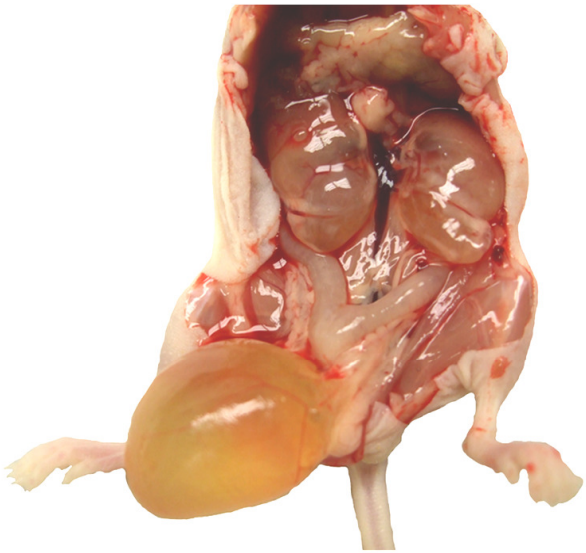
This case report describes similar clinical and pathologic conditions in estrogen-implanted nude mice as observed in the above mice studies; however, it reports for the first time that estrogen implantation is linked to urine retention and hydronephrosis in nude mice.

6.3 History, Gross findings and laboratory results

Twenty-five 4 week old female Nu-FOXn1^{nu} were obtained from Charles River Laboratories (Wilmington, MA, USA). A 1.7 mg 17β-estradiol pellet (Innovative Research of America, Sarasota, Florida), was subcutaneously implanted with a trochar in the subscapular region at 43 days of age. No inflammation/redness around the implantation area was observed after 2-3 days. Three weeks after estrogen implantation, pruritus and ulcerative dermatitis were observed in the perineum in some mice. The lesions then spread to the hindlegs, over the dorsum of the tail head, and in one mouse to the forelegs. After 4 weeks of estrogen implantation, mice looked bloated but otherwise healthy. On physical examination, in living mice, the urinary bladder appeared distended and was easily palpable.

The mice were euthanized by CO_2 asphyxiation and necropsy was performed. Necropsy revealed a variably enlarged urinary bladder filled with clear yellow urine, mild to moderate hydroureter and unilateral or bilateral hydronephrosis (Figure 6.1). The majority of the mice had

variable dilation of the uterus. After euthanasia, blood was collected via cardiocentesis from 14 mice and tested for serum estrogen level (A.3, Figure S13). In the rest of the mice, blood was not collected. 2/14 mice had no estrogen pellet, and were found to be healthy (Figure 6.2) having normal (3-9 pg/ml) serum estrogen level. 6/14 mice had increased estrogen level (36-260 pg/ml).



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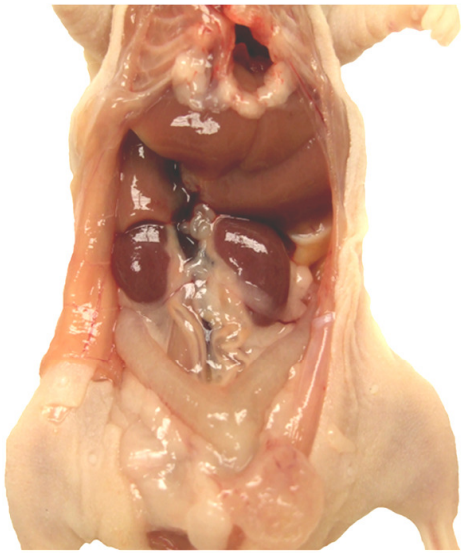

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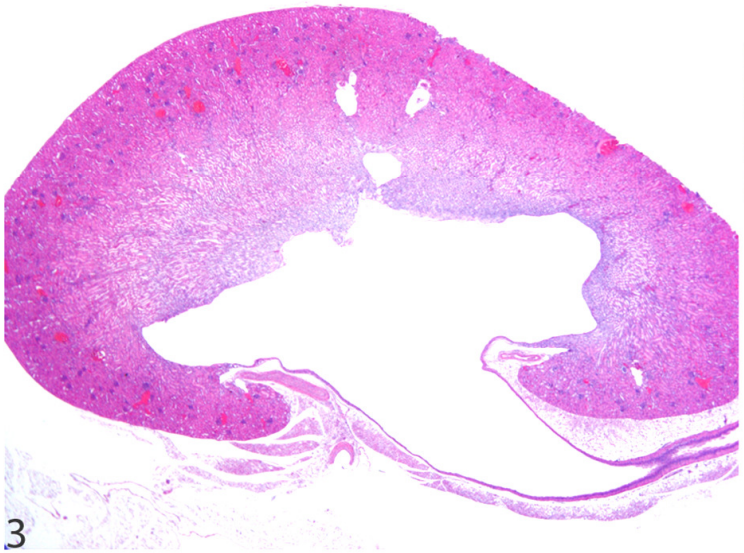

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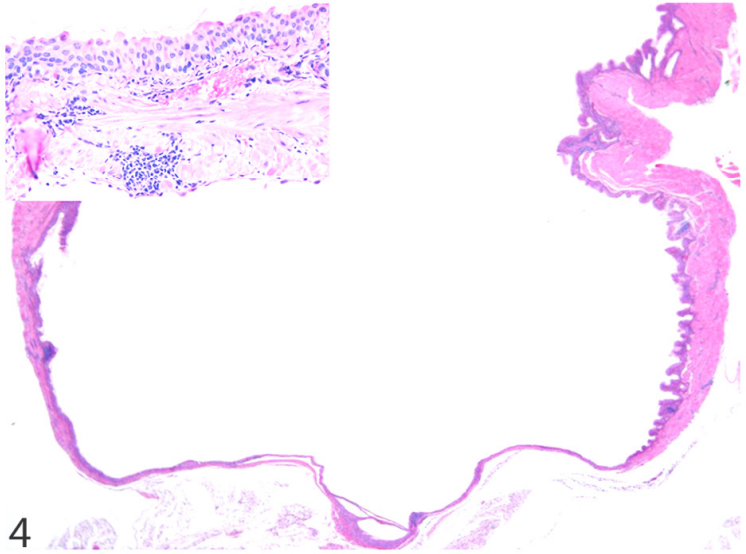

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Figure 6.1 Urogenital tract of an affected nude mouse.

Bilateral hydronephrosis and enlarged bladder filled with urine in a nude mouse.

Figure 6.2 Urogenital tract of an unaffected nude mouse.

Grossly, kidneys and bladder look normal.

Figure 6.3 HE stain of kidney of an affected nude mouse.

There is a dilation of the renal pelvis. The ureter is moderately dilated. Note: flattened renal papillae.

Figure 6.4 HE stain of the urinary bladder.

The urinary bladder is dilated with flattened mucosal epithelium and muscular layers. The lumen is free of inflammatory cells or hemorrhage. The inset reveals multifocal infiltrates with low to moderate numbers of plasma cells within the muscular layer.

6.4 Differential Diagnosis

Differential diagnosis for hydronephrosis in animals includes anything that obstructs the urinary tract at the level of the urethra, urinary bladder or ureters. Urinary calculi and severe cystitis are two of the common causes of urinary tract obstruction in domestic animals. Masses in the caudal abdomen or pelvis such as abscesses may cause external pressure to the urinary tract and cause a functional obstruction. In addition, displacement of the bladder through perineal hernias and acquired urethral or ureteral strictures can block the flow of urine.

Perineal ulcerative dermatitis preceded the clinical signs, therefore, an acquired stricture due to scarring around the perineum as sequelae to dermatitis or a blockage due to cystitis could not be ruled out with initial clinical presentation. There was no evidence of blockage due to scarring, cystitis, urinary calculi or pressure from adjacent masses in any of the mice. Therefore, the most likely cause for the clinical findings was urine retention associated with estrogen administration.

6.5 Microscopic Findings

Microscopically, the distended urinary bladders had flattened epithelium and decreased thickness of the muscular layer (Figure 6.4). All mice (12) that had distended abdomens had microscopically variable degrees of dilation of the urinary bladder, unilateral or bilateral dilation of the kidneys, and mild to moderate hydroureters. Six of the histologically affected mice had multifocal infiltrates with low to moderate numbers of plasma cells within the tunica muscularis (Figure 6.4, inset). There was no evidence of hemorrhage or inflammatory cells within the bladder lumen in any of the mice examined. The mice with ulcerative dermatitis of the perineum had no histologic evidence of bacterial infection, however lesions were not cultured.

Microscopically, 10/23 mice having gross lesions of kidneys showed hydronephrosis. In addition, three mice with no gross lesions had similar histologic lesions. Five histologically affected mice that had hydronephrosis also had unilateral mild to marked dilation of the renal pelvis with flattening of the renal papillae (Figure 6.3). The remaining (eight) mice had mild to marked bilateral renal pelvis dilation. The proximal ureters were moderately to markedly dilated in all mice that had histologic evidence of hydronephrosis.

Two mice had unilateral suppurative nephritis. One of these had multifocal infiltration of large numbers of neutrophils within the tubules. The other mouse had multifocal randomly distributed infiltrates of neutrophils within the cortex and medulla with intralesional colonies of Gram-positive cocci, which was confirmed by Gram's stain.

6.6 Discussion

The current report demonstrates that the subcutaneous implantation of 1.7 mg 17β-estradiol pellet resulted in severe urine retention in the bladder with uni- or bi-lateral hydronephrosis in Nu-FOXn1^{nu}. Hydronephrosis is distention of the kidney with urine, caused by the increased pressure in the renal pelvis when the flow of urine is obstructed. It can be due to any type of urinary obstruction that occurs at any level of the urinary tract, from the urethra to the renal pelvis. This obstruction may be complete or partial and can be acquired, inherited or congenital. Certain inbred strains of mice, such as the C3H, C57L, and DDD mice, have been described to have an higher incidence of hydronephrosis, which are presumed to be inherited (HH, 1988). An autosomal recessive, progressive hydronephrosis has also been described in C57BL/6J mice (Horton *et al.*, 1988). Also, various transgenic mouse strains develop hydronephrosis due to their genetic alterations. In our study, we could not determine the cause of urine retention. However, bacterial infection was not evident in all histological examinations of kidney and bladder except in one kidney of a mouse which had intralesional colonies of Gram-positive cocci. Struvite uroliathiasis in estrogen-treated ovariectomized female nude mice with Staphylococcus intermedius-induced cystitis was observed (Gibbs *et al.*, 2007). However, in our study we did not observe any significant bacterial infection which could have been associated with urine retention. Ascending infections of the urinary tract was documented in female nude mice housed on nonautoclaved corncob bedding and implanted with 0.36 mg estrogen pellets for 9 wk (Simpson JE, 2002). In the present study, there was no effect on changing the corn cob bedding to autoclaved paper bedding. The initial study was to generate tumor growth in Nu-FOXn1^{nu}; however, due to the adverse side effects of estrogen implantation, the study had to be terminated for the physical well being of the mice.

In all, twenty-three mice were assessed grossly or microscopically for urine retention and hydronephrosis. Two mice were not assessed due to untimely death. In 14/23 mice, blood was

collected and serum estrogen level was measured. Normal serum estrogen level is 2-5 pg/ml in mice. 2/14 mice had no estrogen pellet, and were found to have normal serum estrogen level (3-9 pg/ml) and healthy until euthanasia with no gross or microscopic lesions in the kidney or bladder (Figure 6.2). This suggests that estrogen implantation might have caused the urogenital effects observed in estrogen-implanted nude mice. 6/14 mice had increased estrogen level (36-260 pg/ml) and increased bladder volume either grossly or microscopically (Figure 6.5) and 4 mice also had hydronephrosis.

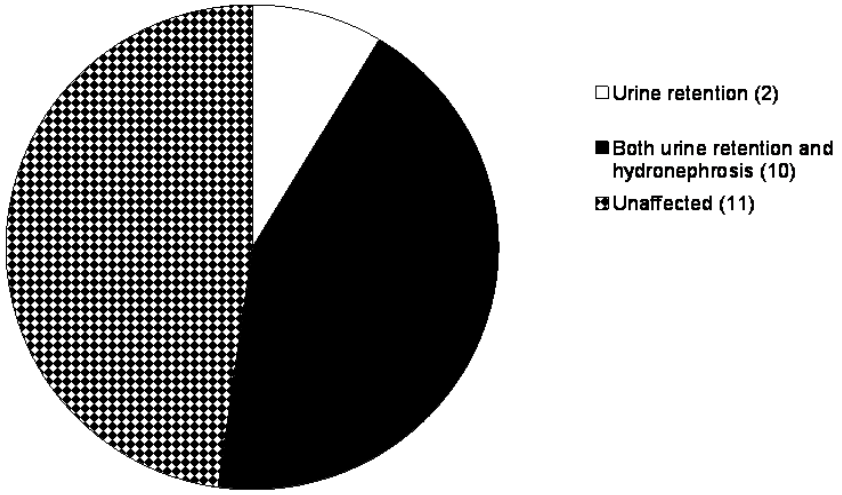

Figure 6.5 Pie chart showing hydronephrosis, urine retention in mice in which estrogen level was measured.

Urine retention was seen in 6 mice, out of which 4 also exhibited hydronephrosis. Six mice were unaffected but high serum estrogen level was observed. Two control animals with normal estrogen level were also found unaffected. Blood serum from 14 mice was sent for the estrogen level measurement. The parenthesis depicts the number of mice in each group followed by the serum estrogen levels. The unit for the serum estrogen levels is pg/ml. Total mice in the group = 14.

Out of these 6 mice, only one had 36 pg/ml; whereas, others had 123-260 pg/ml serum estrogen levels. None of the mice with elevated estrogen levels developed hydronephrosis alone without urine retention in the bladder. This suggests that urine retention preceded

hydronephrosis. 6/14 mice were unaffected with high estrogen level ranging from 43-220 pg/ml, however, only one animal had 220 pg/ml whereas others had 43-102 pg/ml serum estrogen levels. A table showing the estrogen levels in each mouse is provided in Appendix A.3 (Table S12). It should be noted that the estrogen pellet was a 60-day release pellet. According to the manufacturer, estrogen levels should not be high after 60 days. But the estrogen levels were found high in the serum even after 105 days of the estrogen implantation. Out of total 23 mice, 12 were grossly found to have increased urine retention and 10 were grossly found to have either uni- or bi-lateral hydronephrosis (Figure 6.6). Microscopically, 12/23 and 13/23 mice showed dilated bladder and hydronephrosis, respectively. In some mice, hydronephrosis was observed on microscopic examination.

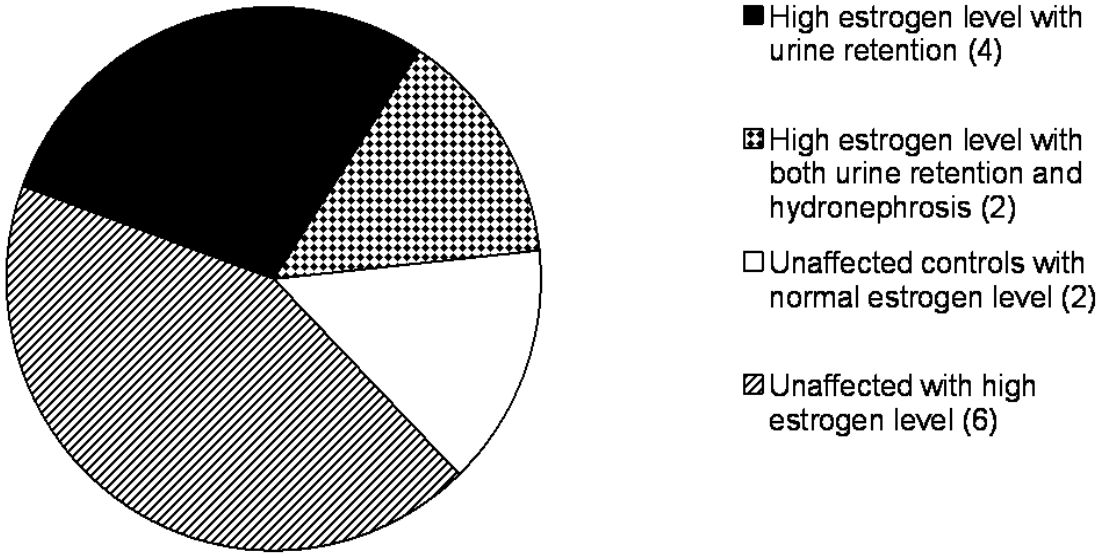

Figure 6.6 Pie chart showing hydronephrosis and urine retention in all 23 mice.
The pie chart is based on the gross examination. Urine retention was observed in 12 mice, out of which 10 mice also showed hydronephrosis. Eleven mice were unaffected and were healthy. The parenthesis depict the number of mice in each group. Total mice in the group = 23.

In conclusion, estrogen implantation can cause urine retention with uni- or bi-lateral hydronephrosis in nude mice. Further studies need to be done to fully understand the relationship

of estrogen implantation to urine retention. An effective safer dose regimen of estrogen needs to be established in nude mice.

Acknowledgements

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6.7 References

Buhl AE, Yuan YD, Cornette JC, Frielink RD, Knight KA, Ruppel PL, and Kimball FA (1985). Steroid-induced urogenital tract changes and urine retention in laboratory rodents. *J Urol.* **134**, 1262-1267.

Game X, Allard J, Escourrou G, Gourdy P, Tack I, Rischmann P, Arnal JF, and Malavaud B (2008). Estradiol increases urethral tone through the local inhibition of neuronal nitric oxide synthase expression. *Am J Physiol Regul Integr Comp Physiol* **294**, 851-857.

Gibbs LK, Hickman DL, Lewis AD, and Colgin LMA (2007). Staphylococcus-induced uroliathiasis in estrogen-treated ovariectomized nude mice. *J Am Assoc Lab Anim Sci* **46**, 61-65.

Horton CE Jr, Davisson MT, Jacobs JB, Bernstein GT, Retik AB, Mandell J (1988). Congenital progressive hydronephrosis in mice: a new recessive mutation. *J Urol*. **140**, 1310-5.

HSU HH (1986). Hereditary hydronephrosis, mouse. In: Urinary System, eds. Jones TC, Mohr U and Hunt RD, pp. 273-275. Springer-Verlag, Berlin.

Kanoh H, Hosono T, Matsui Y, and Okudaira Y (1989). Growth of endometrial cancer hormone. *Gan to Kagaku Ryaho* **16**, 3696-3703.

Kuroda H, Kohrogi T, Uchida N, Imai I, Terada N, Matsumoto K, and Kitamura Y (1985). Urinary retention induced by estrogen injections in mice: an analytical model. *J Urol.* **134**, 1268-1270.

Levin-Allerhand JA, Sokol K, and Smith JD (2003). Safe and effective method for chronic 17 beta-estradiol administration to mice. *Contemp topics in lab anim sci / Am Ass Lab Anim Sci* **42**, 33-35.

Mannen H, Tsuji S, and Goto N (1993). Influence of chronic oestrogen treatment on severity of hydronephrosis in inbred DDD mice. *Lab Anim*. **27**, 124-130.

Nenci I, Marchetti E, and Querzoli P (1988). Commentary on human mammary preneoplasia. The estrogen receptor promotion-hypothesis. *J Steroid Biochem.* **30**, 105-106.

Robinson D, and Cardozo LD (2003). The role of estrogens in female lower urinary tract dysfunction. *Urology* **62** (4), 45-51.

Rozenberg S, Pastijn A, Gevers R, and Murillo D (2004). Estrogen therapy in older patients with recurrent urinary tract infections: a review. *Int J Fertil Womens Med* **49** (2), 71-74.

Shirai T, Tsuda H, Ogiso T, Hirose M, and Ito N (1987). Organ specific modifying potential of ethinyl estradiol on carcinogenesis initiated with different carcinogens. *Carcinogenesis* **8**, 115-119.

Simpson JE, Morrow JE, and Franklin CL (2002). Effect of bedding sterilization and type on the incidence of urogenital disease in the estrogenized nude mouse. *Contemp Topics* **41**, 72 (abstract).

Chapter 7 - General Discussion

In all normal cells, DNA repair mechanisms to some extent can repair the DNA damage caused by carcinogens. If the damage is substantial, it overpowers the cell's repair mechanism capability leading to tumor growth. Therefore, an initiator along with one or multiple promoters facilitates the tumor progression. Promoters, for example, phorbol esters have been shown to exert many cellular effects including cell membrane changes, alteration of growth factor responses and even mutations. Interestingly, an initiated cell despite having mutations in growth regulatory genes still require promoters to cause tumor formation indicating that cellular controls are vital in the body. Therefore, during the cascade of phenotypic changes occurring at the promoter stage, alteration in cellular controls might be rendering an initiated cell towards forming a tumor. Cell-cell (intercellular) and cell-matrix interactions are the key players in achieving cellular control. Considerable evidence suggests that the inhibition of cell-cell communication is one of the critical actions performed by promoters. The inhibition or blockage of transmission between the cells could allow proliferation and emergence of neoplastic cells.

A positive correlation has been found between the restoration of cell-cell communication and decrease in cell proliferation and growth. This suggests that intercellular communication could be transmitting regulatory molecules from one cell to another. Despite having considerable evidence that cell-cell communication affects cell growth, specific growth regulatory molecules passing through gap junction channels have not been established yet. However, small molecules such as ATP, AMP, ADP, Ca^{2+}, inositol triphosphate, and glucose have been shown to pass through gap junctions. The scientific evidence showing decrease in gap junctions and cell-cell communication in tumor cells and tissues led to restoration studies of gap junctions *in vitro* and *in vivo*. So far, studies include transfection of connexin genes in many tumor cell lines and rodent studies. These approaches are informative; however, not feasible to apply in a clinical setting. Currently very few compounds have shown to increase gap junctional activity such as retinoids, lycopene and carotenoids. Thus, there is a great need of gap junctional activators which can open up the gap junction channels and increase cell-cell communication. In my study, I found PQ1, a novel gap junctional activator which also decreases tumor growth.

In the current study, I first examined the gap junctional intercellular communication (GJIC) in breast cancer. Breast cancer is the second leading cause of cancer deaths in North American women. The risk of developing breast cancer in a lifetime of women is one in seven. Breast cancer is caused by many factors: some of them are like hereditary, age, diet, lifestyle, and environment. Hereditary constitutes only about 5-10% of the total breast cancer cases. Therefore, other factors play a critical role in the formation of breast cancer. The effect of 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) on the GJIC in breast cancer cells and normal mammary epithelial cells (HMEC) was observed. HMEC exhibit extensive GJIC and the most predominant Cx present in HMEC is Cx43. A significant amount of Cx43 in HMEC compared to different human breast cancer cells- MCF-7, T47D, ZR-75, MDA-MB-231 and MDA-MB-453 cells was found. Phosphorylation of Cx proteins have been shown to decrease the GJIC. Interestingly, I found an increase in the phosphorylated form of Cx43 in MCF-7 cells while only one unphosphorylated form of Cx43 was seen in the whole cell extract of HMEC.

MCF-7 cells were chosen to conduct the experiments with TCDD because MCF-7 is a well-established human breast cancer cell line used as a representative of breast cancer. GJIC can be measured by using scrape load/dye transfer (SL/DT) assay. Lucifer yellow is a dye which passes through gap junctions; therefore, more the transfer of the green dye from the line of cut, the more is the GJIC. HMEC showed a significant increase in the dye transfer compared to MCF-7 cells. In the presence of 10 and 100 nM TCDD, lucifer yellow dye transfer in MCF-7 cells was decreased while 200 nM caused a tremendous decrease in dye transfer in HMEC. Confocal microscopy showed a decrease in Cx43 plaque formation in MCF-7 cells in the presence of TCDD. Interestingly, confocal microscopy showed a redistribution of Cx43 plaques in HMEC from the membrane to the perinuclear region in the presence of TCDD, also confirmed by protein measurement of nuclear and cytoplasmic extract of Cx43. In the presence of TCDD, MCF-7 cells showed an increase in the phosphorylated form of Cx43. Many kinases are involved in the phosphorylation of Cx43 proteins. Expression of different classical forms of PKC- α, β, and, γ was observed but no effect of TCDD on PKC β and γ was observed. However, an increase and activation in PKC α in the presence of TCDD in MCF-7 cells was seen. Further, immunoprecipitation studies showed a strong interaction between phosphorylated form of Cx43 and PKC α. Calphostin C, an inhibitor of PKC showed to ameliorate the TCDD caused effects on GJIC in a dose-dependent manner.

Restoration of gap junctions has been shown to decrease tumor growth both *in vitro* and *in vivo*. In the present study, we collaborated with Dr. Duy H. Hua, a distinguished chemist at Kansas State University. Dr. Hua's lab synthesized a new class of substituted quinolines (PQs). A computational docking study showed that PQs has a relatively high binding to gap junction protein, Cx43. Thus, PQs were used in this study to find their effect on human breast cancer cells. Screening of PQ1-PQ5 was performed at different doses to observe their effect on GJIC by flow cytometry in different human breast cancer cell lines. The data suggested that compound PQ1 (200 and 500 nM) is a strong candidate to increase GJIC in T47D cells. Furthermore, SL/DT and colony growth assay were performed to measure GJIC and determine the effect of the PQ1 on the colony formation in HMEC and breast cancer cells, respectively. The results showed that PQ1 have a significant antitumor effect in human breast cancer cells compared to control without treatment or HMEC. 200 nM PQ1 showed a 30% increase in the GJIC in T47D cells; however, there was no effect of PQ1 treatment on GJIC in HMEC. In addition to an increase in GJIC, 80-95% growth attenuation was observed by PQ1 in colony growth assay. Since an increase in GJIC has been shown to increase cell death, I investigated the effect of PQ1 on active caspase 3. Indeed, a significant increase in caspase 3 in the presence of PQ1 was seen. The *in vitro* effect was simulated *in vivo* in nude mice. The PQ1-treated animals showed a significant decrease in xenograft tumor growth of T47D cells in nu/nu mice compared to control or tamoxifen-treated animals. The results showed that PQ1 compound have a promising role in exerting antitumor activity in human breast cancer cells.

Combinational effect of PQ1 and tamoxifen was tested in T47D human breast cancer cell line. The outcome of my previous work led to a hypothesis that PQ1, a novel gap junctional activator, might reduce the side effects of tamoxifen by potentiating apoptotic signal and increasing cell communication in breast cancer cells.

A significant increase in BAX expression, caspase 3 activation and DNA fragmentation in combinational treatment of tamoxifen and PQ1 as compared to their individual treatments was observed. Tamoxifen alone and combination with PQ1 showed a decrease in the survivin expression while PQ1 alone shows to be independent of survivin-mediated pathway. The combinational treatment of tamoxifen and PQ1 showed a significant decrease in cell viability compared to tamoxifen treatment alone.

Besides the three objectives, an interesting observation was made while conducting experiments on nude mice. Nude mice were implanted with 17β-estradiol in the subscapular region. After a month of implantation, bloating in some of the animals was seen. At euthanasia, in one of the mice we detected hydronephrosis and urine retention. Further investigation was conducted to establish the source of the enlarged kidneys and bladder by measuring plasma estrogen levels in the nude mice. I reported that 17β-estradiol might be the initiating factor for hydronephrosis and urine retention in nude mice. This was the first study to relate estrogen with hydronephrosis and urine retention in nude mice.

Overall, I have established that an environmental pollutant, TCDD decreases gap junctional intercellular communication (GJIC) in breast cancer cells and also affects GJIC in normal mammary epithelial cells. Further, I found a gap junctional activator, PQ1 and tested its efficacy in breast cancer cells and nude mice. The results obtained in PQ1 study supported the hypothesis that restoration of gap junctions decreases tumor cell growth. In addition, I found an increase in active caspase 3 suggesting the induction of apoptosis. Currently, tamoxifen is a drug of choice for endocrine-responsive breast cancer patients. Patients over time have been shown to develop resistance to tamoxifen. Also, tamoxifen has many side effects, including the risk of endometrial cancer. Tamoxifen is an antiestrogen shown to inhibit estrogen-mediated upregulation of various gene products and induces apoptosis. Therefore, if we combine tamoxifen and PQ1, does it increase the cell death leading to increased apoptosis? The present study demonstrates for the first time that combinational treatment of tamoxifen and PQ1 (gap junctional activator) can be used to potentiate apoptosis of T47D human breast cancer cells. This could either lead to a decrease in the dose or decrease the length of time patients are given tamoxifen, thereby, might reduce the side effects caused by tamoxifen.

Therefore, my dissertation has provided information that GJIC plays an important role in breast carcinogenesis.

Appendix A - Supplemental data

A.1. Supplemental Data for Chapter 3

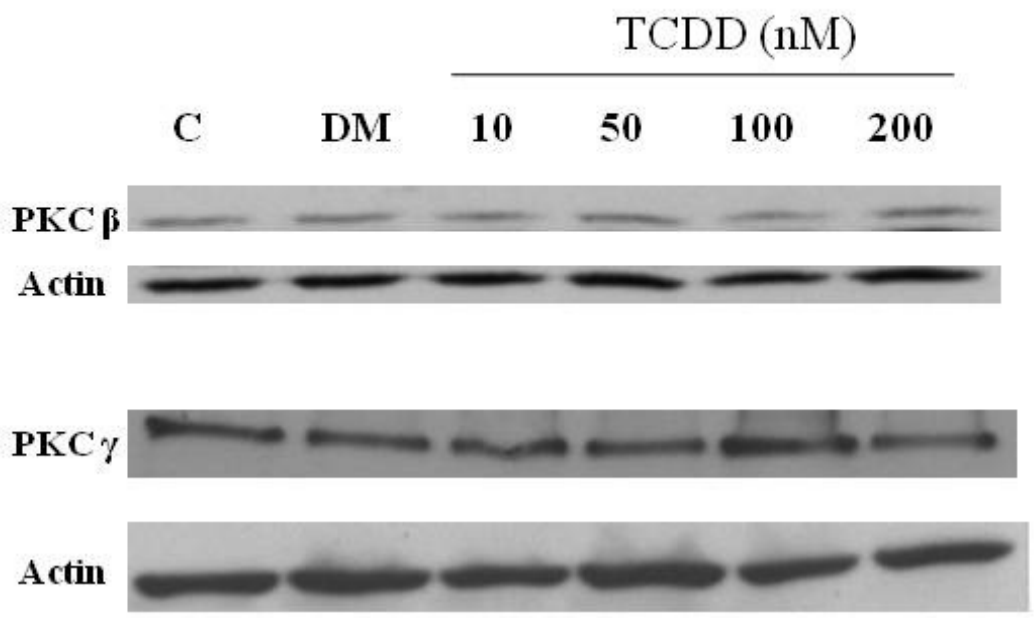


Figure S 1 Effect of TCDD on PKC isoforms.

The figure shows no significant effect of TCDD on PKC β and γ in MCF-7 cells.

A.2. Supplemental data for Chapter 4

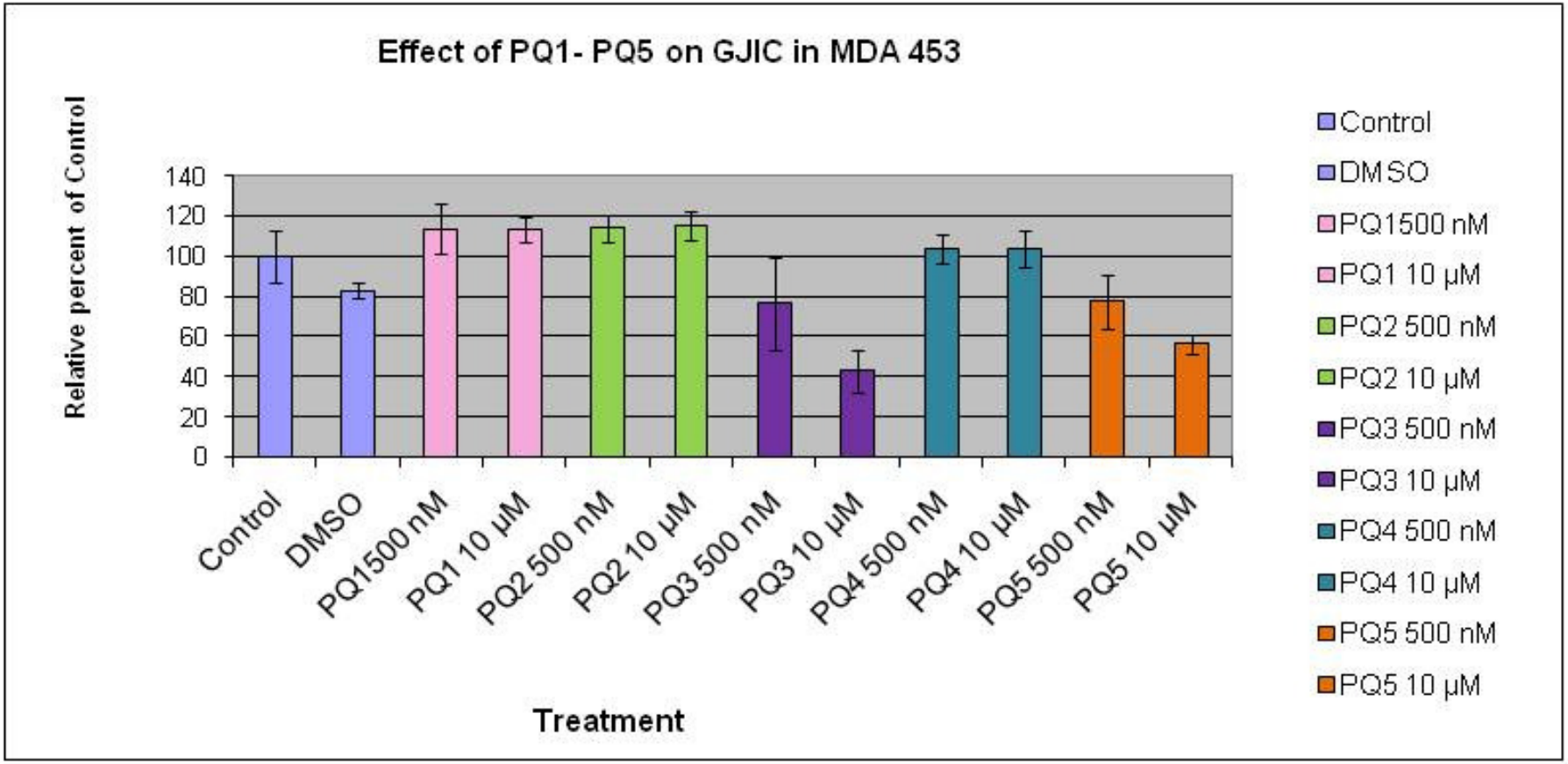


Figure S 2 Effect of PQ1-PQ5 on GJIC in MDA-453 human breast cancer cells.

Materials and Methods for parachute assay: Seed the cells in a six-well plate on the first day. Simultaneously, seed another population of same type of cells in a 25 cm^2 flask. Allow the cells in the six-well plate to reach 85% confluency. Add 4 µl of vibrant-DiD stain in 1 ml DMEM F-12 media in a conical tube. Add the prepared media into each well for 20 min. After 20 min, aspirate out the media and rinse the cells 3X in DMEM F-12 media. Incubate the cells in the media overnight at 37℃. Next day, add 2 µl of calcein-AM (acetoxy-methyl ester) in 1 ml of DMEM-F12 in 25 cm^2 flasks containing the same type of cells as in the six-well plates. Incubate for 30 min at 37℃. Rinse the flasks 3X with PBS. After rinsing, trypsinize the cells and centrifuge at 2,000 rpm for 5 min. Resuspend the cells in DMEM F-12 and add on the top of cells labeled with vibrant DiD (recipient cells) in six-well plate. Dose the cells with appropriate PQ compound and allow the cells labeled with calcein-AM (donor cells) to adhere onto the recipient cells. After 2 hr, trypsinize the cells and after centrifugation rinse the cells in PBS. Aspirate the media and resuspend the cells in PBS at the last wash. Prepare control cells: unstained, calcein-labeled, vybrant DiD-labeled cells and both calcein-AM and vybrant DiD labeled cells. Measure the cells by flow cytometry. Calcein-AM is measured at 488 nm and vibrant DiD at 568 nm.

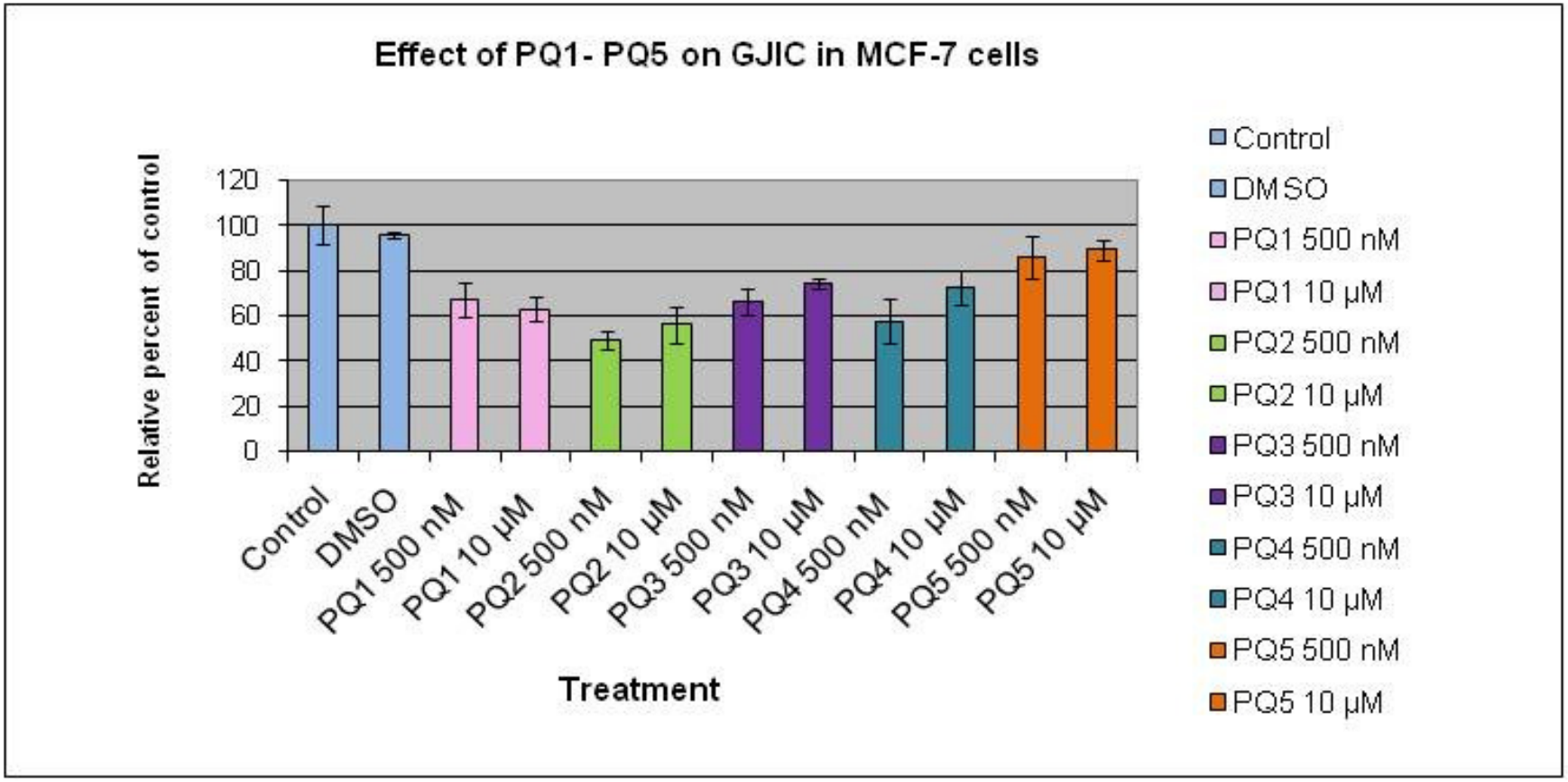


Figure S 3 Effect of PQ1-PQ5 on GJIC in MCF-7 cells.

Parachute assay was performed as described in Figure S2. We did not see any increase in GJIC in MCF-7 cells with compounds PQ1-PQ5.

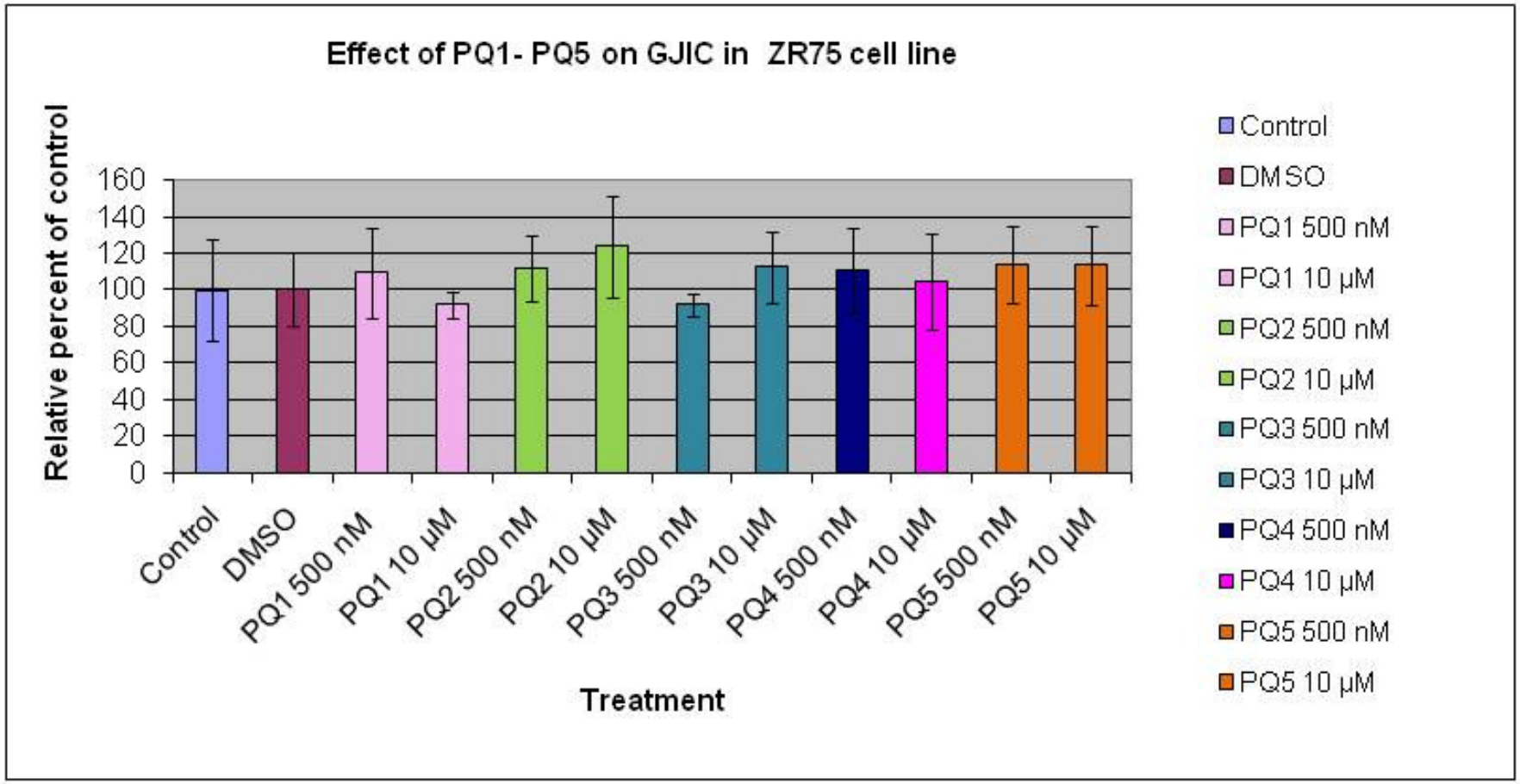


Figure S 4 Effect of PQ1-PQ on GJIC in ZR75 cells.

Parachute assay was performed as described in Figure S2. There was no significant increase found in GJIC in ZR75 cells in the presence of PQ1-PQ5.

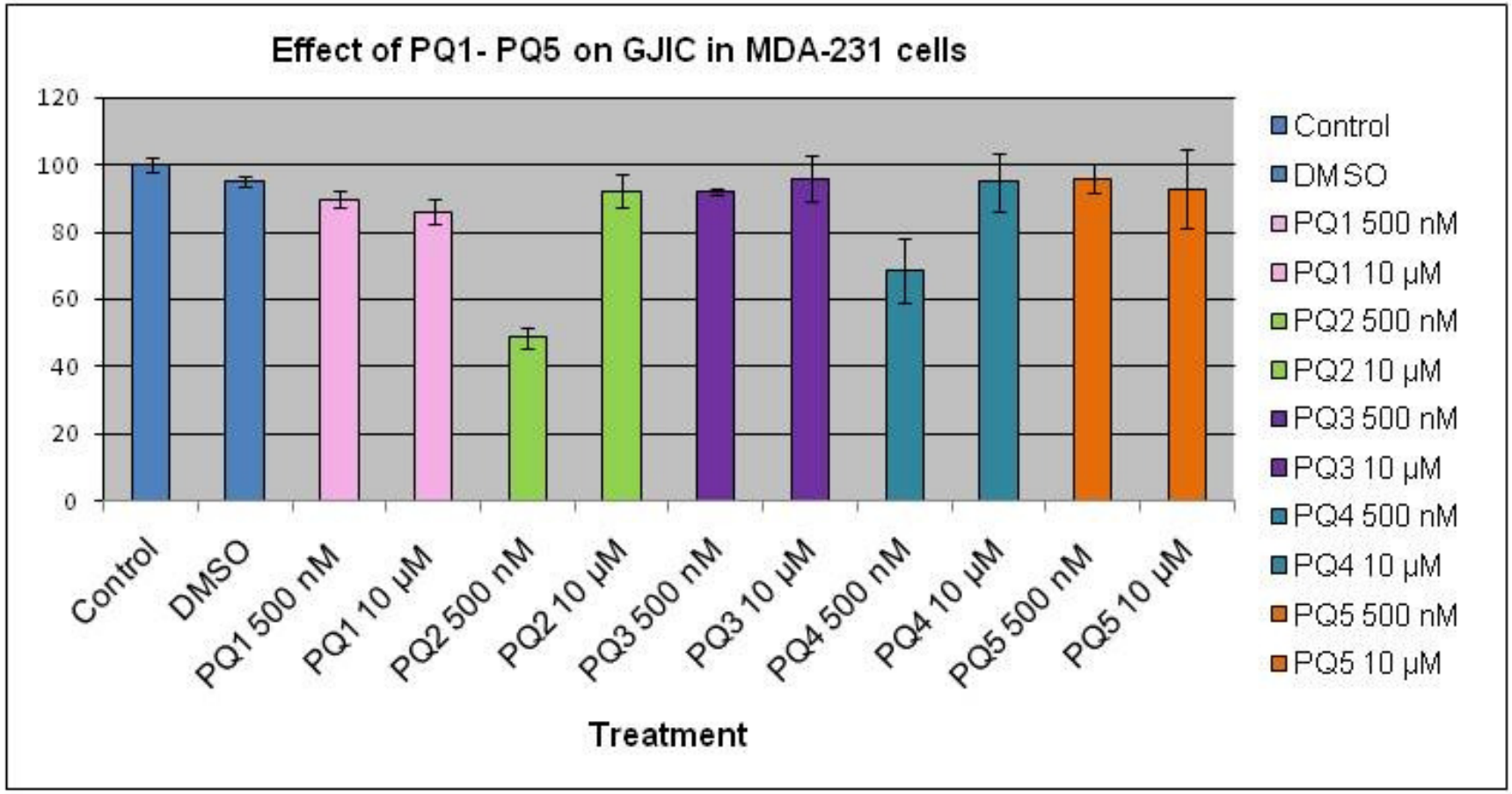
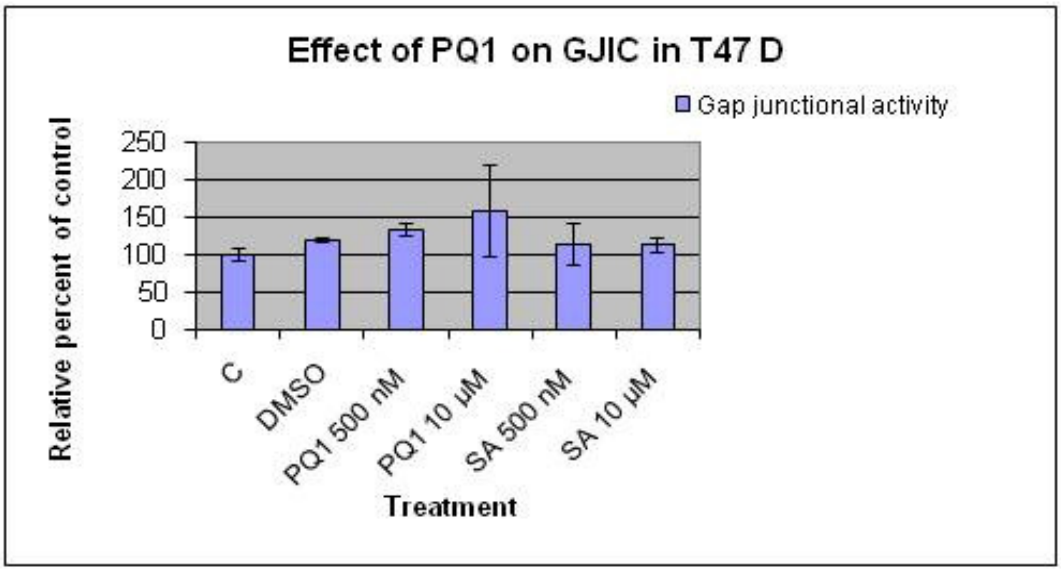


Figure S 5 Effect of PQ1-PQ5 on GJIC in MDA 231 cells.

Parachute assay was performed as described in Figure S2. The graph shows no significant increase in GJIC in MDA 231 cells.

A.



B.

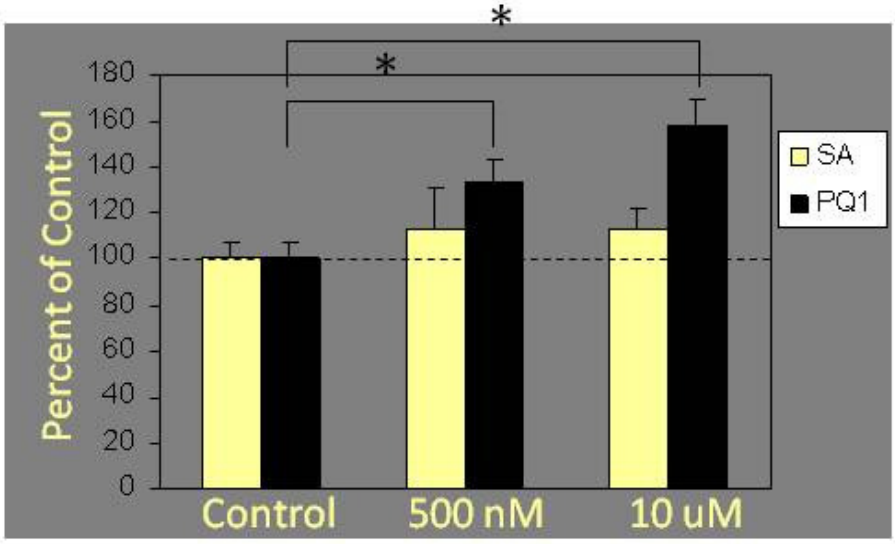


Figure S 6 Effect of PQ1 on GJIC in T47D cells.

A) T47D cells were incubated with PQ1 and gap junctional activity was measured by flow cytometry. Succinic acid (SA) was used as a control. We found a 35-40% increase in GJIC by parachute assay. **B)** Histogram showing a significant increase in GJIC by PQ1 in T47D cells by parachute assay.

PQ compounds	MDA-MB 453	MCF-7	ZR 75	MDA-MB 231	T47D
PQ1					
500 nM	Slight ↑	↓*	↓ (20 %)	no effect	Slight ↑
10 µM	Slight ↑	↓*	no effect	no effect	Slight ↑
PQ2					
500 nM	Slight ↑	↓*	no effect	↓*	no effect
10 µM	no effect	↓*	no effect	no effect	no effect
PQ3					
500 nM	↓	↓*	no effect	no effect	no effect
10 µM	↓*	↓*	no effect	no effect	no effect
PQ4					
500 nM	no effect	↓*	no effect	↓*	no effect
10 µM	no effect	↓	no effect	no effect	no effect
PQ5					
500 nM	↓	↓	no effect	no effect	no effect
10 µM	↓*	↓	no effect	no effect	no effect

Figure S 7 Chart showing effect of PQ1-PQ5 in different human breast cancer cell lines.

A. B.

Figure S 8 Xenograft tumor formation in nude mice.

The picture depicts the formation of xenograft T47D tumors in the inguinal region of nude mice. **A)** Shows the tumor formation in a control nude mouse **B)** Shows the tumor formation in a PQ-treated nude mouse.

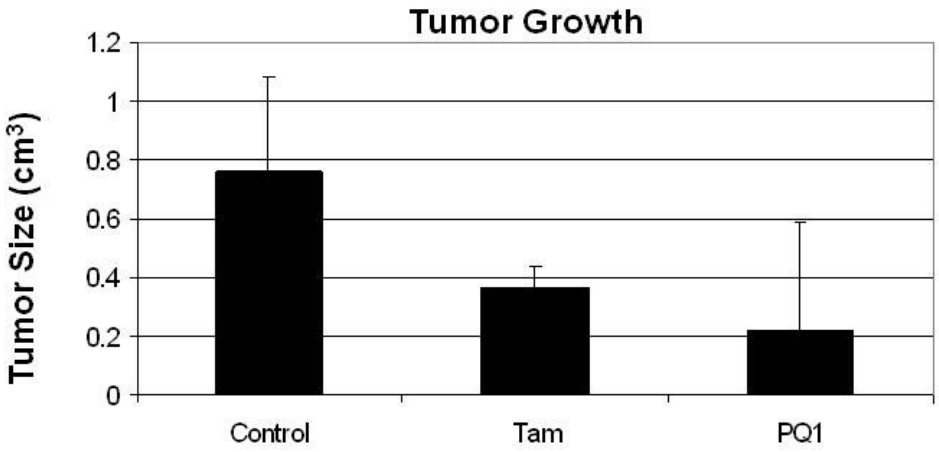
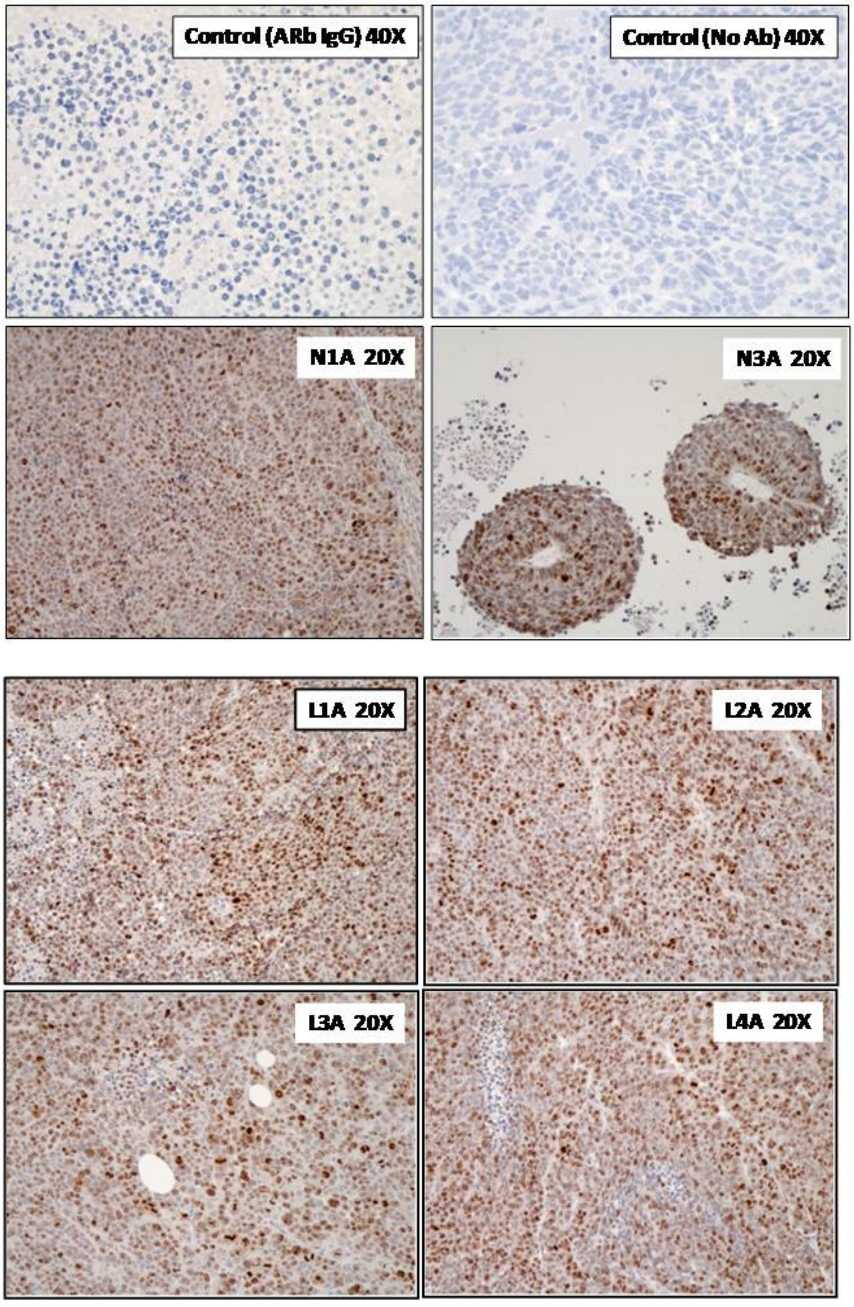


Figure S 9 Xenograft Tumor Growth of T47D Cells in Nu/Nu Mice.

Mice were implanted with 17 β-estradiol pellets (1.7 mg/pellet) subcutaneously into the inguinal region before the injection of 1 x 10^7 T47D cells. Animals received treatment at 1 µM PQ1 or 10 µM tamoxifen. The results after 6 days of injection show a decrease in tumor growth of PQ1-treated animals compared to control or tamoxifen.


Control (ARb IgG) 40X
Control (No Ab) 40X
N1A 20X
N3A 20X
L1A 20X
L2A 20X
L3A 20X
L4A 20X

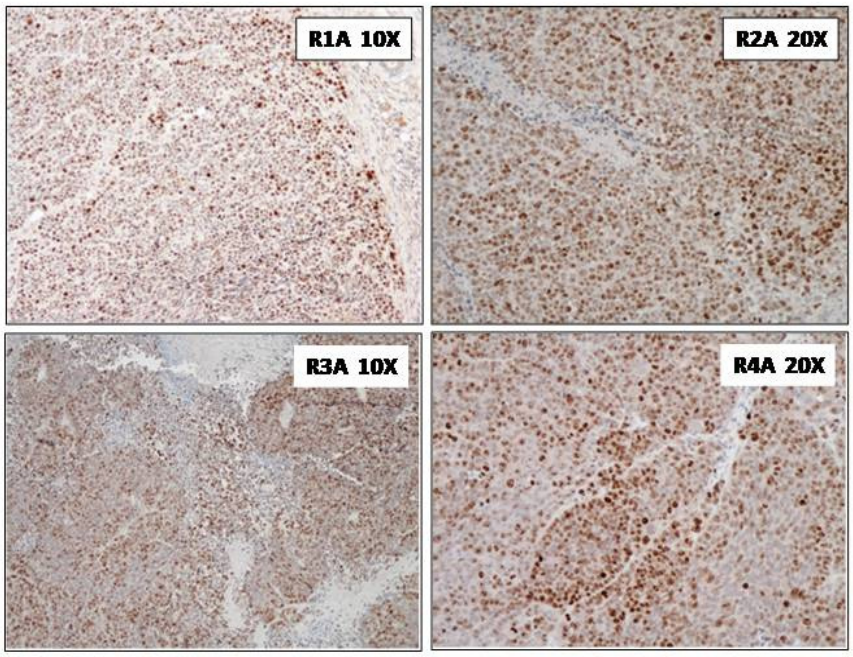
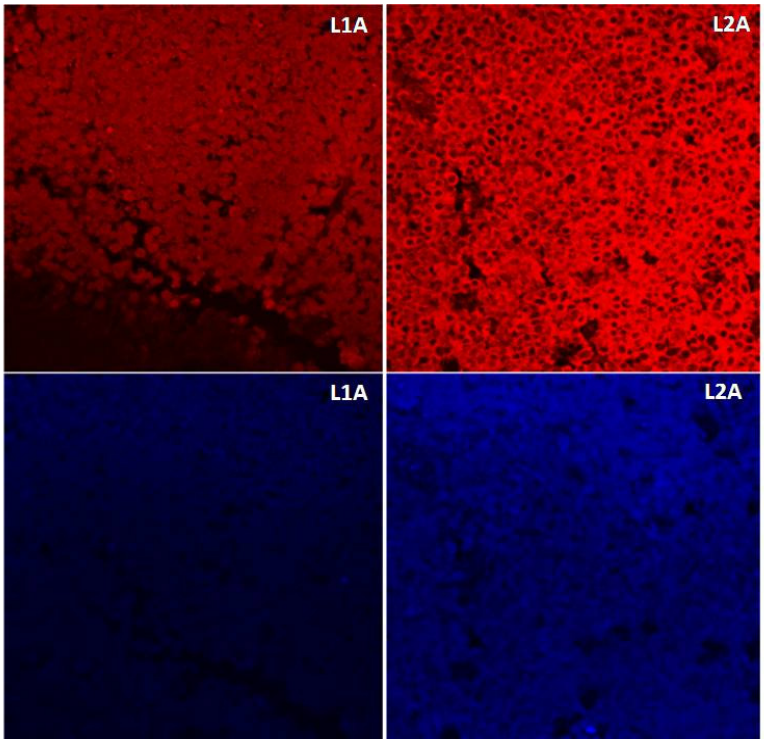
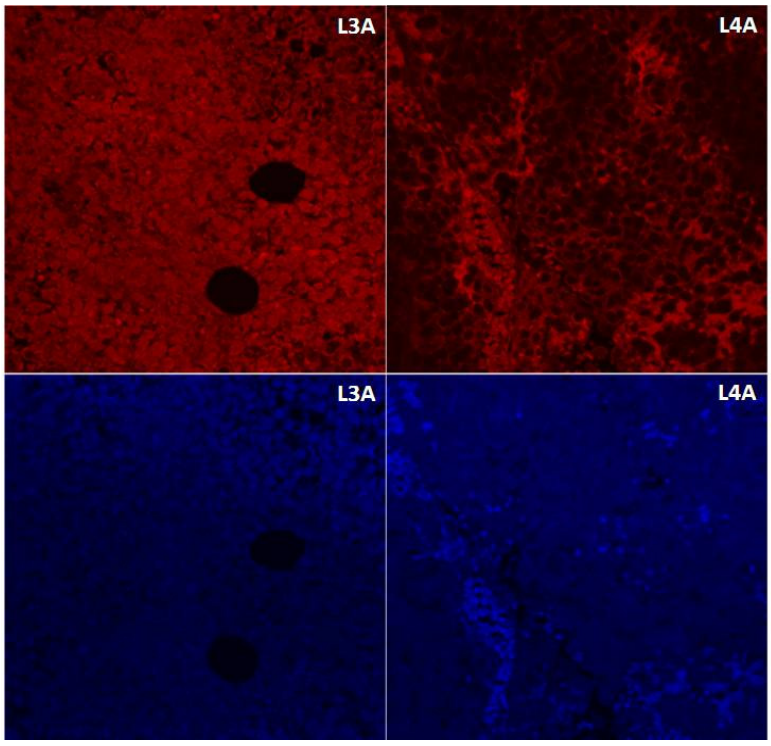


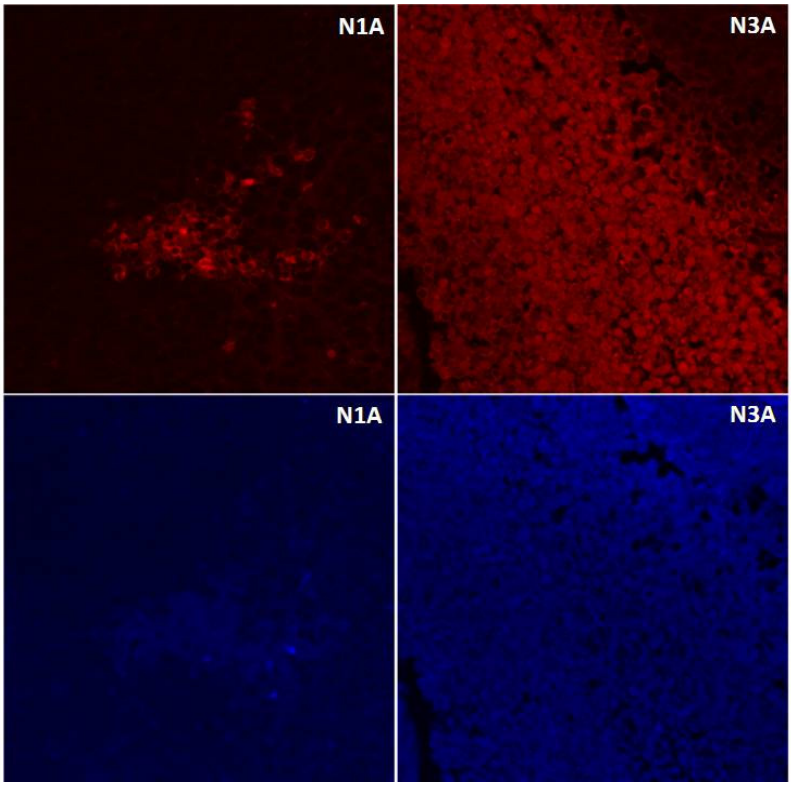
Figure S 10 Effect of PQ1 on Ki67 expression in Xenograft T47D tumor formation in nude mice.

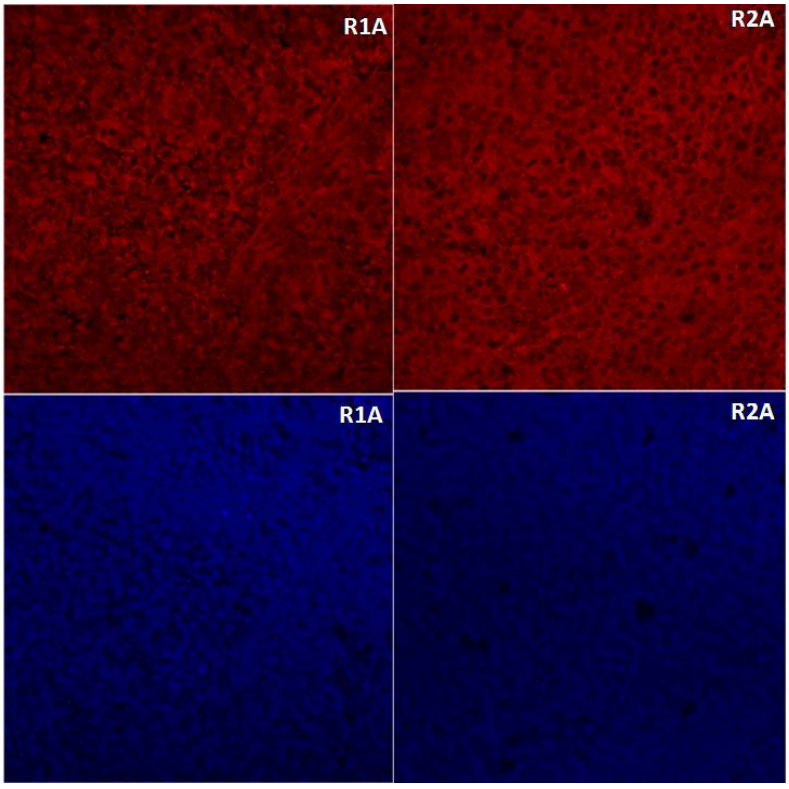
Tumors were harvested from the inguinal region of nude mice after euthanasia at the end of the study. Harvested tumors were fixed in 10% neutral buffered formalin for minimum 24 hr. After fixation, immunohistochemistry (IHC) was performed. We did not find any difference in Ki67 expression in control versus PQ1-treated tumor tissues harvested from nude mice. This could be due to increased tumor growth. The tumor size became so huge before the first injection of PQ1 (1 µM). We believe that the increased tumor size in the beginning did not allow PQ1 to either reach effectively to all the cells or the dose of PQ1 was not sufficient enough for such a big size tumor. Ear notching was performed to distinguish between the animals. R= Right notch, L= Left notch, N= No notch. The numbering denotes the cage number. A denotes the tumor, as I was interested in collecting brain also; therefore, I used A= tumor, B= brain. Three mice were housed per cage. R1A, R2A, L2A= tamoxifen-treated animals. L1A, R3A, N3A= PQ1-treated animals. N1A, L3A, R4A, L4A= control animals. L4A was euthanized at an early stage of the study because of humane reasons. N2A, N4A did not show any tumor formation. Tamoxifen (1 µM) was used as a positive control for tumor attenuation. **Materials and Methods for IHC:** Formalin fixed tumor tissue was cut into 5 µ size before paraffin-embedding them onto the

slides. Slides were baked for 25 min at 55℃. Slides were put in the xylene wash three times for 5 min each followed by graded alcohol baths- in 100% ethanol 2 times for 5 min each, 95% ethanol one time for 5 min, 80% ethanol one time for 5 min, distilled water for 5 min. Slides were pretreated with sodium citrate, pH 6.0 for the antigen retrieval method in a steamer for 20 min followed by cooling of the slides for 15 min. Blocking was done for one hr in 3% BSA in PBS at room temperature. Slides were incubated with Ki67 antibody (Santa Cruz, CA) for two hr at room temperature. Slides were washed in 0.1% PBS/T for 15 min. Secondary biotin labeled anti-rabbit antibody (1:200) was used for 15 min at room temperature. Slides were again washed in 0.1% PBS/T for 15 min. ABC elite enzyme reagent (Vector Lab) was applied for 15 min at room temperature. Slides were washed for 15 min in 0.1% PBST. Slides were immersed in distilled water for 5 min prior to the addition of substrate-chromogen (3, 3 diaminobenzidine-DAB). Slides were observed under the microscope and when color development was observed, slides were immediately transferred to distilled water for 5 min. Counter-staining was done with hematoxylin. Slides were rehydrated again in xylene and ethanol before mounting them with the mounting media.


L1A
L2A
L1A
L2A


L3A
L4A
L3A
L4A


N1A
N3A
N1A
N3A


R1A
R2A
R1A
R2A

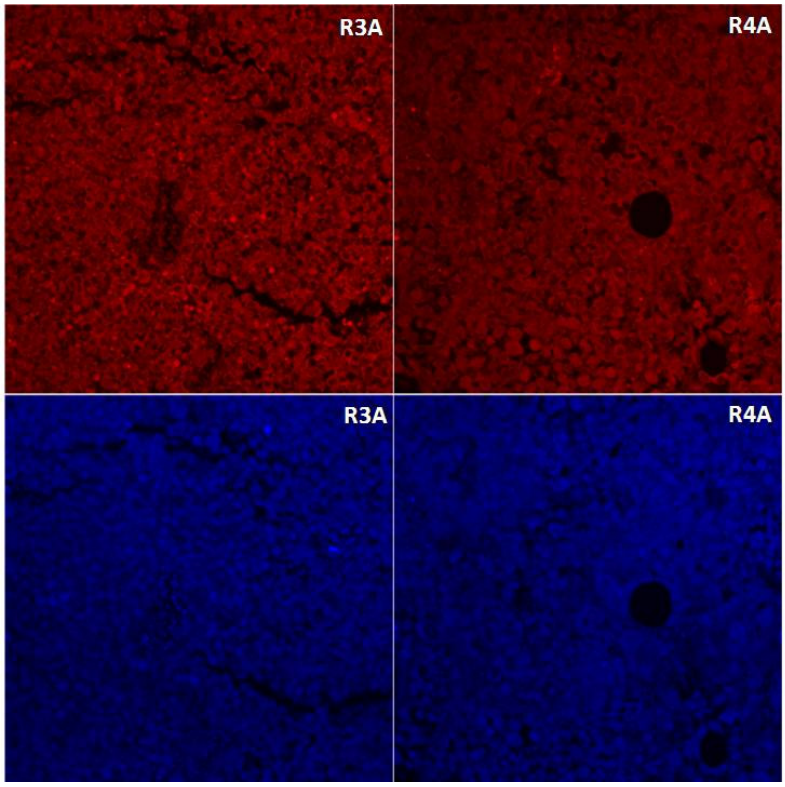


Figure S 11 Effect of PQ1 on Cx43 in xenograft T47D tumors harvested from nude mice. We did not find any significant differences in the expression of Cx43 in the control versus PQ1-treated nude mice. Ear notching was performed to distinguish between the animals. R= Right notch, L= Left notch, N= No notch. The numbering denotes the cage number. A denotes the tumor, as I was interested in collecting brain also; therefore, I used A= tumor, B= brain. Three mice were housed per cage. R1A, R2A, L2A= tamoxifen-treated animals. L1A, R3A, N3A= PQ1-treated animals. N1A, L3A, R4A, L4A= control animals. L4A was euthanized at an early stage of the study because of humane reasons. N2A, N4A did not show any tumor formation. Tamoxifen (1 µM) was used as a positive control for tumor attenuation. **Materials and Methods for confocal microscopy:** Formalin fixed human breast tissue was cut into 5 µm size before paraffin-embedding them onto the slides. Slides were baked for 25 min at 55℃. Slides were put in the xylene wash three times for 5 min each followed by graded alcohol baths- in 100% ethanol 2 times for 5 min each, 95% ethanol one time for 5 min, 80% ethanol one time for 5 min, distilled water for 5 min. Slides were pretreated with sodium citrate, ph 6.0 for the antigen retrieval method in a steamer for 20 min followed by cooling of the slides for 15 min. Blocking

was done for one hour in 3% BSA in PBS at room temperature. Slides were incubated with anti-mouse Cx43 (1:300) overnight at 4℃. After washing three times in PBS, slides were incubated with Alexa Fluor 568 nm for 3 hr at 4℃. Slides were stained with DAPI (nuclear stain) for 1 min and washed 3X with PBS. Slides were coversliped and observed under confocal microscope (Carl Zeiss LSM 510 META, Narashige, MN).

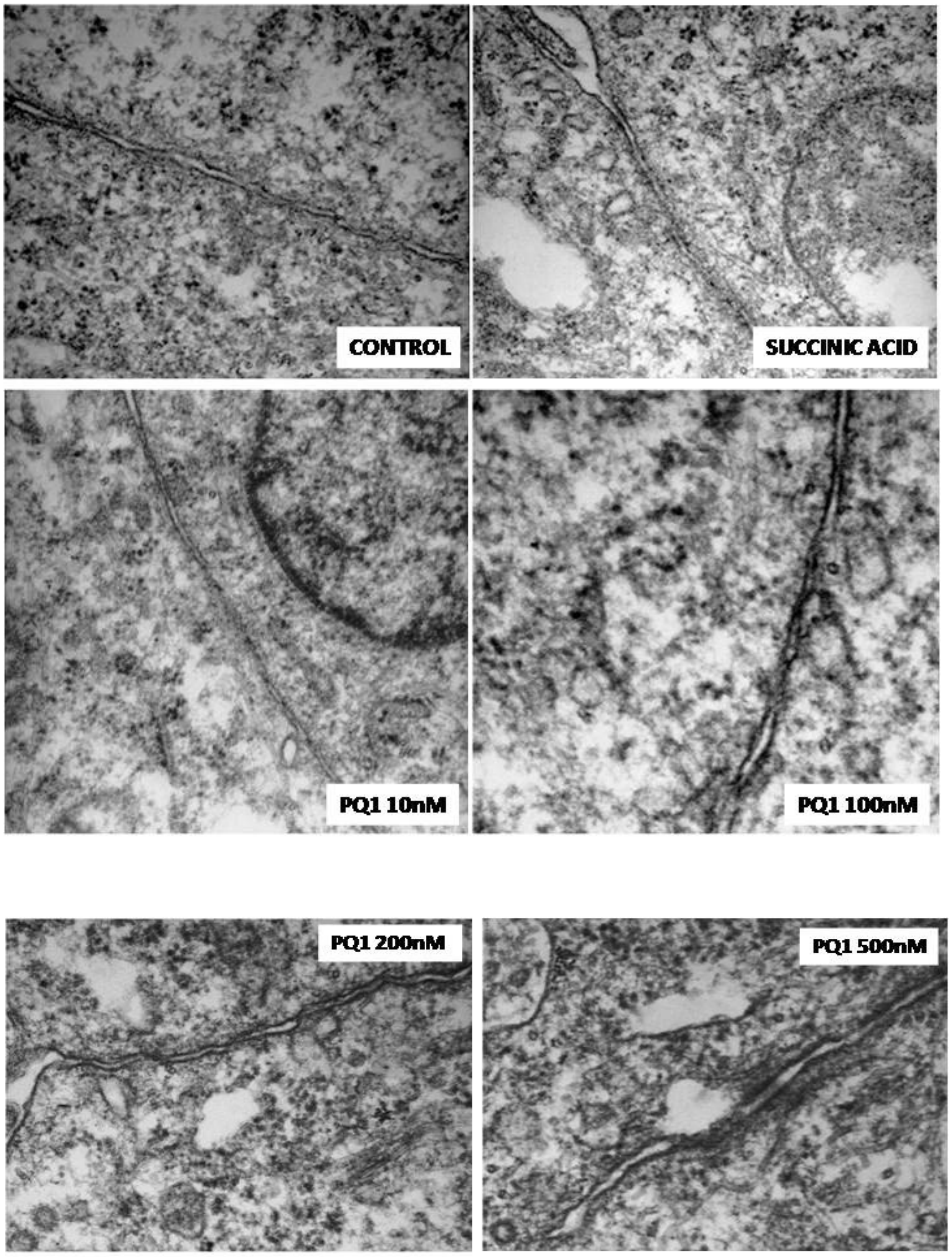


Figure S 12 Effect of PQ1 on gap junctions observed by Electron Microscopy.

T47D cells were dosed with PQ1 for 1 hr and ultrastructural study was performed to examine the effect of PQ1 on gap junctions. We found an increase in number of gap junctions in PQ1-treated cells compared to control cells. **Materials and Methods for Electron microscopy:** T47D cells were grown in a monlolayer in a trans-well plate, incubated with 10, 100, 200, and 500 nM PQ1. Cells were fixed in Karnosvsky's-cacodylate fixative (a 50/50 solution of 2.0%

paraformaldehyde and 2.5% gluteraldehyde in 0.1M cacodylate buffer) at 4℃ for approximately 2 hr. Fixative was replaced with fresh cold modified Karnosvsky's-cacodylate fixative, for approximately 2 hr. The cells were rinsed three times in chilled 0.1M cacodylate buffer and post-fixed in cold 1% osmium tetroxide-0.1Mcacodylate buffered fixative solution for 30 and 60 min, respectively. After three 5 min washes in cold doubled distilled water, the samples were then dehydrated in a series of increasing strength ethyl alcohol solutions (50%, 70%/ uranyl-acetate, 70%, 70%, 95%, 100%, 100%). The cells were stained for 60 min with uranyl acetate added to the first 70% alcohol solution. Dehydration was completed with two 20-minute rinses in 100% acetone. The specimens were then infiltrated with three increasing concentrations of resin in acetone ending in 100% resin for periods of 2-12, 2-12, 8-12 hours respectively. All samples were then embedded in Epon LX112 embedding medium then polymerized at 45℃ for 24 hr then 60℃ for another 24 hr. Embedded tissues were trimmed and sectioned on an Ultracut E-Reichert-Jung ultramicrotome (C. Reichert Optische Werke AG, 219, A-1171 Wien, Austria). Thick sections (0.5 microns) were cut initially from blocks, stained with Toluidine Blue, and examined with light microscopy. Silver and gold thin sections (approx 90 nm thickness) were taken from each specimen, retrieved to copper grids, allowed to dry, and then stained with uranyl acetate and lead citrate. The sections were then examined and viewed with a Hitachi H-300 electron microscope utilizing Kodak 4489 electron microscope film. Standard dark room procedures were used to print all micrograph film.

A.3. Supplemental data for Chapter 6

Animal Number	Estrogen level (pg/ml)	Gross findings	Microscopic findings
C1-RLN	173	Bladder and uterus distended	Marked bladder, one kidney ok, other with dilated ureter
C1-RTN	220	OK, only uterus distended	Ok
C1-LLN	123	Bladder ok, uterus distended	Ok
C1-LTN		Bladder and uterus distended, kidneys distended	moderate to marked hydronephrosis X2
C1-NN		Uterus distended, kidneys looked normal	1 ok other marked dilate ureter and mild pelvis-kidney, moderate with m/f plasma cell infiltrate
C2-RLN	91	Bladder ok, uterus distended	Ok, mild dilation of bladder
C2-RTN	36	Hydronephrosis, uterus and bladder moderately distended	mod X2 , mild with plasma cells
C2-LTN		Big Kidney, bladder and uterus distended	
C2-LLN		Bladder was normal, Kidneys looked normal	1 is mild, other OK- kidney, bladder mod with plasma cell in muscle
C2-NN		Severe hydronephrosis seen, bladder and uterus distended	Unilateral hydronephrosis, 3 m/f suppurative nephritis
C3-RLN		Kidneys hydronephrosed, bladder distended	3 kdneys, PM change and moderate hydronephrosis of 1
C3-RTN		Animal was grossly bloated bladder was enlarged, uterus highly distended	Mild-mod kidney, bladder dilated w mucin
C3-NN	161	everything ok, uterus distended	OK, mild dilation of bladder
C3-LLN	0	no pellet seen, uterus was swollen	Normal, no bladder
C3-LTN	102	everything normal, uterus distended	Ok
C4-NN	260	lesions around anus, kidneys normal	Bladder moderately dilated
C4-RTN	202	Kidneys enlarged, bladder distended	1 marked enalarged other OK- kidney, mod. Bladder w plasma cell in muscle
C4-RLTN	43	pellet was little out, kidneys were normal, uterus distended	Both kidneys mod. dilated, bladder ok
C4-LLN		Left kidney hydronephrosed, uterus and bladder distended	Kidneys -1 mod. dilated, other supp nephritis w bacteria
C4-LTN		Animal died, no sample taken	
C5-NN	9	no pellet seen, uterus normal	Ok
C5-RLN	222	Hydronephrosis, Uterus and bladder distended	1 moderate other enlarged ureter, marked bladder
C5-LLN	427	submandibular growth was seen on left side	Ok
C5-RTN		Kidneys highly enlarged, uterus and bladder distended	Marked hydronephrosis with hem. and infarct
C5-LTN		Kidneys hydronephrosed, uterus and bladder distended	Marked X2, bladder marked

Table S13 Gross and microscopic findings in individual mice associated with hydronephrosis and urine retention.

Mice were housed five per cage. Ear notching was performed to distinguish between the animals. C5= Cage and number of the cage. RTN= Right top notch, LTN= Left top notch, RLN= Right low notch, LLN= Left low notch, NN= No notch, Hem= haemorrhage, Mod= moderate, PM= post-mortem. Gross findings were observed at the time of euthanasia and were mostly described based on the appearance of kidney and bladder. Cardiocentesis was performed immediately after animal was euthanized. Estrogen levels were measured in pg/ml. Microscopic findings were investigated by a pathologist.

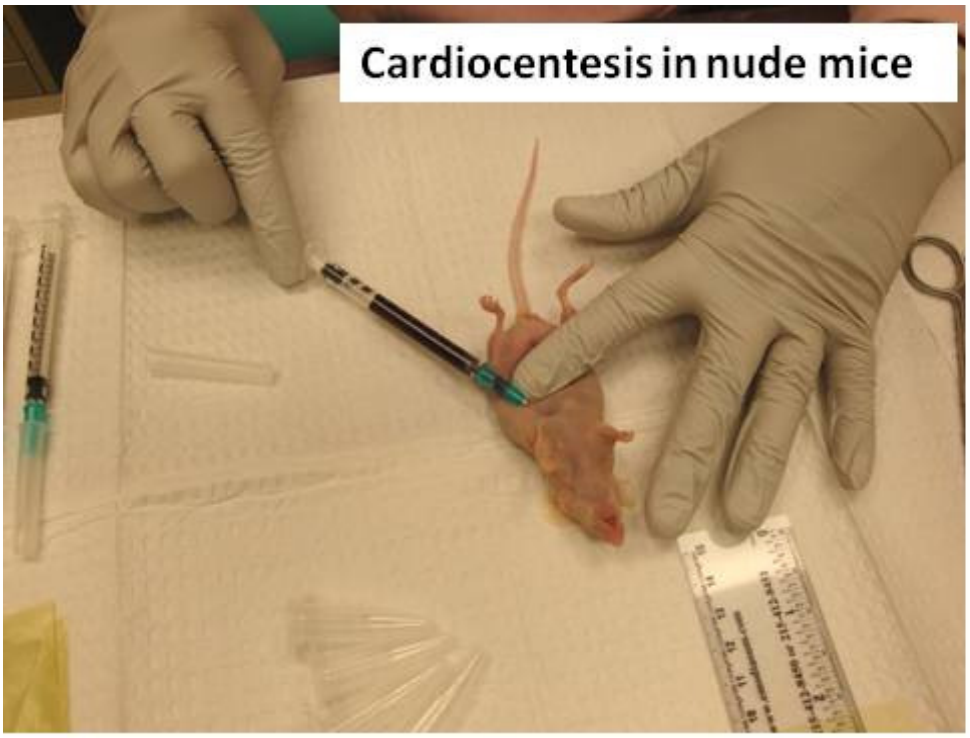


Figure S 14 Cardiocentesis in a nude mouse.

In mice, cardiocentesis should be performed immediately within 1 min after euthanasia.