GAP JUNCTION ENHANCER AS AN ANTI-CANCER AGENT VIA GJIC-INDEPENDENT AND -DEPENDENT PATHWAYS

by

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B.E., Tianjin University, Tianjin, China, 2004
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AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Biochemistry and Molecular Biophysics
College of Arts and Sciences

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Abstract

Gap junctions (GJ) are intercellular channels connecting adjacent cells, allowing small molecules to transport between cells, thereby maintaining all homeostasis. Loss of gap junctional intercellular communication (GJIC) and/or connexins, the gap junction proteins, is a hallmark of cancer. Restoration of GJIC and/or increase of connexin expression have been related to the reduction of tumorigenesis. Connexins have been reported as tumor suppressors due to both GJIC-independent and -dependent mechanisms. Therefore, development of effective agents or methods to enhance GJIC and restore connexin expression in cancer cells is a new strategy in cancer treatment. PQ1, 6-Methoxy-8-[(3-aminopropyl)amino]-4-methyl-5-(3-trifluoromethyl-phenyloxy)quinoline, has been demonstrated to increase GJIC, restore connexin expression, and exert anti-cancer effects on T47D breast cancer cells. Studies of apoptotic pathways showed that PQ1 activated both extrinsic and intrinsic apoptotic pathways, indicating that PQ1 exerts its anti-cancer effects via a GJIC-independent mechanism through the induction of apoptosis. Combinational treatment of PQ1 and cisplatin showed that PQ1 counteracted cisplatin-induced inhibition of GJIC and reduction of connexin expression, thereby increasing the efficacy of cisplatin in T47D cancer cells via a GJIC-dependent mechanism. Further studies of drug distribution and toxicity revealed that administration of PQ1 by oral gavage can be achieved with low toxicity to normal vital organs. All the results suggest that PQ1, a gap junction enhancer, can function as an anti-cancer agent and potentiate the efficacy of antineoplastic drugs via both GJIC-independent and -dependent pathways.

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Dedication

I would like to dedicate my dissertation to my loving family- Dad, Mom, and my dear husband Yang.

Chapter 1 - Review of Literature

1.1 Gap Junctions and Connexins

Gap junctions are intercellular channels connecting adjacent cells, allowing small molecules of less than 1.2 kDa in size to transport between cells. Gap junctions are formed by the gap junction protein known as connexins. Six connexins oligomerize into a hexameric structure known as connexon. Connexon docks with another connexon from the adjacent cell to form a gap junction. Gap junctions have been found in nearly all animal organs and tissues. In normal cells and tissues, gap junctions play an important role in controlling cell growth, regulating cell differentiation, and maintaining homeostasis.

1.1.1 Nomenclature and Distribution of Connexins

Connexins are found only in vertebrates. Recently, the screening of the mouse and human genomic databases has revealed 21 human and 20 mouse connexin genes, each encoding a protein [1-4]. Two systems have been used for the connexin nomenclature. The most widely used nomenclature system is based on the predicted molecular mass of connexin polypeptide [5]. For example, connexin 43 (Cx43) is a connexin with a predicted mass of 43 kDa. The other system, the Greek nomenclature system, is based on evolutionary considerations [6]. In this system, connexins are divided into subgroups (α, β, γ or δ) with respect to a combination of gene structure, overall sequence homology, and matching of specific sequence motifs [7], such as $\alpha 1$ represents for Cx43, $\beta 1$ represents for connexin 32 (Cx32), and so on. Connexins have a near-ubiquitous distribution in the tissues of vertebrates. However, different connexins have been observed in different tissues, indicating the tissue specific expression of connexins [1]. The connexin genes, the nomenclature of connexins based on two systems, and the representative tissues in human are shown in Table 1.

Gene	Molecular Mass Nomenclature	Greek Letter Nomenclature	Representative Tissues/Organs

GJA1	Cx43	α1	Heart, skin, breast
GJA3	Cx46	α3	Lens
GJA4	Cx37	α4	Blood vessels, endothelium
GJA5	Cx40	α5	Heart, endothelium
GJA6 (GJA6P)	Cx33	α6	Pseudogene in humans
GJA8	Cx50	α8	Lens
GJA10	Cx59	α9[8]	Lens (retina)[9]
GJA10	Cx62	α10	Lens (retina)[9], ovary
GJB1	Cx32	β1	Liver, brain
GJB2	Cx26	β2	Liver, breast, Cochlea, skin
GJB3	Cx31	β3	Skin, placenta
GJB4	Cx30.3	β5	Thymus, Skin
GJB5	Cx31.1	β4	Skin

GJB6	Cx30	β6	Skin, cochlea, brain
GJB7	Cx25	β7[8]	N.D.
GJC1/GJA7	Cx45	α7 or γ	Pancreas, small intestine
GJC2/GJA12	Cx47	γ	Oligodendrocytes
GJC3	Cx30.2	γ	Ear
GJD2/GJA9	Cx36	α9 or γ	Pancreas, neurons
GJD3/GJC1	Cx31.9	β	Liver, spleen, kidney, heart, colon
GJD4	Cx39	α2	Developing muscle
GJD4	Cx40.1	δ	N.D.
GJE1	Cx23	δ5[8]	N.D.
GJE1	Cx29	δ	Brain

N.D.= Not determined

Table 1. Summary of published human connexin genes, connexin nomenclatures, and representative organs

1.1.2 Structures of Connexins and Gap Junctions

Examination of amino acid sequences and the locations of different domains revealed the structure of connexins [10-13]. The members of connexin family usually weigh between 26 and 60 kDa with an average length of 380 amino acids. Despite the variations in weight and length, several features of connexins are common to all. Each connexin has four hydrophobic transmembrane domains (M1- M4) (Figure 1.1). Studies of Cx43 showed that the transmembrane domains are α-helical, on the basis of a 7 Å structure [14]. Two loops, between M1 and M2, as well as M3 and M4, are accessible from extracellular side [12]. A hydrophilic loop between M2 and M3, the carboxy-domain (C-terminus) and the amino-domain (N-terminus) are accessible from the cytoplasmic side [15]. The conserved regions are located in transmembrane domains and extracellular loops [3]. The length of the C-terminus varies among connexins, and is responsible for the molecular weight of the connexin. The C-terminus contains several sites related to connexin phosphorylation, chemical gating, and protein binding [16-18].

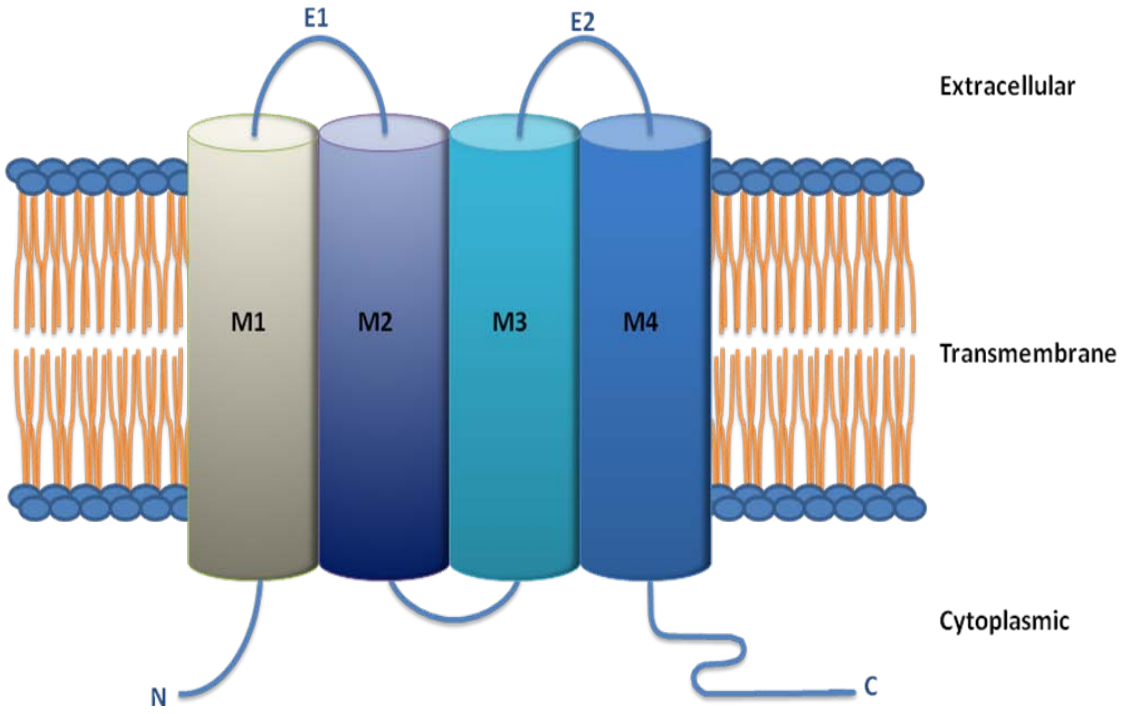

Figure 1.1 Structure of connexin.

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). Both the C and N cytoplasmic termini are shown in the figure.

Connexins oligomerize into hexamers, the connexons. After formation, connexon stays at plasma membrane or docks with another connexon from the adjacent cell to form a complete gap junction channel [19]. The two extracellular loops (E1 and E2) are considered to be involved in the docking of connexons. In each of the extracellular loops, a set of three cysteine residues exists with high reserved order (except for Cx31): [C-X_6-C-X_3-C] for the first loop and [C-X_5-C-X_5-C] for the second loop. Opposing cysteines in both loops were reported to form disulfide-bridges enabling two connexons to dock with each other [7]. Connexons can be formed with uniform connexins (homomeric) or differing connexins (heteromeric) (Figure 1.2). The gap junctions channels contain two identical connexons are called homotypic, whereas gap junctions with differing connexons are called heterotypic (Figure 1.2). Based on different combinations, gap junctions show great variations of channel subtypes. Co-expressions of two compatible connexins in the same cells make it possible to assemble heteromeric connexons. Many heteromeric connexons have been reported in different organs. Cx26/Cx32 connexons have been shown to exist in the liver [20], Cx26/Cx30 connexons in the cochlea[21], Cx46/Cx50 connexons in the lens [22], and Cx43/Cx45 connexons in the myocardium [23, 24]. However, not all co-expressed connexins can form heteromeric connexons. For example, Cx26 is able to co-oligomerize with Cx32 but unable to co-oligomerize with Cx43 [20, 25]. The imcompatibility between connexins is suggested to be related to the second extracellular domain (E2) [26, 27] and tempers the diversities of gap junction channels.

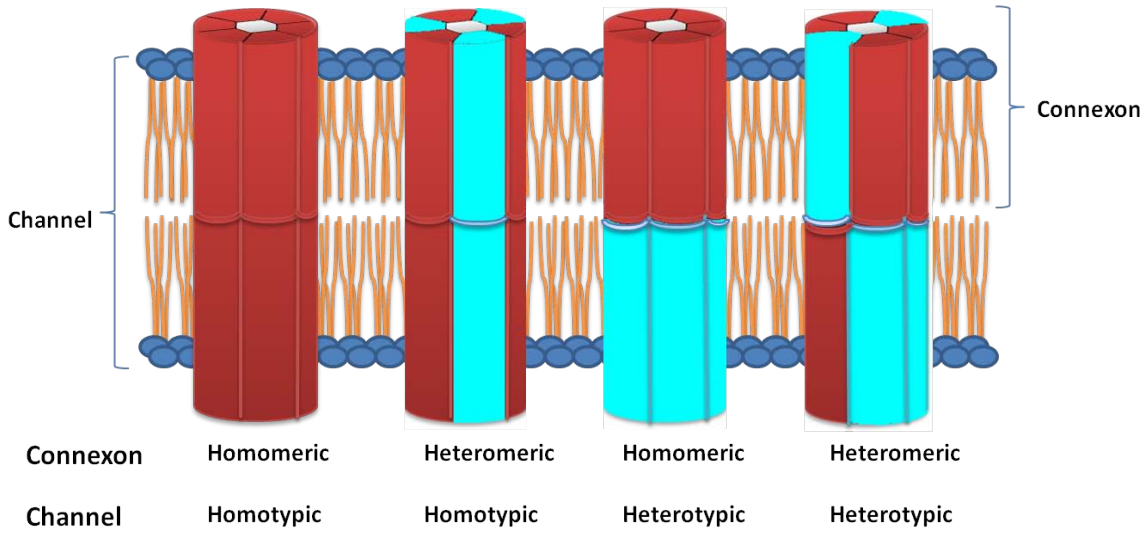

Figure 1.2 Possible arrangements of connexons and gap junctions.

Connexons are composed of six identical connexin subunits (homomeric) or different species of connexins (heteromeric). Connexons dock with each other to form gap junction channels. Gap junction channels formed with identical connexons are called homotypic, while those with different connexons are called heterotypic. Connexins are indicated by cylinders (red and blue).

1.1.3 Regulation Pathways of Connexins

1.1.3.1 Biosynthesis of Connexin

1.1.3.1.1 Connexin Genes

Some connexin genes are clustered together within the genome [28]. Human chromosome 1 codes for eight connexins, chromosome 6 for four connexins, chromosome 13 for 3 connexins, chromosome 17 for two connexins, and chromosomes 7, 10, 15, and the X chromosome for one connexin each [29]. The general structure of connexin genes is very simple. The gene is composed of a separate 5'-untranslated region (5'-UTR) which is designated as exon 1, as well as an exon 2 which contains complete protein coding sequence and the 3'-untranslated region (3'-UTR) (Figure 1.3 A) [29]. Subsequently, exceptions from the initial structure have been described. For example, *GJB1* (Cx32) has multiple 5'-UTR exons that are spliced in an alternate manner due to

transcription from tissue-specific promoters (Figure 1.3 B) [29]. *GJC1* (Cx45) has three exons, including two 5'-UTR exons with one coding exon. *GJC1* transcripts can be differentially spliced so that 5'-UTR can be generated from exon 1 and 2 or only from exon 2 after transcription driven from a single promoter (Figure 1.3 C) [29]. Additionally, in some connexin genes, such as *GJD2* (Cx36) and *GJE1* (Cx23), the coding region can be interrupted by introns (Figure 1.3 D) [29].

1.1.3.1.2 Gene Transcription of Connexin

The transcription of connexin DNAs is regulated by a variety of transcription factors, biological substances, and signal transduction pathways, which are cell type-independent (ubiquitous) or –dependent. The investigations of all factors that regulate expression of connexin genes have been reviewed previously [30]. In most connexin genes, the basal promoter P1 is located 300 bp upstream from the transcriptional initiation site in exon 1. Within this region, binding sites for transcription factors have been identified in several connexin genes. For instance, it contains binding sites for cell type-independent transcription factors, including TATA box-binding protein, Sp1/Sp3 and AP-1, as well as for cell type-dependent factors, containing cardiac-specific transcription factors (Nkx2-5, GATA4, Tbx5) and HNF-1 [30]. Binding of these factors is not only important for basal expression of connexins, but also important for large changes in expression. In addition to the transcription factors, regulation of connexin transcription is related to many biological substances and signal transduction pathways. For example, cyclic adenosine monophosphate (cAMP) and retinoids have been reported to active connexin expression [31-33]. Over-expressions of Wnt1 and H-Ras increase expression of Cx43 mRNA and protein, indicating that Wnt pathway and Ras-Raf-MAPK pathway are involved in the regulation of Cx43 expression [31, 34].

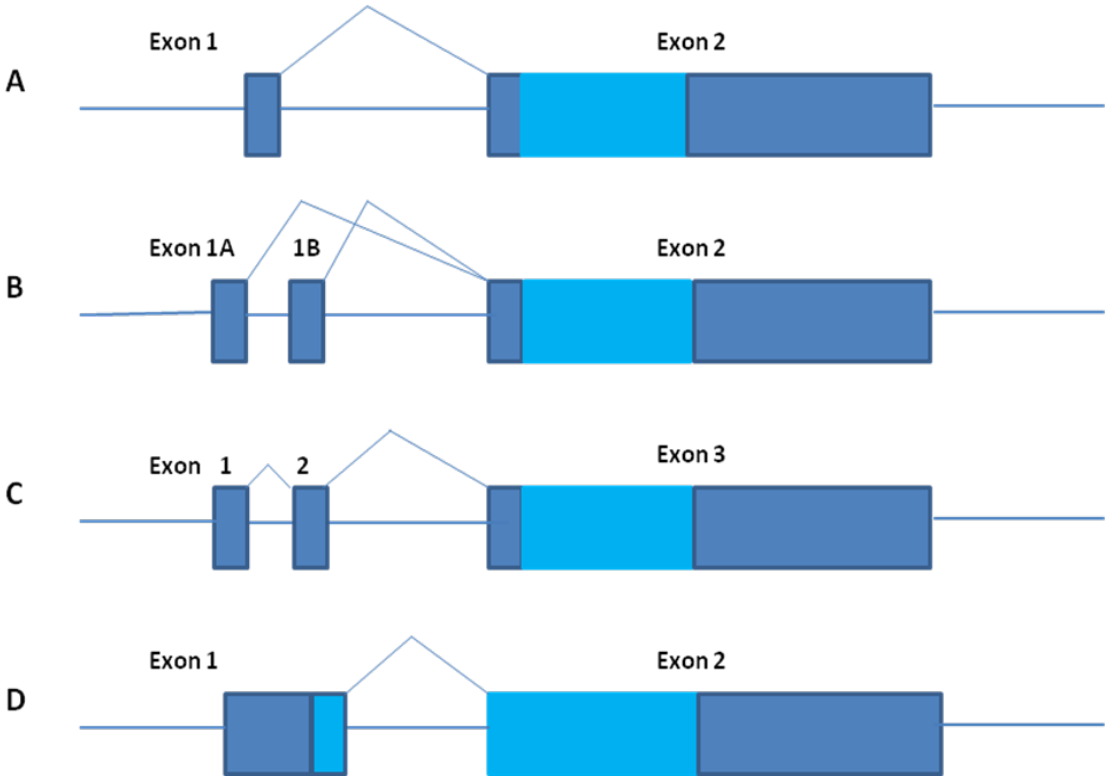

Figure 1.3 Structure of the connexin genes.

Diagrams show the structures of different connexin genes with the noncoding regions (dark blue boxes) and coding regions (light blue boxes). The variations occur in the 5'-UTR. **A.** The general structure of connexin gene contained only one 5'-UTR exon. **B.** Some connexin genes contain two or more 5'-UTR exons (1A and 1B) that are alternatively utilized due to transcription from tissue-specific promoters. **C.** Connexin genes contain two or more 5'-UTR exons (1 and 2) that may be present with the coding exon (3) in the mature mRNA or that may be alternatively spliced to generate multiple mRNA variants. For these connexin genes, multiple mRNAs can be generated after transcription driven from a single promoter. **D.** In a few connexin genes, the coding region is interrupted by an intron.

1.1.3.1.3 Translation of Connexin

After transcription, the connexin protein is synthesized by translation in the ribosomes that are bound to the endoplasmic reticulum (ER) membrane. The encoded hydrophobic domains are recognized by a signal recognition particle (SRP) to form the SRP/ribosome/nascent-polypeptide-chain/mRNA complex [35]. The complex dock to a

protein-channel in the ER membrane which allows the translation of the nascent chain to proceed until polypeptide is synthesized [36]. Once complete, connexins are translocated from the hydrophilic channel lumen of the translocon to the hydrophobic ER membrane environment [37]. The charged residues within the hydrophobic transmembrane regions of connexins might be shielded from the hydrophobic bilayer environment through oligomerization.

Although connexin gene expression is considered to be dominantly regulated at the transcriptional level, many studies found that alternative regulation at the translational level could be in place as well [38]. It has been reported that in addition to the Cap-dependent translation using the 5'-Cap recognition site, some mRNAs containing internal ribosome entry site (IRES) are translated through the Cap-independent translation in cells [39, 40]. IRESs have been identified in the 5'-UTRs of some connexin genes, including Cx43, Cx32, and Cx26 [41-43], highlighting the potential role of Cap-independent translation of connexins especially under the conditions when Cap-dependent translation is suppressed. The IRES-mediated translation makes it possible to regulate connexin expression at the translational level. For example, Lahlou et al. reported that the existence of IRES in Cx26 mRNA permits connexin expression in density-inhibited or differentiated cells, where Cap-dependent translation is generally reduced [43]. They also found that Cx26 expression is increased at the level of translation in density inhibited human pancreatic cells, and the increased translation is not sensitive to rapamycin, an inhibitor of Cap-dependent translation, suggesting that the IRES-dependent synthesis is an important mechanism of the endogenous expression of Cx26 [43]. In addition to the IRES-mediated translational control, regulation of connexin expression through short upstream open reading frames (uORFs), which are located between 5' end of the mRNA and the beginning of the main ORF is another type of translational control. The uORF on an mRNA generally decreases translation of the downstream genes. uORFs have been identified in mRNAs of *Xenopus laevis* Cx41 as well as mouse Cx31, Cx45, Cx46, and Cx47 [44, 45]. Meijer et al. reported that mutation of each of three uAUGs into AAG codons of *Xenopus laevis* Cx41 significantly enhanced translation compared to the wild-type Cx41 5'-UTR, indicating that the three uORFs play an important role in the regulation of Cx41 expression [44]. Pfeifer et al. found that among various mouse Cx43

transcripts, the Cx43 5'-UTRs lacking uAUG have higher translation efficiencies than those with uAUGs.

1.1.3.1.4 Connexin Assembly and Gap Junction Formation

The newly synthesized polypeptides of connexins are cotranslationally inserted into the ER membranes and transported via the Golgi apparatus and the *trans*-Golgi network to the plasma membrane [46]. During this process, connexin peptides are posttranslationally modified and oligomerized into connexons. For example, pulse-chase studies show that Cx43 is initially synthesized as a 40 to 42 kDa polypeptide which is subsequently posttranslationally modified by phosphorylation of serine residues in a serine-rich sequence near the carboxy terminus [47, 48]. The phosphorylation occurs soon after translation, even before the Cx43 exit from the Golgi apparatus [49, 50].

Six connexins oligomerize into one connexon before they transport to the plasma membrane. The intracellular location where connexins assemble into connexons is connexin-specific. Das Sarma et al. provided evidence for the assembly of Cx32 in the ER [51]. Musil and Goodenough reported that the assembly of Cx43 occurs after exit from the ER, probably in the trans-Golgi network [52]. Ahmad and Evans demonstrated that Cx26 hemichannels are integrated directly into plasma membranes in a posttranslational manner [53]. For the heteromeric connexons, Diaz et al. provided evidence for the assembly of Cx26 and Cx32 in Golgi membranes, while the oligomeric intermediates of Cx26 are in the ER-Golgi-intermediate subcellular fraction [54]. In the cells expressing two connexins, it is possible to form either homomeric connexons or heteromeric connexons or both. Not any connexins are compatible to form heteromeric connexons. Until now, all reported heteromeric connexons are composed of two members of the same subgroup. For example, Cx46 hetero-oligomerize with Cx50 (all α-group) [22], Cx32 hetero-oligomerize with Cx26 (all β-group) [55], and Cx43 hetero-oligomerize with Cx40, Cx37, and Cx46 (all α-group), but not with Cx32 (β-group) [56-58]. It is still unclear that how the selective compatibility between different connexins is achieved. Results of co-immunoprecipitation of full-length and truncated Cx43, Cx32, and Cx26 polypeptides indicated that hetero-oligomerization might be based on intrinsic signals [59, 60]. The signals that allow connexins isoforms to recognize each other might

be located in the C-terminus, while the signals that regulates subunit compatibility might be located in the N-terminus of the connexin polypeptides. Recent studies using the CLUSTAL W algorithm of the OMIGA sequence analysis package suggested that the N-terminal amino-acid residues at position 12/13 are involved in the oligomerization compatibility of α and β connexins [61].

The trafficking of connexin polypeptides from ER to Golgi is through membrane vesicles. Transportation of connexins follows the general intracellular transport route. The membrane vesicles containing polypeptides is budded from the ER and fused with subsequent intracellular membrane compartments, such as Golgi stacks, [49]. After transportation, the membrane vesicles shuttle back to the membrane compartments that they originated [62]. The delivery of connexons to plasma membrane has also been studied. Lauf et al. reported that Cx43 connexons were delivered in vesicular carriers that traveled along microtubules from the Glogi to the plasma membrane [63]. Martin et al. reported that Cx26 connexons traffic in a microtubule independent pathway [64]. The observations of very short distance between the locations of ER membranes and plasma membrane suggested that connexons might be able to transfer directly into the plasma membrane from the ER. In addition to microtubules, intact actin filaments have also been reported to be involved in the plasma membrane delivery of Cx26 [65].

After trafficking, the connexons are ready to insert into the plasma membrane. Connexons are inserted into the plasma membrane in a closed configuration [66]. Freeze-fracture electron microscopic studies showed that the insertion is through fusion of particle-bearing cytoplasmic vesicle couriers [67]. Lauf et al. reported that insertion of Cx43 connexons in non-polarized HeLa cells were distributed over the entire non-junctional plasma membrane surface [63]. The results of Fluorescence Recovery After Photo-bleaching (FRAP) experiments indicated that the inserted connexons can move laterally in the plasma membrane. The connexons are distributed in the plasma membrane as single particles, or small groups, but not aggregates.

After insertion, the connexons stay in the plasma membrane as hemichannels or dock with another connexon on the adjacent cell to form double-membrane spanning gap junction channels (as described in section ***1.1.2***). The gap junction channels aggregate to form plaques instead of staying as single channels on the plasma membrane, and the

plaques keep growing by addition of individual gap junction or fusion of small aggregates [68, 69]. The number of individual channels in a single plaque varies from less than a dozen to several thousands. The newly synthesized gap junctions are added along the outer margins of plaques [63, 70]. When removal of older gap junctions in the center of plaques and accrual of newly synthesized gap junctions to the margins of plaques reach a balance, the size of the plaques keep constant. The mechanisms of the gap junction aggregation into plaques are not completely clear. 1.1.3.2 Gap Junction and Connexin Degradation

The half-life of connexins is very short, ranging from 1 to 5 hours [71-73]. Gap junctions cannot be separated into connexons during the internalization [74]. Morphological and biochemical studies showed that the entire junctions were internalized into one of the adjacent cells via endocytosis, and formed double-membrane vacuoles named “annular gap junctions” or “connexosomes” [75, 76]. Following internalization of gap junctions, connexins are degraded through lysosomal or proteasomal degradation pathways [71]. Many regulations, including phosphorylation and ubiquitination have been reported to be involved in the degradation process [77, 78].

1.1.3.2.1 Phosphorylation of Connexins

All connexin isoforms are phosphoproteins, except Cx26 [79, 80]. Connexin phosphorylation plays an important role in multiple steps in the life cycle of gap junctions, including connexin biosynthesis, trafficking, assembly, membrane insertion, channel gating, internalization, and degradation [1]. Several protein kinases, such as mitogen-activated protein kinase (MAPK), protein kinase C (PKC), protein kinase A (PKA), cdc2/cyclinB, casein kinase 1, v-src and c-src have been identified to target different phosphorylation sites on connexins [81]. Most of the consensus sites for phosphorylation on connexins are located in the carboxy terminus. For example, on Cx43, the phosphorylation sites are Ser-255, Ser-279, and Ser-282 for MAPK, Ser-368 and Ser-372 for PKC, and Tyr-247 and Tyr-265 for v-src [82-85].

Cx43 is the most widely studied connexin. The activation routes of protein kinases in Cx43 phosphorylation can be generally divided into three pathways (Figure 1.4) [16, 82, 85]:

1. Epidermal growth factor (EGF) binds to its receptor (EGF-R), which induce the dimerization and autophosphorylation of EGF-R. The phosphorylated EGF-R interacts with Grb2 adaptor protein and SOS guanine nucleotide exchange protein. Membrane-localized SOS activates ras from GDP-bound form ($p21^{ras}$-GDP) to GTP-bound form ($p21^{ras}$-GTP), initiating the phosphorylation of raf-kinase (c-Raf). Phosphorylation of c-Raf activates MAPK kinase (MEK ½) and MAPK. Active MAPK directly phosphorylates Cx43 on serine residues (Ser-255, Ser-279 and Ser-282). Binding of lysophosphatidic acid (LPA) to its receptor (LBP) also leads to the activation of ras through interaction with a heterotrimeric G-protein (G_i), and initiates activation of c-Raf, MEK 1/2, and MAPK.

2. Other ligands (L) bind to their membrane receptors (R), which activates G_q proteins. Activation of G_q proteins induces activation of phospholipase C (PLC), and subsequently converts phosphatidylinositol 4,5-bisphosphate (PIP_2) to diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). DAG directly activates PKC, while IP_3 activates PKC through induction of Ca^{2+} release from ER. PKC can directly phosphorylate Cx43 on serine residues, or activate MEK ½ and MAPK to phosphorylate Cx43.

3. Expression of v-src tyrosine kinase can also phosphorylate Cx43. The tyrosine kinase v-src interacts directly with Cx43 via its SH3 and SH2 domains, and phosphorylates Cx43 on two tyrosine residues.

In general, connexin phosphorylation is not a generic prerequisite for connexin degradation as evidenced by the fact that Cx26 is not phosphorylated but still appears to be degraded in a short time [86]. However, increasing number of reports provide evidence that for some connexins, notably Cx43, phosphorylation may trigger its internalization and degradation. It has been reported that phosphorylation is correlated with Cx43 internalization when cells exit the G_2 phase and entering mitosis. At the onset of mitosis, $p34^{cdc2}$ protein kinase-dependent phosphorylation of Cx43 correlated with cells rounding and gap junction internalization into large structures which resemble internalized annular junctions [87, 88]. In addition to $p34^{cdc2}$ protein kinase, PKC activation and EGF-induced MAPK activation have also been shown to be tightly associated with Cx43 internalization and eventual degradation. Ruch et al. reported that

in WB-F344 rat liver cells, PKC-induced phosphorylation was related to gap junction internalization [89]. Lampe found that the PKC activator TPA had a significant inhibition of Cx43 assembly in Novikoff cells and caused the Cx43 half-life to decrease from 3.1 to 2 h [90]. Leithe and Rivedal provided evidence to show that EGF-induced phosphorylation of Cx43 in IAR20 rat liver epithelial cells resulted in the increased ubiquitination of Cx43 which may act as a signal of Cx43 internalization [91]. Although the role of phosphorylation in connexin degradation is still controversial, it is reasonable to propose that the internalization of connexins, especially Cx43, is regulated by select and specific phosphorylation events. Furthermore, the phosphorylation may also regulate the ubiquitination of connexins.

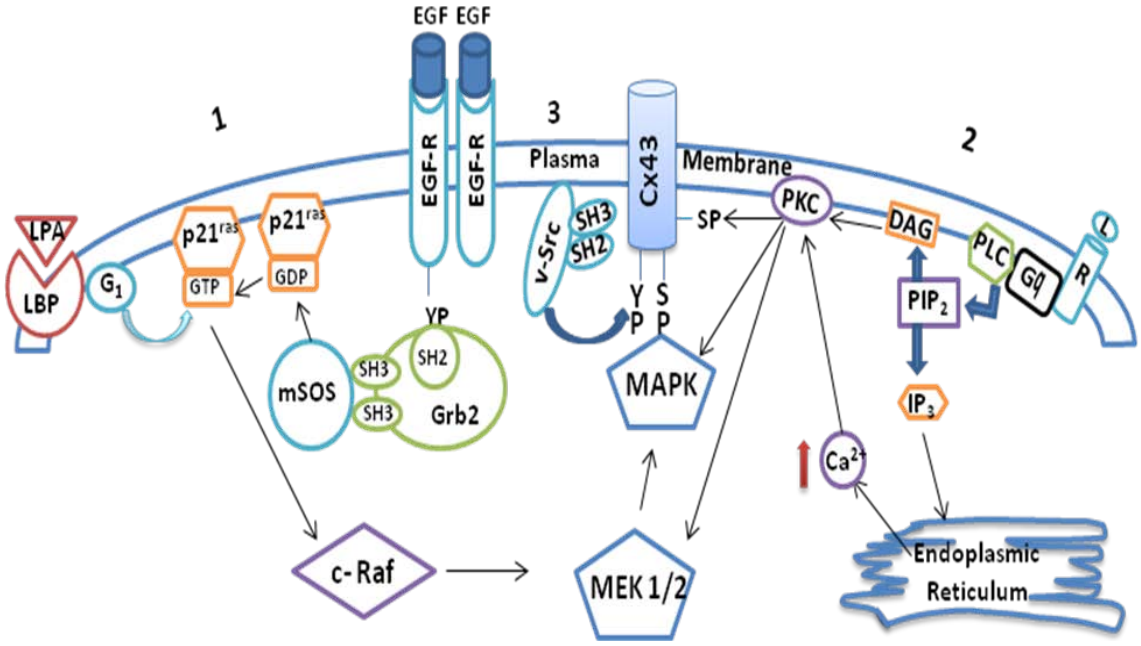

Figure 1.4 Schematic presentation of three phosphorylation pathways of Cx43.

1. Binding of EGF to EGF-R induces the receptor dimerization, autophosphorylation and interaction with Grb2 and SOS. Membrane-bound SOS transforms ras from GDP-bound form ($p21^{ras}$-GDP) to GTP-bound form ($p21^{ras}$-GTP). Binding of LPA to its receptor LBP which is coupled to G_i protein also activates ras. After activation, ras phosphorylates c-Raf and subsequently activates MEK ½ and MAPK. MAPK phosphorylates Cx43 on serine residues. 2. Other ligands L bind to their receptors R, which induces activation of G_q proteins. G_q proteins activate PLC which subsequently converts PIP_2 to DAG and IP_3.

DAG directly activates PKC, while IP_3 induces Ca^{2+} release from ER which also promotes PKC activation. Active PKC phosphorylates Cx43 directly or indirectly through activation of MEK ½ and MAPK. 3. Tyrosine kinase v-src interacts directly with Cx43 through its SH3 and SH2 domains, and phosphorylates Cx43 on tyrosine residues.

1.1.3.2.2 Ubiquitination of Connexins

Some reports suggest that Cx43 is a substrate for ubiquitination [91, 92]. Ubiquitination of Cx43 is important in regulating Cx43 degradation [78]. Ubiquitination of Cx43 occurs at the plasma membrane, indicating that ubiquitin might be involved in the internalization and intracellular trafficking of Cx43. Since Laing and Beyer reported the involvement of ubiquitin in Cx43 degradation in 1995 [92], many studies have provided evidence for the role of ubiquitination in Cx43 degradation. Laing and Beyer used the Chinese hamster ovary cell line CHO-ts20 with the expression of a thermolabile E1 ubiquitin-activating enzyme. They found that Cx43 was increased when the ubiquitin-activating enzyme is defective, indicating the involvement of ubiquitin in Cx43 degradation [92]. They also provided evidence that the ubiquitinated Cx43 is mainly degraded via the proteasomal pathway [92]. Subsequent experiments performed by Leithe and Rivedal suggested that EGF-induced phosphorylation is a signal for conjugation of ubiquitin to Cx43 [91]. The EGF-induced hyperphosphorylation, ubiquitination, internalization and degradation of Cx43 were found to be mediated by MAPK pathway [91]. By using the 12-O-tetradecanoylphorbol-13-acetate (TPA), a tumor-promoting PKC activator, to study the effects of PKC on Cx43 ubiquitination, Leithe and Rivedal found that TPA-induced degradation of Cx43 was associated with strongly increased ubiquitination of Cx43 [93]. Results of coimmunoprecipitation and Western blotting showed that the TPA-induced ubiquitination involved both PKC and MAPK pathways [93]. Leykauf et al. reported that in the rat liver epithelial cell line WB-F344, Cx43 binds to the WW domains of E3 ubiquitin ligase Nedd4 through the PY motif, a proline-rich region on the Cx43 C-terminal sequence [94]. Depletion of Nedd4 by RNA interference caused accumulation of Cx43 gap junctions at the plasma membrane, indicating that ubiquitination may play a role in the internalization of Cx43 [94]. Girao et al. further reported that Nedd4-mediated ubiquitination of Cx43 is required

to recruit Eps15, an endocytic adaptor containing ubiquitin-binding domains, through its ubiquitin-interacting motif, and targets ubiquitinated Cx43 to the endocytic pathway [95].

Based on all the evidence, the degradation pathway of Cx43 can be described: Activation of MAPK or PKC phosphorylates Cx43, which subsequently recruits E3 ubiquitn ligase, such as Nedd4. Nedd4 binds to Cx43 via WW domains and ubiquitinates Cx43. In the ubiquitination process, the recruitment of Eps15 is required. After ubiquitination, Cx43 is degraded through proteasomal pathway. Nedd4 can also bind to Cx43 independently of the Cx43 phosphorylation state. In addition to a ubiquitin-dependent pathway for Cx43 endocytosis, a ubiquitin-independent pathway exists, in which the YXXΦ motif in the Cx43 C terminus is important.

No direct evidence exists that other connexins are ubiquitinated prior to internalization and degradation, except Cx43. Henzl et al. have reported that OCP1 (organ of Corti protein1) harbors a consensus F-box motif which is a subunit of a SCF E3 ubiquitin ligase and that OCP1 binds to Cx26 in the Corti [96, 97]. However, further studies are needed to determine the role of this interaction in Cx26 trafficking and degradation.

1.1.3.2.3 Degradation of Connexins

Both lysosomal and proteasomal degradation pathways have been reported to be involved in connexin degradation [98]. The role of lysosomes in degrading connexins is prominent. Electron microscopic studies showed that connexosomes are able to fuse directly with lysosomes, providing convincing evidence that lysosomes play a critical role in connexin degradation [76, 99, 100]. Based on inmmunoelectron microscopic experiments, Leithe et al. suggested that internalized Cx43 gap junctions undergo a maturation process from double-membrane vacuoles to multivesicular endosomes with a single limiting membrane [101]. This transformation is associated with trafficking of Cx43 from early endosome to late endosome, prior to lysosomal degradation of Cx43 [101]. Proteasomal degradation pathway plays a critical role in some cell types as evidenced by the fact that lysosomal inhibitors, such as chloroquine, leupeptin, and NH_4Cl, do not cause as much of an increase in the half-life of Cx43 as proteasomal inhibitors [102]. For example, in CHO cells, lysosomal inhibitors have modest effects on

the half-life of Cx43 while proteasomal inhibitors are more potent at extending the life of connexins [103]. In some cells types, such as MDA-MB-231 breast cancer cell line, both lysosome and proteasome play distinct roles in the life cycle of Cx43. Qin et al. demonstrated that in MDA-MB-231 cells, secretory Cx43 and internalized gap junctions were degraded by lysosomes, while destabilization of phosphorylated gap junctions at the plasma membrane were associated with proteasomes [104]. How the cells select the degradation pathways of connexins is still not clear. Girao and Perira showed that phosphorylation of Cx43 stimulates proteasome-dependent degradation in lens epithelial cells [105]. Thomas et al. found a tyrosine-based sorting signal in the C-terminus of Cx43 that appears to be a prime determinant of Cx43 stability by targeting Cx43 for degradation in the endocytic/lysosomal compartment [106].

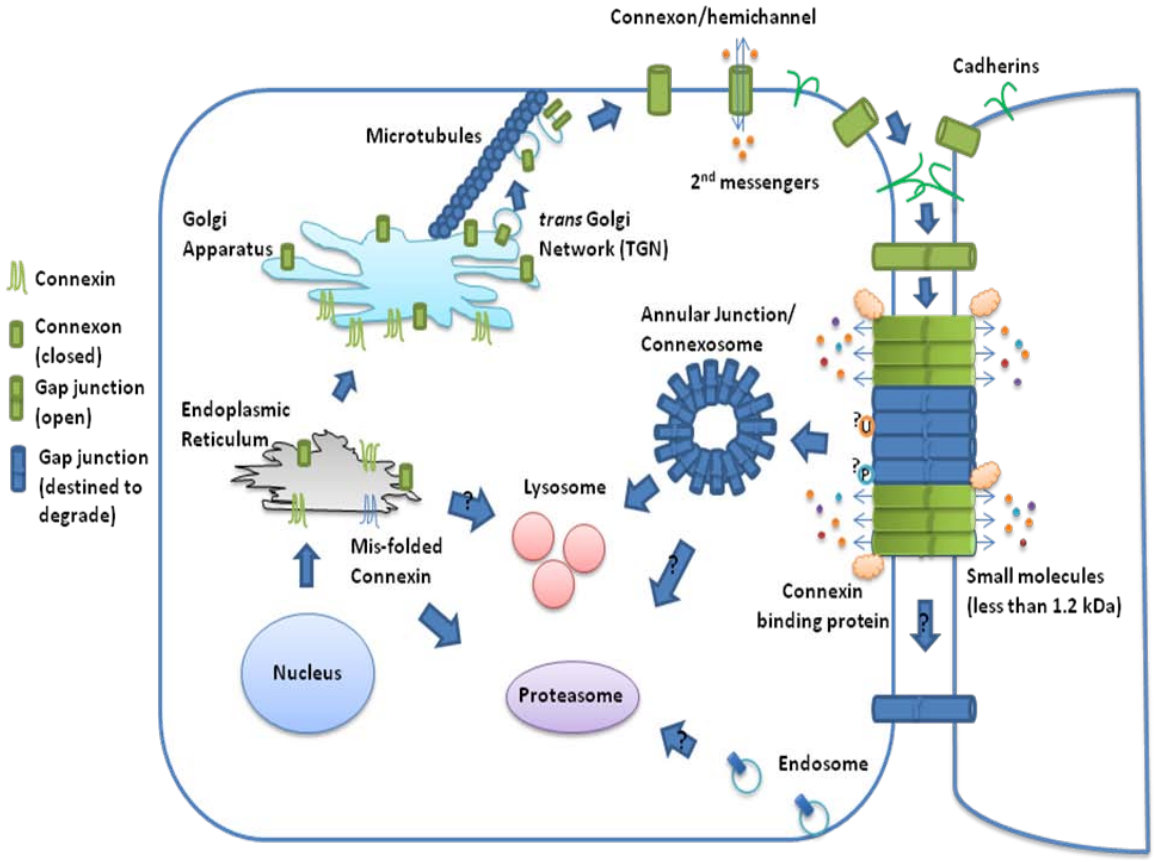

Figure 1.5 The life cycle of a connexin.

The connexin is synthesized in the ribosomes that are bound to the endoplasmic reticulum (ER) membrane and entered the classical secretory pathway. Connexin

topology is established in the ER where the protein is observed traversing the membrane four times. Connexin transport vesicles bud from exit sites of ER and fuse with the *cis* Golgi network of the Golgi apparatus. Oligomerization of connexins into connexons occurs in the ER, Golgi or *trans* Golgi network (TGN). Mis-folded connexins are translocated from ER membranes and degraded by proteasome. In some transformed cells with defective protein trafficking characteristics, connexins may be able to enter lysosomes for degradation. Closed connexons are delivered by transport vesicles to the cell surface with the help of microtubules. Connexon remains closed and diffuse throughout the plasma membrane until docking with another connexon in the adjacent cell to form a gap junction channel. The docking of connexons is facilitated by cadherin-based cell adhesion. Connexin binding proteins are proposed to play a role in regulating gap junction assembly and function. Gap junctions aggregate into plaques. The gap junction plaque is assembled from the outer rim and the inner gap junctions become internalized as double-membrane structures termed annular junctions or connexosomes. Other mechanisms of gap junction disassembly and internalization using clathrin, caveolae and endosomes have not been ruled out. Degradation of gap junctions is complex and evidence suggests roles for both proteasomes and lysosomes. Gap-junction-like membrane fragments have been identified in lysosomes. Connexin phosphorylation and ubiquitination have been reported to play important role in the proteasomal-degradation of Cx43. The life cycle of connexins has a very short life-time, ranging from 1 to 5 h.

1.1.4 Functions of Gap Junctions

1.1.4.1 Function of Gap Junction Channels

Gap junctional channels connect the cytoplasm of two cells, and provide a means for adjacent cells to exchange small molecules of less than 1.2 kDa, including H_2O, ions (K^+ and Ca^{2+}), second messengers (cAMP, cGMP, and inositol 1,4,5-triphosphate (IP_3)), and small metabolites (glucose), allowing electrical and biochemical coupling between cells [107, 108]. According to the properties of the molecules transported by the gap

junctional cell-cell communication, the functions of gap junctions in tissues can be categorized as follows:

1) Rapid exchange critical ionic electrical signals, such as Ca^{2+}, and their regulators. By gap junctional communication, waves of Ca^{2+} ions can move from a disturbance point in a field of cells through cells to neighboring cells in order to signal the disturbance, so that the regional tissue reaction can occur in coordinated manner [109].

2) Allow the distributions of critical metabolites, such as cAMP, among tissue cells. Because all of the cells in a tissue do not possess the same metabolic capacity, gap junctional communication can help the cells of lower capacity benefit from those with higher capacity [110]. Therefore, gap junction-mediated sharing of essential metabolites is beneficial to the tissue as a whole.

3) Nourish the sick or deprived cells by healthy neighboring cells. When the gap junctional intercellular communication is not compromised and the toxicity limit is not exceeded, the cell injured is remediated with assistance from healthy neighboring cells, so that a tissue can recover after a toxic insult, keeping the homeostasis of the tissue.

4) Alleviate the harmful effects of xenobiotic chemicals. By dispersing xenobiotic molecules from the exposure entrance point into the tissue via gap junctions, the local concentration of offending xenobiotics are diluted and easy to be metabolized in more cells and in a steady-state fashion.

5) Eliminate unwanted byproducts. By gap junctional communication, byproducts are eliminated from tissue interior cells to the vascular system in a gradient fashion for purpose of excretion.

1.1.4.2 Function of Hemichannels

In addition to gap junctional intercellular communication, connexin-forming hemichannels function as independent entities between intra- and extracellular milieus, which play critical roles in modulating cellular functions. Many functions have been reported to be related to hemichannels, including isosmotic cell volume regulation, inhibition of the activity of Ca^{2+}-channels and glutamate release in the retina, rescue from apoptosis, regulation of glutamate and aspartate release in astrocytes, and differentiation

of teratocarcinama progenitor cells into neuronal and nonneuronal cells [111-116]. Hemichannels regulate cellular functions probably by the factors released through the open hemichannels. The released factors bind to the receptors on cell surface, which accordingly lead to the activation of intracellular signaling pathways and regulation of cellular function and physiology through gene transcription, translation, or post-translation. Recent studies showed that connexin hemichannels release molecules like PGE_2, ATP and NAD^+ in response to stimuli such as mechanical stimulation, lower extracellular calcium, lower pH or metabolic inhibition [117]. ATP mediates cochlear homeostasis, ischemic preconditioning, and cell cycle through G-protein coupled purinergic receptor signaling [117]. PGE_2 activates β-catenin, and subsequently induces Cx43 transcription [117]. NAD^+ mediates cADPR, a factor related to cell cycle progression [117]. Detailed signaling pathways associated with hemichannel communication are still under investigation.

1.2 Gap Junctions and Diseases

1.2.1 Overview

Mutations in connexins and dysregulation of gap junctions are associated with many diseases. At present, ten distinct diseases have been linked to gene mutations of connexin family members, such as demyelinating neuropathies, various skin disorders, cataracts, sensorineural deafness, oculodentodigital dysplasia (ODDD), and so on. The first discovered human disease caused by gene mutation in connexin is chromosome-X-linked Charcot-Marie-3 Tooth disease, a peripheral neuropathy [118]. This disease is related to over 270 mutations in Cx32 and clinically manifested by progressive peripheral axon demyelination and limb weakness [119-121]. After that, a broad scope of human diseases has been reported to be induced by connexin mutations. Up to 40-50% of all cases of inherited neurosensory deafness are associated with mutations in the genes encoding Cx26, Cx30, Cx31, Cx32 and Cx43 [122]. Pelizaeus-Merzbacher-like disease is associated with gene mutations of Cx47 [123]. Skin disorders, including keratitis-ichthyosis and palmoplantar keratoderma are linked to mutations in Cx26, Cx30, Cx30.3, Cx31, and Cx43 [124]. The skin diseases caused by the connexin mutations are always

accompanied by other diseases, like deafness, erythrokeratodermia variabilis, hidrotic ectodermal dysplasia and Clouston's syndrome [124]. Congenital cataracts are related to mutations of Cx46 and Cx50 in lens [125]. ODDD which is occasionally accompanied by palmoplantar keratoderma is associated with Cx43 mutations [126]. Lastly, atrial fibrillation is reported to be associated with somatic mutations in Cx40 [127]. The connexin-linked diseases and the related connexin mutations are listed in Table 2.

Connexin-linked diseases	Gene mutations in connexins
Peripheral neuropathy (X-linked Charcot-Marie-Tooth disease)	Cx32β1
Deafness	Cx26β2, Cx30β6, Cx31β3, Cx32β1, and Cx43α1
Skin disorders	Cx26β2, Cx30β6, Cx31β3, Cx30.3, and Cx43
Cataracts	Cx50α8 and Cx46α3
Oculodentodigital Dysplasia (ODDD)	Cx43α1
Atrial fibrillation	Cx40
Pelizaeus-Merzbacher-like disease	Cx47

Table 2 Connexin-linked diseases and the connexins with related gene mutations

Apart from the connexin-linked disease associated with gene mutations, many diseases, termed connexin-dysregulated diseases, are related to changes in connexin expression level, assembly state, localization, and deficiency in gap junctional intercellular communication. One example of the connexin-dysregulated diseases is illustrated by cardiovascular disease which is caused by the changes in the gap junction distribution and remodeling. In advanced ischemic disease, the normal distribution of gap junctions in the narrow zone consisting of 5 layers of cells bordering healed myocardial

infarctions was disrupted with a shift of Cx43-containing spots to the lateral cell borders [128]. In a guinea pig model of congestive heart failure, a 37% reduction of Cx43 was observed at the congestive heart failure stage [129]. Matsushita et al. found that in rat ventricular cells bordering healed infarcts, many of the lateral gap junction plaques have no contributions to cell-to-cell communication, suggesting that lateralization of gap junctions is a prominent feature of diseased myocardium [130]. Other studies showed that protein kinases and phosphatases play an important role in disease state. In myocardial ischemia, Cx43 becomes increasingly less phosphorylated, redistributes to the lateral cell surfaces and accumulates in intracellular compartments [131]. Later studies by Solan et al. revealed that Cx43 phosphorylation at S365 was lost in ischemic hearts, while phosphorylation at S368 was increased [132]. Kieken et al. reported that scaffolding to Cx43 by ZO-1 is competed by active c-Src binding to ZO-1, leading to Cx43 relocalization to lateral cell surface and finally result in arrhythmogenesis [133]. All these examples reveal that cardiac pathologies are closely related to connexin redistribution and remodeling.

Another important example of connexin-dysregulated diseases is cancer. Numerous reports have provided evidence to establish the relationship between connexin dysregulation and cancer.

1.2.2 Gap Junctions and Cancer

Cancer is a complicated disease which is caused by multiple different mechanisms related to cell differentiation, regulation and growth control. Because gap junctional intercellular communication (GJIC) has been associated with control of cell growth, development, differentiation and homeostasis, it is supposed that there may be a link between cancer and GJIC. The assumption first originated from Loewenstein and his colleagues who found the lack of cell coupling in rat and human hepatoma cells, tumors of the thyroid in rodents, and carcinoma of the stomach in humans [134-136]. Based on several studies in 1960s, Loewenstein hypothesized that lacking of gap junctions or GJIC is a prerequisite to the dysregulation of growth control, and is related to tumorigenesis. This hypothesis was formulated and documented into a prescient review in 1979 [137].

Since then, a tremendous accumulation of results have been reported to demonstrate the important roles of connexins and GJIC in cancer.

Through the years, a phenotypically heterogeneous population of neoplastic cells generates, forming a tumor. Considering the progression of solid tumors, cancer development can be schematically separated into three fundamental stages: growth of the primary tumor, invasion, and dissemination (metastasis). These three fundamental stages of solid tumor progression are representative of three very different phenotypes of cancer cells: deregulated growth (tumor formation), motility (invasive tumor), and interaction with both the endothelial barrier of blood or lymphatic vessels and the cells of the colonized organs (metastatic tumor). Because of the different phenotypes, it is possible that the junctional behavior of cancer cells is different at every stage. For example, disruption of intercellular junctions may be necessary for cell detachment and motility during cancer invasion, whereas establishment of cell-cell contact between cancer cells and endothelial cells would be needed during cancer metastasis. Thus, the role of gap junctions might be different depending on the cancer stage.

1.2.2.1 Gap Junctions and Primary Tumor Growth

Over the past four decades, a considerable amount of studies exploring the link between gap junctions and tumor growth have reported that gap junctions and/or connexins play a tumor suppressing role. Using various tumor cell lines and transgenic mouse strains, many kinds of evidence have been provided to support the idea of a tumor suppressor function for gap junctions and/or connexins. For example, gap junctions and/or connexins are absent or greatly reduced in many primary tumors as well as in cell lines that are derived from primary tumors [138-140]. Additionally, it has been known for a long time that multiple tumor-promoting agents (such as 12-*O*-tetradecanoylphorbol-12-acetate (TPA) and cigarette components) and oncogenes (such as ras, v-mos, neu, src, little and large T, and E1A) down-regulate connexin expression and inhibit GJIC in cancer cells, whereas antitumor agents (such as retinoic acid and retinol) up-regulate GJIC [141-144]. Furthermore, reintroduction of connexins into tumor cells reduces cell proliferation and tumor growth, and partially re-differentiates transformed cells [145-148]. Transfection of connexin 32 cDNA into communication-deficient human

hepatocellular carcinoma SKHep1 cells significantly retarded xenograft tumor growth *in vivo* [149]. Differential or subtractive gene profiling, as well as gene knockdown studies, also identified connexins as potential tumor suppressors [150, 151]. Lastly, recent studies using transgenic and mutant mouse models showed that connexin-knockout mice, as well as mice expressing reduced or dominant-negative connexins, have an increased incidence of tumor onset when challenged with a carcinogen [152-155]. The maintenance of a full complement of connexins is likely to be protective against tumor onset. This might be illustrated by the evidence that mice lacking Cx32 are more susceptible to chemical- and radiation-induced liver and lung carcinomas [152, 153], as well as the evidence that $Cx43^{+/-}$ mice have an increased incidence of chemically induced lung neoplasm [154]. Collectively, these studies provide evidence that connexins and/or gap junctions exhibit tumor suppressive properties in tumorigenesis.

The mechanisms by which connexins play a tumor suppressing role in tumorigenesis were originally proposed to be GJIC-dependent [156, 157]. The primary role of connexins is linked to the formation of gap junction channels which mediate the intercellular exchange of small molecules. Therefore, it is not surprising that the mechanism associated with tumor suppressing role of connexins is supposed to be related to the specific molecules that are exchanged among healthy cells through GJIC compared with the lack of such exchanges in cancer cells without GJIC. This leads to the studies, identifying molecules that need to be exchanged for cells to maintain a normal cell cycle and inhibit the transformation of cells into invasive cell types. However, so far, few molecules have been identified to transport through gap junctions. Among them, some molecules are involved in very fundamental and metabolic pathways of the cells, such as adenosine diphosphate, and cannot help to understand any putative role of connexins in carcinogenesis [158], whereas other molecules play a more informative role. For example, the glutathione, a tripeptide, has high permeability through gap junction channels. Its antioxidant properties protect cells from reactive oxygen species, thus protecting cells from DNA damage, as well as detoxifying carcinogens [159, 160]. In addition, the gap junctions transport second messengers, such as cAMP and IP3, suggesting that gap junctions are involved in the tissue homeostasis and in the intercellular diffusion of hormonal information [161, 162].

Studies using inhibitors of GJIC and connexin mutants lacking the ability to form gap junction channels suggest that at least in some cases, connexins exhibit the tumor suppressing functions through a GJIC-independent pathway [163-166]. For example, Moorby and Patel reported that wild-type Cx43 inhibited growth of Neuro2a cells under conditions where gap junctions were unable to form [167]. Moreover, the C-terminus of Cx43 which could not form gap junctions was as effective as the wild-type in suppressing the growth of Neuro2a cells [167]. This study provide direct evidence suggesting that growth regulation by Cx43 is independent of gap junction formation in Neuro2a cells. The mechanism of GJIC-independent function of connexins remains unknown. Several possible mechanisms have been hypothesized. First, the carboxyl terminus of Cx43 interacts with transcription factors and regulates gene expression. The connexins that exhibit cell growth suppression function have been localized to the cytoplasm or nucleus and the putative nuclear-targeting sequence encoded within the carboxyl terminus [167, 168]. Second, the inhibition of cell and tumor growth by connexins could be related to the involvement of connexins in the cell cycle regulation. Zhang et al. reported that over-expression of Cx43 contributed to an accumulation of the lypophosphated retinoblastoma (Rb) protein and an increase in the level of cyclin-dependent kinase inhibitor p27 [169]. The same group also reported that Cx43 inhibited expression of S phase kinase-associated protein 2 (skp2), a human F-box protein that promotes the ubiquitination of p27 [170]. Both Skp2 and p27 were required for Cx43 to inhibit cell proliferation, because Cx43 barely inhibited cell proliferation of $Skp2^{-/-}$ and $p27^{-/-}$ cells [170]. Furthermore, inhibition of skp2 expression was observed by using C-terminal domain of Cx43, a truncated Cx43 that cannot form gap junctions, and the inhibitory effect cannot disrupted by the coupling inhibitors, indicating the GJIC-independent mechanism of Cx43 in the cell cycle [163, 170]. Third, phosphorylation of connexins may be another factor associated with the GJIC-independent mechanism. Phosphorylation of Cx43 on S262 by protein kinase C (PKC) counteracted the inhibition of HEK-293 cell growth by Cx43 through a GJIC-independent pathway [171]. Studies of Cx43 transfection in human glioblastoma cell revealed that transfected Cx43 normalized cell phenotype through an accumulation of the unphosphorylated Cx43 in the cytoplasm and even in the nucleus [172]. Last, the GJIC-independent effect of connexins may be related to the interactions

with tumor-suppressing molecules responsible for important signaling pathways. For example, Cx43 has been reported to interact with tumor-suppressing molecule, caveolin 1, in keratinocytes and suppress tumor growth [173, 174]. Cx43 has also been reported to interact with nephroblastoma overexpressed (NOV), a matricellular protein involved in cell signaling and communication that has been shown to suppress tumorigenesis [175, 176].

1.2.2.2 Gap Junctions and Cancer Invasion

The strong non-covalent links between docked connexons affords adhesive properties, suggesting that connexins may have effects on cell adhesion and migration [177, 178]. In the rat C6 glioma model, the level of Cx43 expression has been shown to be related to the mobility capacity of glioma cells. Results of wound healing and transwell assays demonstrated that cell migration was increased by the over-expression of Cx43 [179]. Lin et al. reported that Cx43 expression induced the morphological transformation of glioma cells into an epithelial phenotype, increased the competence of glioma cells to aggregate, enhanced the cell adhesivity, and mediated the invasion of malignant glioma cells [177]. The use of antibodies specific against the extracellular domain of Cx43 reduced cell aggregation in glioma cells and restored the connexin-deficient phenotype [177]. Further studies by Zhang et al. showed that when co-culture Cx43-C6 glioma cells and astrocytes, the astrocytic phenotype was modified [180]. It was suggested that this phenotypic transformation of astrocytes may contribute to their susceptibility to Cx43-C6 glioma invasion [180]. The acquired migration capacity is related to the presence of matrix metalloproteinases (MMP-2 and MMP-9) in culture medium from Cx43-C6 glioma cells [181]. Overall, a model using glioma cells provides evidence that connexins are associated with cancer cell migration and invasion.

In skin and prostate cancer cells, cell migration is associated with gap junctions and Cx26 expression [182, 183], suggesting that the connexin-mediated migration is not cell type or connexin isoform specific. Collectively, although the mechanism is not clear, all the studies provide evidence that increase of connexin expression and gap junctions can enhance cell migration and invasion in many cancer cell types. There are also some exceptions. One of the exceptions is that in Hs578T breast cancer cells, silencing Cx43

increased the cell migration capacity [151]. These studies add to the complexity in understanding the relationship between connexin expression and cancer cell motility.

1.2.2.3 Gap Junctions and Cancer Metastasis

In addition to the functions of connexins in tumorigenesis, a role for connexins in tumor metastasis is also suggested by many studies; however, they are controversial. One of the first steps of metastasis is that cancer cells interact with endothelial cells to pass through the vascular barrier and transport into lymph or blood flow. In this step, gap junction channels may be needed for the invasive cells to interact with endothelial cells. Actually, GJIC has been observed between vascular endothelial cells and malignant cells from various tumor types, such as glioma, melanoma, and breast cancer cells [184-186]. These observations indicate that the connexins and gap junctions, which are frequently lost in the primary tumors, may appear at later stage of cancer progression. This has been illustrated by the evidence that in mouse skin carcinogenesis, the reduced Cx26 at the early stage is restored in tumor metastases in lymph nodes [187]. Same results were also observed in breast cancer. During the progression of human breast cancer, Cx26- and Cx43-negative primary tumors were found to develop Cx26- and Cx43-positive metastases in lymph nodes [188].

Angiogenesis is a preliminary and crucial step of the metastatic process. The role of connexins in angiogenesis remains controversial. Studies using breast cancer cells indicate that connexin expression plays a protective role against angiogenesis. For example, Shao et al. reported that the decreased expression of Cx43 in Hs578T breast cancer cells resulted in decreased levels of thrombospondin-1 (TSP-1, an antiangiogenesis molecule) and increased expression of VEGF [151]. Further studies by Qin et al. showed that Cx26 down-regulated the expression of connective tissue growth factor (CTGF), an angiogenesis-related gene, and increased TSP-1 [189]. These data suggest that connexins are function as inhibitors of angiogenesis in breast cancer cells. However, studies using glioma cells have a completely different result. Cx43 expression in Cx43-transfected T98G human malignant glioma cells has been reported to significantly increase tubulogenesis of co-cultured human umbilical vascular endothelial cells (HUVECs) [184]. The reason why Cx43 has opposite effects on angiogenesis in

breast cancer cells and glioma cells is not clear. However, even if they are contradictory, these results provided evidence for the complex functions of connexins in angiogenesis.

Cancer metastasis in part is involved in the interactions between metastatic cells and the cells of the targeting colonized tissue. Studies exploring cell-to-cell communication between MDA-MB-435 breast carcinoma cells and hFOB1.19 human osteoblastic cells indicated that gap junctions may play a role in these interactions. Kapoor et al. reported that MDA-MB-435 cells formed heterotypic gap junctions with osteoblastic cells [190]. This heterotypic GJIC was even quantitatively greater than the homotypic communication in bone [190]. Furthermore, the heterotypic GJIC was not significantly affected by the transfection of BRMS1, a breast cancer metastasis-suppressor gene [190]. Further studies are needed to check whether the heterotypic GJIC between metastatic cells and cells of the target tissue play a role in the dormancy of metastasis.

1.2.3 Gap Junctions and Breast Cancer

Breast cancer is one of the most malignant diseases that threaten the health of women all over the world, especially in western countries. It ranks the second leading cause of cancer deaths among women in the United States [191]. Mammary development is a process with the contributions of many factors. Recent studies showed that gap junctions play an important role in the mammary development and breast carcinogenesis.

1.2.3.1 Development of Mammary Gland

Human mammary glands and mouse mammary glands have similar structure, except the relative abundance of connective tissue to epithelial cells [192]. This similarity as well as the accessibility makes the mouse mammary gland as a good model to study the development and differentiation of human mammary glands. Studies showed that mammary gland development can be generally divided into four stages [193]. The first stage is from birth to puberty. In this stage, the gland is only composed of a rudimentary collection of small ducts, and the growth of the rudimentary tree is restricted until puberty. The second stage is from puberty to pregnancy. At the onset of puberty, systemic hormones induce the ducts to branch and elongate into the surrounding adipose tissue. Upon pregnancy, the third stage, full differentiation of the gland induces further

ductal branching and proliferation. The alveologenesis, in which lobulo-alveolar milk-secreting buds sprout at the forefront of the ducts, occurs in this stage. In the mature gland, a series of alveoli join up to form a group known as lobules, and each lobule drains into openings in the nipple through a common ductal system. The ducts and alveoli are lined by single layers of mammary epithelial cells. An outer layer of myoepithelial cell surrounds the alveoli and provides contractile strength for milk ejection when the alveolar luminal cells secrete milk into the lumen during lactation. The fourth stage is post-weaning. After weaning, the structure of the glands returns to the pre-pregnancy architecture due to the extensive apoptosis and autophagy. The third and fourth stages are cyclic with each pregnancy and birth. Figure 1.6 shows all the stages of mammary glands development.

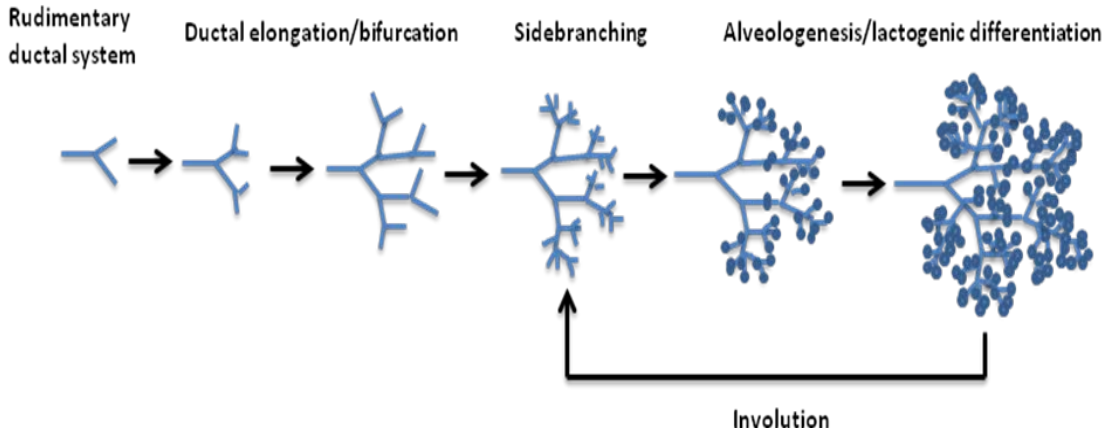

Figure 1.6 Scheme of mammary gland development.

Four stages are included in mammary gland development. Before puberty, the gland mainly consists of rudimentary ductal system. On the set of puberty, ductal elongation and bifurcation occur. At pregnancy, the gland undergoes alveologenesis and lactogenic differentiation, changing the mammary gland to a structure that is suitable for lactation. After weaning, during the involution, the gland remodels to regain a pre-pregnancy structure.

1.2.3.2 Role of Gap Junction in the Development of Mammary Gland

Many factors contribute to the regulation of mammary gland development and differentiation, including systemic hormones and paracrine factors, tight and adherens junctions, and gap junctions. Both the function of gap junction channels and connexin

expression are regulated throughout the development of mammary gland. The functions of gap junctions in mammary development and differentiation are not only related to the GJIC, which is important to the cross-talk between cells, but also concerned with the signaling cascades regulated by connexin-associated molecules.

1.2.3.2.1 Connexin Expression in Mammary Gland

Up to date, only two connexins, Cx26 and Cx43 were identified in human mammary gland [194]. Both the Northern analysis and immunocytochemistry showed the expressions of Cx26 and Cx43 in normal mammary epithelial cells [195]. Further studies of dye transfer indicated a functional channel formed by connexins between epithelial cells [195]. Monaghan et al. reported a localization of Cx43 in myoepithelial cells and a predominant expression of Cx26 between the luminal cells of ducts [196]. Cx43 was also reported to express in normal human mammary fibroblasts [195].

Unlike the human mammary gland, in addition to Cx26 and Cx43, another two connexins, Cx32 and Cx30, were reported in the mouse mammary gland [197]. In the mouse mammary gland, Cx43 channels were identified between myoepithelial cells and Cx26 channels were found between luminal cells, which are similar to the locations in human mammary cells. However, Cx30 and Cx32 were also reported to express in luminal cells, peaking during lactation [194]. *In vitro* studies showed that Cx32, Cx30 and Cx26 had abilities to form heteromeric connexons [198]. In the study by Talhouk et al., Cx43 was also found to form gap junction between myoepithelial cell and epithelial cell at several developmental stages; however, this finding is somewhat controversial [197].

1.2.3.2.2 Connexin Function in the Stages of Mammary Gland Development

The expression profiles of various connexins in different stages of mammary gland development suggest the possible functions of connexins. For example, Cx43 is expressed throughout development, indicating it may play a role in the growth and differentiation of myoepithelial cells. Cx43 is down-regulated at mid-pregnancy and almost disappears during lactation but is re-expressed after involution [199]. However, expressions of Cx26, Cx30, and Cx32 increase throughout pregnancy, peak at the onset of lactation, and then decline in involution, suggesting that these connexins may be

essential to milk production and/or secretion [197]. To further study the distinct functions of connexins and gap junctions in the mouse mammary gland, knockout and transgenic mouse models are used. Cx43 knockout mice were lethal; however, the Cx43KI32 mice, in which Cx43 was replaced with Cx32, sheds some light on Cx43 function. The Cx43KI32 mice had normal mammary gland development and milk production, but had impaired milk ejection, indicating that Cx43 played a role in providing contractile force to the myoepithelial cells for milk ejection [200]. The mouse model (Gja $1^{Jrt/+}$), which has an autosomal dominant mutation in Cx43 and is used to mimic the human disease oculodentodigital dysplasia (ODDD), also provided a sight to the role of Cx43. In this model, Cx43 mutation induced a delay in mammary gland development, a failure in milk delivery, but no changes in morphology of the gland, indicating the involvement of Cx43 in milk ejection. Cx26 knockout mice were lethal too. However, a conditional knockout of Cx26 in the mammary epithelium before puberty led to deficiency in lobuloalveolar development and function during lactation, whereas ablation of Cx26 during the pregnancy had no effects on alveolar development and function [201]. Similarly, Cx32-null mice had normal development and function of mammary gland, indicating that Cx26 and Cx32 may compensate for each other during later stages of pregnancy and lactation [201]. Further studies by Locke at al. showed that Cx26 and Cx32 formed heteromeric Cx26-Cx32 connexons in the luminal epithelial cells of the mouse mammary gland [198, 202]. Locke and his colleagues found that at the onset of parturition, Cx26 was predominant in the heteromeric connexon; however, the Cx32 ratio increased throughout the lactation and heteromeric connexons were finally shifted into homomeric Cx32 channels, which have wider pores than homomeric Cx26 channels [202]. Cx30 can form heteromeric channels with both Cx26 and Cx32. Compared to Cx26 channels, both Cx32 homomeric channels and Cx26-Cx30/Cx30-Cx32 heteromeric channels are more insensitive to taurine, an amino acid used for osmolytic balance during milk protein synthesis, indicating an important role for Cx30 or Cx32 at the lactation stage [203].

Both the GJIC-dependent and GJIC-independent functions of connexins have been found to involve in the regulation of mammary gland development. El-Sabban et al. reported that enhanced GJIC induced partial differentiation of mammary epithelial cells, in the absence of exogenouly provided basement membrane [204]. The same group also

reported that heterocellular interaction between SCg6 cells and SCp2 cells increased the association of connexins with α-catenin and ZO-2, leading to the recruitment of β-catenin into the gap junctional complex, which prevented translocation of β-catenin to the nucleus, stabilized gap junctions, and contributed to the GJ-induced functional differentiation of mammary epithelial cell [205].

Collectively, all these findings indicate that connexins have stage-specific roles in the development and differentiation of normal mammary glands.

1.2.3.3 Role of Connexin in Breast Cancer

Subtypes of Breast Cancer

Human breast tumors are diverse in the epidemiological risk factors, the development histories, and the responses to therapies. The tumors could be classified into subtypes by genetic array testing or immunohistochemistry [206-209]. Based on global gene expression analyses, four molecular intrinsic subtypes of breast cancer, including Luminal A, Luminal B, HER2-enriched (HER2 is also known as ERBB2), and Basal-like, have been identified and intensively studied [206]. These subtypes are different in genomic complexity including key genetic alterations and clinical prognosis [210, 211]. Although genetic array testing is a powerful tool to recognize the subtype of breast cancer, it is not always feasible to obtain gene expression array information. Recently, a simplified classification proposed by Cheang et al. has been adopted [212]. The new classification is based on clinicopathological criteria, using immunohistochemical definition of estrogen receptor (ER) and progesterone receptor (PR), the detection of overexpression and/or amplification of the human epidermal growth factor receptor 2 (HER2) oncogene, and Ki-67 labeling index, a marker of cell proliferation, to identify tumor subtypes. Breast cancer subtypes defined by this method are similar to but not identical to intrinsic subtypes. Intrinsic breast cancer subtypes and their clinicopathologic definitions are listed in Table 3.

The diversity of the subtypes results in different metastatic behaviors. Follow-up studies of patients with early-stage breast cancer showed that the median durations of survival with distant metastasis were 2.2 years for Luminal A, 1.6 years for Luminal B, 1.3 years for Luminal/HER2 (Luminal B/HER2 positive), 0.7 years for HER2-enriched,

and 0.5 years for Basal-like [213]. The metastatic sites vary among different subtypes. Bone was the most common metastatic site in all subtypes except Basal-like [213]. Both HER2-enriched and Basal-like tumors have higher rate of brain and lung metastases, and HER2-enriched tumors has high rate of liver metastasis [213].

Because the factors of breast cancer, including histological grade, invasiveness and metastasis, hormone receptor and HER2 status, and proliferation, are determinants for the chemotherapy, the diversity of these factors in subtypes makes the response to chemotherapy differed by subtype. Recently, it has been widely accepted that Luminal A subtype is less responsive to chemotherapy [214]. For Luminal B disease, both anthracyclines and taxanes have been considered to be used in the chemotherapy. These anti-cancer agents were also recommended to the treatment of HER2 positive disease. Moreover, the use of trastuzumab, a specific antibody which targets HER2/neu protein on the surface of HER2 positive cells, is a standard adjuvant treatment for patients with HER2 positive disease [215]. One year of trastuzumab therapy has been regarded as an optimal adjuvant therapy for HER2 positive patients [215]. For Basal-like (triple negative) disease, in addition to the anthracyclines and taxanes, an alkylating agent is also used, especially for triple negative tumors with BRCA1 mutation [216]. Treatment recommendations for all subtypes are listed on Table 3.

Intrinsic Subtypes	**Clinicopathologic Definition**	**Type of Therapy**
Luminal A	Luminal A ER and/or PR positive, HER2 negative, Ki-67 low	Endocrine therapy alone
Luminal B	Luminal B (HER2 negative) ER and/or PR positive, HER2 negative, Ki-67 high	Endocrine ± cytotoxic therapy
	Luminal B (HER2 positive) ER and/or PR positive, HER2 over-expressed or amplified, any Ki-67	Cytotoxics + anti-HER2 + endocrine therapy

HER2-enriched	HER2 positive (non luminal) ER and PR absent, HER2 over-expressed or amplified	Cytotoxics + anti-HER2
Basal-like	Triple negative (ductal) ER and PR absent, HER2 negative	Cytotoxics

Table 3. Intrinsic subtypes of breast cancer, clinicopathologic definitions, and relative type of therapy.

Breast Cancer and Connexin

Because gap junctions and connexins play stage-specific functions in the development and differentiation of normal mammary glands (as discussed above), it is not surprising that loss of connexin expression and deficiency of GJIC may contribute to breast carcinogenesis. Mammary gland a valuable model for the investigations of gap junction and/or connexin functions in every stage of cancer, from onset to progression and metastasis.

In breast carcinogenesis, connexins have long been considered as tumor suppressor genes, due to the aberrant expression or localization of connexins and the deficiency of GJIC in breast cancer cell lines and tissues. However, recent data reported that invasive breast carcinomas express high levels of connexins, indicating that connexins may play a different role at the late stage of carcinogenesis [188, 217, 218]. Until now, it is generally accepted that aberrant connexin expression and impaired GJIC promote tumorigenesis at the initial steps of this process [194, 219], whereas, although some contradictory results have been reported [220, 221], expression of connexins and functional GJIC are found to be crucial, at later stages, for migrating cells to invade, interact with endothelial cells, and metastasis [186, 217, 219].

Several studies using both primary breast cancer cell lines and breast cancer patient tissue samples reveal a loss of overall connexin protein expression or a re-localization of the connexin to intracellular compartments, resulting in a loss of GJIC compared to normal mammary cells and breast tissues [139, 150, 222, 223]. For example, Cx26 and Cx43 have been reported to be down-regulated in primary cells derived from human breast tumors as well as in rat mammary tumors and breast cancer cell lines [139,

224]. Over-expression of Cx26 in GJ-deficient human MCF-7 breast cancer cells, which maintain the phenotype of early-stage cancers, restored cell-cell communication and reduced cell malignant properties, including the proliferation rate, the saturation density, and the anchorage-independent growth capacity [225]. Retroviral delivery of Cx26 and Cx43 to HBL100 breast cancer cell line that lacks connexin expression resulted in a dramatic inhibition of tumor growth *in vivo* [226]. Interestingly, this inhibition is independent of the formation of gap junctions [226]. All these results suggest a tumor-suppressive role for connexins at the early stage of carcinogenesis.

Contrary to a tumor-suppressive role for connexins, several studies have reported an increased connexin expression in breast cancer tissue. Cx26 and Cx43 expression was detected in more than 50% of the invasive breast carcinomas, as compared to normal tissue samples, although the location of connexins were cytoplasmic [227]. In addition, phosphorylated form of Cx43 was up-regulated in all cells of invasive breast carcinomas as well as in myoepithelial cells and transformed luminal cells of *in situ* carcinomas [228]. Furthermore, the levels of cytoplasmic Cx43 and Cx26 expressions have been correlated to advanced histological grade of the tumor, larger tumor size and poor prognosis in breast cancer patients [217, 229]. These results challenge the notion of connexins as tumor suppressors and lead to re-evaluation of connexin functions at the late stage of carcinogenesis. At metastatic stage, connexins have been shown to be highly implicated at intravasation and extravasation sites. For instance, Cx43 expression increased in tumor cell/endothelial cell contact areas *in vitro* and *in vivo*, where it marked the sites of metastases to the lungs [230]. In addition, GJIC via Cx43 increased the adhesion of breast cancer cells to lung endothelial cells. However, the adhesion was decreased in breast cancer cells with a dominant-negative Cx43 mutation, which has non-functional GJIC, indicating the importance of Cx43 and GJIC in metastasis [230]. Other studies showed that expressions of Cx26, Cx32 and Cx43 were up-regulated in lymph nodes metastases compared to primary tumors [188, 231]. 70% of Cx26, Cx32 and Cx43-negative primary tumors developed Cx26, Cx32, and Cx43-positive lymph node metastases, suggesting an increase of connexin expression during breast cancer progression resulting in a more malignant phenotype [231].

Collectively, all these reports indicate that connexins and gap junctions can function as either suppressor or enhancer in breast tumorigenesis, depending on the stage of tumor formation. The stage-specific roles of connexins and GJIC suggest that restoration of connexin expression and GJIC at primary stages of tumorigenesis might have beneficial effects in breast cancer therapy. However, considering the promotion function of connexins and GJIC on the late-stage disease, down-regulation of connexin and reduction of GJIC should be applied to inhibit the metastasis.

1.3 Connexins and Gap Junctions as Therapeutic Targets

As discussed above, connexins have both GJIC-dependent and GJIC-independent functions in the formation and regulation of connexin-related diseases, especially cancer, suggesting that they are potential therapeutic targets. Therefore, development of molecules and methods that can modulate connexin expression and GJIC has become a strategy in cancer treatment.

1.3.1 Therapeutic Approaches Related to GJIC

Although GJIC has paradoxical roles in carcinogenesis, either as a suppressor or facilitator, increasing evidence showed that up-regulation of GJIC at primary stages of tumorigenesis had beneficial effects in cancer therapy. The main strategy in GJIC-based cancer therapies relied on the “bystander effect”, a mechanism by which cytotoxic molecules or signals are transferred from target cells to neighboring cells. When there is lack of GJIC in cancer cells, the effects of drugs are limited to the target cells, whereas bystander effect provides a route for drugs to spread throughout a tumor mass and induce more cell death. It is an important factor which is related to drug sensitivity and efficacy. Bystander effect has been applied in gene therapy, radiation therapy, immunotherapy and chemotherapy. For example, in gene therapy, after enhancing connexin 43 (Cx43) and GJIC by 8-bromo-cyclic-AMP treatment, gene therapy effect was strengthened by herpes simplex virus thymidine kinase/gancyclovir (HSV-TK/GCV) system [232]. In radiation therapy, the bystander effect amplified cells’ responsiveness to radiation therapy through the transmission of damage signals (such as superoxide radicals, reactive nitrogen species, and other DNA damage molecules) from irradiated cells to non-irradiated cells

[233]. In immunotherapy, MCL (melanoma cell lysate)/TNF-stimulated human dendritic cells (hDCs) allowed melanoma antigen transfer via bystander effect, which facilitated antigen cross-presentation and dendritic-cell-mediated melanoma-specific T cell response [234]. The MCL/TNF-stimulated hDCs have been used in melanoma immunotherapy in patients and triggered tumor-specific immune responses [235]. In chemotherapy, bystander effect has been shown to potentiate the efficacy of anti-cancer drugs not only through transduction of the drugs, but also through the transmission of drug-induced death signals. A cisplatin-induced death signal involving the Ku70, Ku80 and DNA-dependent protein kinase complex, has been reported to be transmitted from the target cells to neighboring cells through gap junctions [236].

Several molecules, natural or synthesized, have been reported to up-regulate GJIC. The natural molecule resveratrol reversed the GJIC-blocking effects of TPA and DDT [237]. (-)-Epicatechin, a flavonoid found in green tea, stimulated GJICand counteracted GJIC inhibition by TPA in rat liver epithelial cells [238]. Combination of dibutyryl-cyclic AMP (db-cAMP) and all-*trans*-retinoic acid (tRA) enhanced GJIC and increased Cx43 expression [232]. 4-phenylbutyrate (4-PB), a HDAC inhibitor (HDACi) increased GJIC in pancreatic cancer cells [239]. In addition, diverse carotenoids have been reported to induce GJIC in fibroblast cells [240].

1.3.2 Therapeutic Approaches Related to Connexin Expression

Based on the anti-tumor growth effect of connexins in cancer, especially in primary cancer, restoration of connexin expression has been considered as a therapeutic approach in cancer treatment. Over-expression of connexins by molecules targeting transcriptional and post-transcriptional regulation pathways has been achieved in some cancer cell lines.

On the transcriptional level, inhibitors of HDAC, a group of enzymes involved in chromatin remodeling, have been reported to restore Cx43 expressions as well as GJIC. For example, The HDAC inhibitor Trichostatin A (TSA) restored Cx43 expression and GJIC in prostate cancer cells [241]. Another factor that correlates with connexin gene regulation is DNA methylation. The DNA demethylating agent 5-aza-2’-deoxycytidine (5-aza-C), was reported to restore Cx32 expression in human renal cell carcinoma (RCC),

and subsequently inhibit the xenograft tumor growth [242]. Connexin transcription is also regulated by proteins via specific signaling pathways. For instance, the AML1-ETO fusion protein was shown to transcriptionally up-regulate Cx43 expression through JNK signaling pathway [243]. Furthermore, a protein complex containing HSP90 and c-Myc was reported to interact with Cx43 promoter and up-regulate Cx43 through Ras-Raf-MAPK pathway [34].

On the post-translational level, studies of connexin restoration focus on the phosphorylation and degradation. BQ123, an antagonist of ET_A receptor (ET_AR), was shown to counteract Cx43 phosphorylatin and GJIC inhibition induced by endothelin-1 (ET-1), a ligand for the ET_AR, indicating the role of phosphorylation in connexin expression and GJIC function. [244]. Treatment with the proteasome inhibitor MG132 decreased Cx43 degradation, increased Cx43 expression, and sensitized cells to the pro-apoptotic effect of MG132, suggesting that inhibition of degradation pathways is an alternative strategy to restore connexin expression.

1.3.3 PQ1 as a Gap Junction Enhancer

In search of effective gap junction enhancers, we collaborated with Dr. Duy H. Hua in the Department of Chemistry at Kansas State University who synthesized a series of quinoline derivatives (named PQs). Computational docking studies showed that PQ1, 6-methoxy-8-[(3-aminopropyl)amino]-4-methyl-5-(3-trifluoromethylphenyloxy)quinoline, interacts with the partial crystal structure of connexon, indicating the potential role of PQ1 in gap junction [245]. Further biological studies showed that PQ1 increased GJIC and had cytotoxicity in human breast cancer cells. 200 nM PQ1 significantly increased GJIC in T47D breast cancer cells, but had no effect on the GJIC in normal mammary epithelial cells [245]. In addition to the effect on GJIC, PQ1 decreased cell viability, inhibited colony growth, and attenuated xenograft tumor growth of T47D cells; however, no cytotoxic effects of PQ1 were detected on HMECs [245, 246]. Combinational treatment of T47D cells with Tamoxifen and PQ1 synergistically decreased cancer cell proliferation, viability and colony growth [246]. All these reports suggest that PQ1 is a gap junction enhancer as well as a potential anti-cancer agent. However, there are still many questions which need to be answered before

we have a comprehensive understanding of the mechanism and function of PQ1: How PQ1 exerts its anti-cancer function? Can PQ1 potentiate the efficacy of anti-cancer drugs through bystander effect? Does PQ1 have side effects? What is the mechanism of PQ1 in the regulation of gap junction? Further studies are needed to answer these questions.

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Chapter 2 - Hypotheses and Objectives

2.1 Hypotheses

1. The anti-cancer effects of PQ1, 6-methoxy-8-[(3-aminopropyl)amino]-4-methyl-5-(3-trifluoromethylphenyloxy)quinoline, on T47D breast cancer cells have been reported; however, the mechanism of the anti-cancer effect is unclear. Many quinoline derivatives have been shown to induce apoptosis in cancer cells. PQ1 is a quinoline derivative; therefore, the hypothesis is that the anti-cancer effect of PQ1 is mediated via the apoptotic pathways in T47D breast cancer cells.
2. Further studies demonstrated that PQ1 acts as a gap junction enhancer by increasing GJIC in T47D cells. Therefore, the second hypothesis is that PQ1 can be used in the combinational treatment with anti-cancer drugs to increase the efficacy of anti-cancer drugs through bystander effect.
3. Minimal toxicity of PQ1 on normal human mammary epithelial cells (HMECs) was observed within the effective doses of anti-cancer effects and GJIC-mediated responses. Thus, the hypothesis is that PQ1 can be administered with low toxicity to normal organs.

2.2 Objectives

1. To determine the mechanism of PQ1 anti-cancer effect in T47D breast cancer cells
2. To evaluate the combinational effects of PQ1 and cisplatin
3. To examine the effects of PQ1 on normal organs

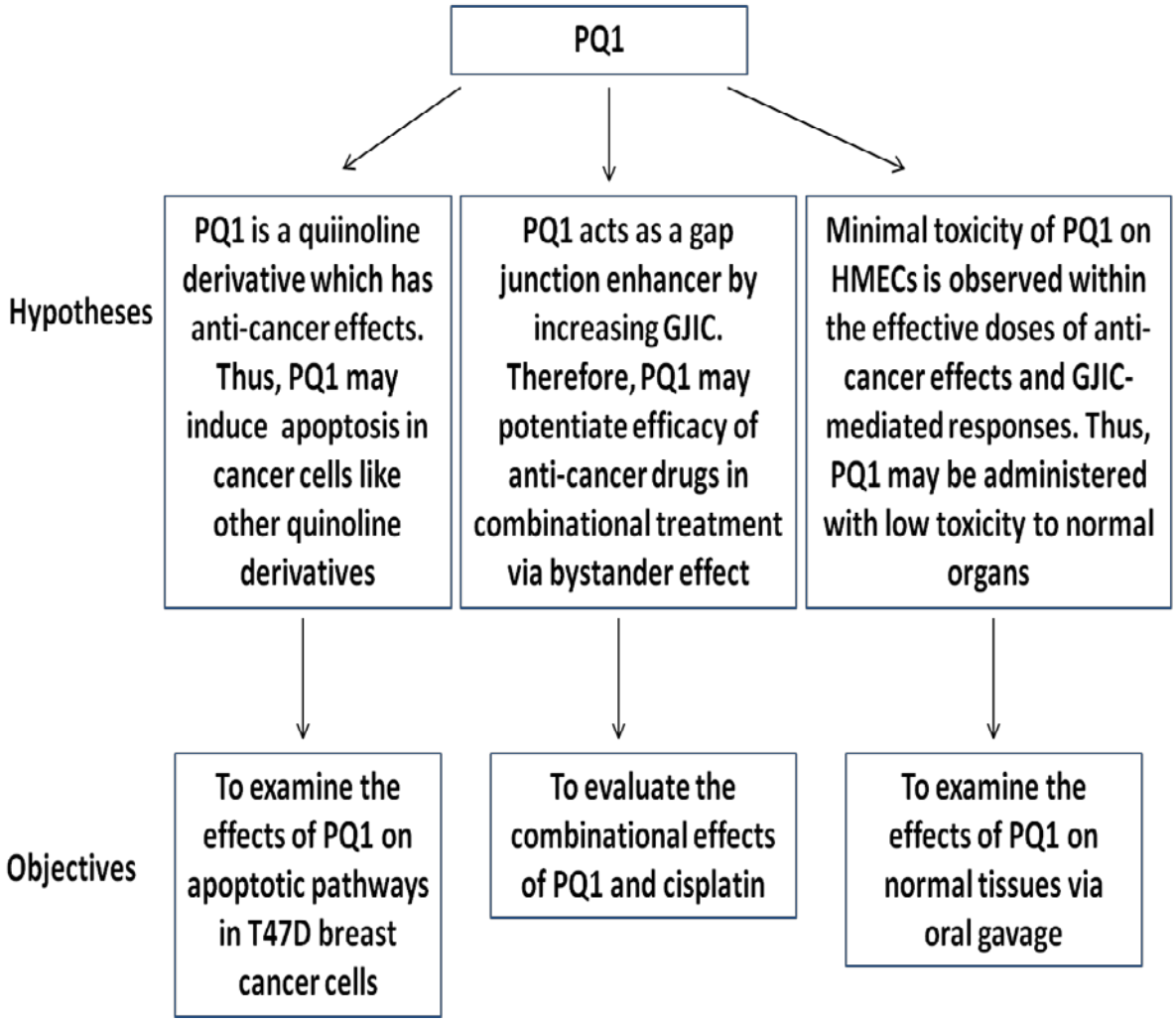


Figure 2.1 Schematic presentation of hypotheses and objectives

Chapter 3 - PQ1, a Quinoline Derivative, Induces Apoptosis in T47D Breast Cancer Cells through Activation of Caspase-8 and Caspase-9

3.1 Abstract

Apoptosis, a programmed cell death, is an important control mechanism of cell homeostasis. Deficiency in apoptosis is one of the key features of cancer cells, allowing cells to escape from death. Activation of apoptotic signaling pathway has been a target of anti-cancer drugs in an induction of cytotoxicity. PQ1, 6-methoxy-8-[(3-aminopropyl)amino]-4-methyl-5-(3-trifluoromethylphenyloxy)quinoline, has been reported to decrease the viability of cancer cells and attenuate xenograft tumor growth. However, the mechanism of the anti-cancer effect is still unclear. To evaluate whether the cytotoxicity of PQ1 is related to induction of apoptosis, the effect of PQ1 on apoptotic pathways was investigated in T47D breast cancer cells. PQ1-treated cells had an elevation of cleaved caspase-3 compared to controls. Studies of intrinsic apoptotic pathway showed that PQ1 can activate the intrinsic checkpoint protein caspase-9, enhance the level of pro-apoptotic protein Bax, and release cytochrome c from mitochondria to cytosol; however, PQ1 has no effect on the level of anti-apoptotic protein Bcl-2. Further studies also demonstrated that PQ1 can activate the key extrinsic player, caspase-8. Pre-treatment of T47D cells with caspase-8 or caspase-9 inhibitor suppressed the cell death induced by PQ1, while pre-treatment with caspase-3 inhibitor completely counteracted the effect of PQ1 on cell viability. This report provides evidence that PQ1 induces cytotoxicity via activation of both caspase-8 and caspase-9 in T47D breast cancer cells.

3.2 Introduction

Quinoline is a heterocyclic aromatic nitrogen compound which is often used for the design of many synthetic compounds with diverse medical benefits [1]. Recent studies found that numerous quinoline derivatives display potent anti-cancer activity by targeting different cellular pathways, including multidrug resistance, proliferation, and apoptosis [2-4]. Apoptosis is a programmed cell death, an important control mechanism of normal cell physiology [5, 6]. Deficiency in apoptosis is one of the key features of cancer cells [7]. Restoring and activating apoptosis in cancer cells is a major target of cancer treatment [8]. By using cell- and caspase-based high-throughput screening assays, Kemnitzer *et al.* found a new series of apoptosis inducers, the 1-benzoyl-3-cyanopyrrolo[1,2-α]quinolines, among which the compound 1-(4-(1*H*-imidazol-1-yl)benzoyl)-3-cyanopyrrolo[1,2-α]quinoline displayed high cytotoxic activity in T47D human breast cancer cells, HCT116 human colon cancer cells, and SNU398 hepatocellular carcinoma cancer cells [9]. Sharma *et al*. reported that a quinoline derivative, 8-methoxy primido[4',5':4,5]thieno(2,3-b)quinolin-4(3H)-one (MPTQ), induces apoptosis in K562 myeloid leukemia cell line and inhibits tumor progression in mice bearing different types of tumors [4]. These reports indicate that quinoline derivatives are potential anti-cancer drugs, targeting the induction of apoptosis.

Caspases, a class of proteases, play an essential role in the induction and execution of apoptosis. Caspase-dependent apoptosis can be generally divided into two signaling pathways: the intrinsic pathway and the extrinsic pathway [10]. The intrinsic pathway is initiated from within the cell and depends on the balance of the pro- and anti-apoptotic members of Bcl-2 family proteins. The internal signal, like DNA damage or severe cellular stress, causes pro-apoptotic protein, Bax, to migrate to the surface of the mitochondrion, where it inhibits the protective effect of anti-apoptotic protein Bcl-2. Bax generates holes in the mitochondrial membrane, which permits the leakage of cytochrome c to the cytoplasm. Cytochrome c activates caspase-9 and subsequently results in the cleavage of caspase-3 as the cell death executioner [11]. In contrast, the extrinsic pathway is triggered by a signal outside of the cells. These signals are pro-apoptotic ligands that bind to their specific receptors on the plasma membrane and activate the checkpoint protein caspase-8 [12]. Caspase-8 signal transduction eventually activates the caspase-3 to initiate cell death [12]. Some compounds trigger only one apoptotic pathway,

while others can activate both pathways. For example, the MPTQ is reported to trigger two signaling pathways by activating both caspase-8 and caspase-9 [4].

PQ1, 6-methoxy-8-[(3-aminopropyl)amino]-4-methyl-5-(3-trifluoromethylphenyloxy)-quinoline, has been reported to possess anti-cancer activity in breast cancer cells but not in normal human mammary epithelial cells (HMECs) (Fig. 3.1). Previous studies showed that one µM of PQ1 decreased cell viability to 50% in T47D breast cancer cells and attenuated 70% of T47D xenograft tumor in nude mice [13]. In the present study, we investigated whether the cytotoxicity induced by PQ1 is mediated through apoptosis. The results of Western blotting and immunofluorescence assays showed that PQ1 activated mitochondrial intrinsic pathways by increasing Bax, releasing cytochrome c from the mitochondria to cytosol, and subsequently activating caspase-9. Furthermore, PQ1 increased the level of active caspase-8. This report demonstrates that PQ1 induces cytotoxicity in T47D cells via both caspase-8 and caspase-9 mediated apoptotic pathways.

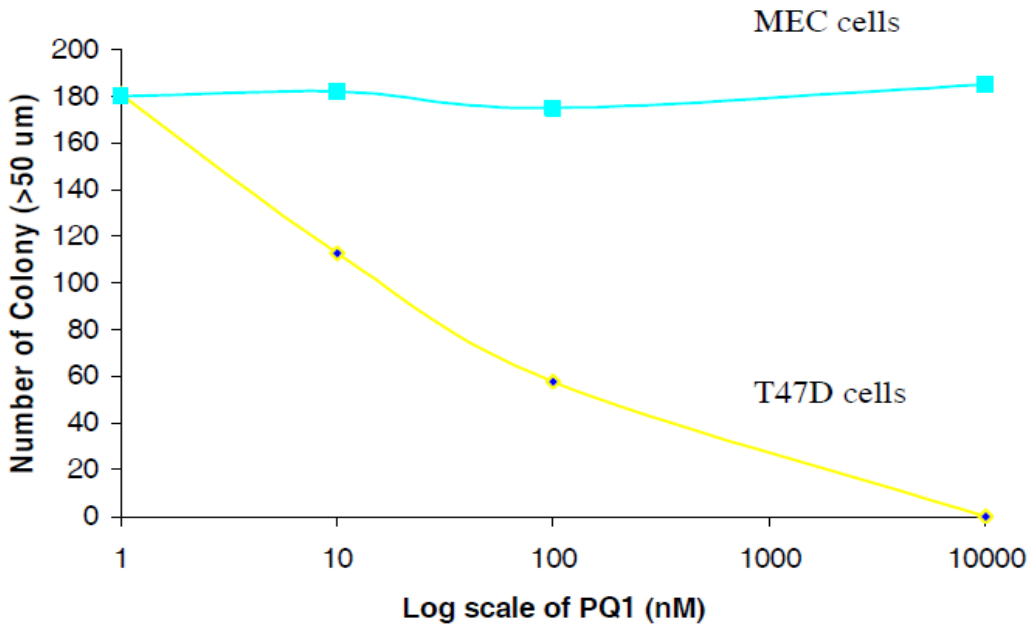


Figure 3.1 Effects of PQ1 on T47D breast cancer cell colony and human mammary epithelial cell (HMEC) colony

Base agar plates were prepared containing 0.8% agar and 0.4% agar in Ham's F12. HMECs and T47D cells (5 x 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°C for 7 days and examined for the presence of colonies. Individual colonies of 50 µm or greater were examined. HMECs and T47D cells were

treated with 1, 10 and 100 nM PQ1 and SA (succinic acid). Individual colonies of 50 μm or greater were examined. Statistical significance, $*p<0.05$, of at least three experiments. (Cited from dissertation of Aibin Shi, Kansas State University, http://hdl.handle.net/2097/1638)

3.3 Meterial and Methods

3.3.1 Reagents and Antibodies

PQ1, 6-methoxy-8-[(3-aminopropyl)amino]-4-methyl-5-(3-trifluoromethylphenyloxy)-quinoline, was obtained as described by Shi at al. [14] and graciously provided by Dr. Duy Hua (Kansas State University). Acridine orange/propidium iodide (AO/PI) dye for viability assay was purchased from Nexcelom Bioscience (Lawrence, MA, USA). Anti-cleaved caspase-3, anti-caspase-8 p18 (H-134), anti-caspase-9 p35 (H-170), anti-Bax, and anti-Bcl-2 antibodies were all obtained from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Rabbit anti-cytochrome c, HRP-linked anti-rabbit/mouse, anti-cleaved caaspase-8 (Asp391), and anti-cleaved caspase-9 (Asp315) antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Mouse anti-cytochrome c and rabbit anti-Cox IV antibodies were obtained from Abcam (Cambridge, MA, USA). Alexa-568-conjugated anti-rabbit IgG and Alexa-488-conjugated anti-mouse IgG antibodies were obtained from Invitrogen (Camarillo, CA, USA). Caspase-3 inhibitor (Ac-DMQD-CHO), caspase-8 inhibitor (Ac-IETD-CHO), and caspase-9 inhibitor (Ac-LEHD-CHO) were purchased from Enzo Life Sciences (Enzo Life Sciences, Farmingdale, NY, USA).

3.3.2 Cell Line and Cell Culture

T47D human breast cancer cell line was purchased from American Type Cell Culture (ATCC) (Manassas, MA, USA). Cells were grown in RPMI-1640 (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal bovine serum (Atlanta Biological, Lawrenceville, GA, USA), 1 mM sodium pyruvate, 10 mM hepes, 4.5 g/L glucose, 2 g/L sodium bicarbonate, and 0.2 units/ml bovine insulin. Cells were maintained in T-75 flasks at 37 °C with 5% CO_2 and cultured in 6-well plates or T-25 flasks for experimental analysis.

3.3.3 Cell Morphology

T47D cells were cultured in T-25 flasks until 80% confluent state. Cells were treated with DMSO and 100, 200, and 500 nM PQ1 for 24 and 48 hours. Cells without any treatment were used as controls. Cell morphology was captured using Nikon 80i light microscope.

3.3.4 Cell Viability Assay

Cell viability was measured using Acridine Orange/Propidium Iodide (AO/PI) staining method. T47D cells were cultured into 6-well plates until 80% confluent state. Cells were treated with DMSO and 100, 200, and 500 nM PQ1 for 24 and 48 hours. Cells without treatment were used as controls. Detached and trypsinized cells were combined to access the total cell viability after treatment. Samples were centrifuged at 2,000 rpm using a HERMLE Z300K centrifuge with rotor 221.05 V01 (Labnet, Woodbridge, NJ, USA) for 5 minutes. Media and trypsin supernatant were discarded and cell pellet was resuspended in 1 X phosphate-buffered saline (PBS). A cell suspension was mixed with AO/PI dye (5 μg/mL AO and 100 μg/mL PI in PBS) at 1:1 ratio. Viable and dead cells were visually examined by the Cellometer Auto 2000 (Nexcelom Bioscience, Lawrence, MA, USA). AO is a nuclear stain that is used to stain live cells and emits in the "green" range. PI is a fluorescent stain that only penetrates dead cells and emits in the "red" range. After taking both "green" and "red" fluorescent images, all fluorescent cells in each channel were counted and the concentration of live (green) and dead (red) cells as well as viability were determined.

3.3.5 Flow Cytometry

Apoptotic cells were analyzed by flow cytometry using the Alexa Fluor 488 Annexin V/Dead Cell Apoptosis Kit (Invitrogen, Camarillo, CA, USA). T47D cells were treated with DMSO and various concentrations of PQ1 for 24 and 48 hours. Cells were harvested and washed with cold PBS. After centrifugation, supernatant was discarded and cell pellets were resuspended in 1 X annexin-binding buffer to the final concentration at 1 x 10^6 cells/ml. After adding 1 μL of Alexa Fluor 488 annexin V and 1 μL of 100 μg/ml PI working solution into each 100 μL cell suspension, cells were incubated at room temperature for 15 minutes. 400 μL of 1 X annexin-binding buffer were added into the cell suspension after incubation. Stained cells were detected by flow cytometry measuring the fluorescence emission at 530 nm and 575 nm.

3.3.6 Mitochondria Isolation

Mitochondria were isolated by using Mitochondria Isolation Kit from Thermo Scientific (Rockford, IL, USA). Followed the manufacturer's instructions, control and treated cells were trypsinized and centrifuged at 850 x *g* using an Eppendorf centrifuge 5415R with rotor F-45-24-11 for 2 minutes. Cell pellets were treated with mitochondria isolation reagents. Cytosol and

mitochondria fractions were isolated by two consecutive centrifugation steps (700 x *g* and 12,000 x *g*, respectively) at 4 °C. After isolation, expressions of cytochrome c in mitochondria and cytosol were examined by western blot analysis.

3.3.7 Western Blot Analysis

T47D cells were cultured in T-25 flasks until 80% confluent state and then treated with DMSO and PQ1 as indicated for 48 hours. Cells without treatment were used as controls. Cells were washed with PBS for three times and harvested in lysis buffer (Cell Signaling Technology, Danver, MA, USA). Cell lysates were sonicated using Vibra-Cell sonicator (Sonics & Materials Inc, Danbury, CT, USA) and then centrifuged at 13,000 rpm using an Eppendorf centrifuge 5415R with rotor F-45-24-11 for 30 minutes at 4 °C. Supernatants were collected and measured for its total protein concentration. Thirty µg of samples were separated by 4-20% gradient SDS-PAGE for 35 minutes at 200 Volts, and transferred to nitrocellulose membranes (Midwest Scientific, Saint Louis, MO, USA). After blocked with 5% milk for 30 minutes, membranes were immunoblotted against protein of interest. Immunoreactions using chemiluminescence were visualized by FluoChem E Imaging Instrument (ProteinSimple, Santa Clara, CA, USA). Intensities of the bands were digitized using Un-Scan-It software (Silk Scientific Inc., Orem, Utah, USA).

3.3.8 Immunofluorescence and Confocal Microscopy

T47D cells cultured on coverslips in 6-well plates were treated with DMSO and PQ1 for 48 hours. Cells were rinsed by warm PBS and fixed with 2% paraformaldehyde for 20 minutes at room temperature. Fixed cells were washed 3 times with PBS and then permeabilized with 0.1% Triton X-100 for 8 minutes. Cells were washed 3 times with PBS again, and blocked with 2.5% BSA in PBS for 1 hour at room temperature. After blocking, cells were incubated with primary antibodies overnight at 4°C and Alexa-conjugated secondary antibodies for 1 hour at room temperature. DAPI was used for nuclei stain. The slides were mounted with prolong-antifade reagent (Invitrogen, Camarillo, CA, USA), sealed and observed under a confocal microscope (Carl Zeiss LSM 700 META, Narashige, MN, USA).

3.3.9 Caspase Inhibitor Assay

T47D cells were cultured into 6-well plates until 80% confluent state. Cells were pretreated with caspase-3 inhibitor (Ac-DMQD-CHO), caspase-8 inhibitor (Ac-IETD-CHO), or caspase-9 inhibitor (Ac-LEHD-CHO) at concentration of 20 μM for 1 hour, and then treated with 500 nM PQ1 for 23 hours. Cells without any treatments or treated with 500 nM PQ1 for 24 hours were used as controls. After treatment, cell viability was assessed by trypan blue method. Cells floated in the media were collected and cells attached to the wells were trypsinized. Two parts of cells were combined together, centrifuged and resuspended. A cell suspension was mixed with trypan blue dye (0.2% in PBS) at 1:1 ratio and viable cells were examined by the Cellometer Auto 2000 (Nexcelom Bioscience, Lawrence, MA, USA).

3.3.10 Statistical Analysis

Statistical analysis of data was performed using student's t-test Data presented were expressed as mean ± standard deviation (S.D.) of at least three independent experiments. Significance was considered at $p\text{-value} < 0.05$.

3.4 Results

3.4.1 PQ1 Changes Cell Morphology and Induces Cell Death in T47D Breast Cancer Cells

Cytotoxicity of PQ1 on T47D breast cancer cells was determined by observed morphological change and dual-fluorescence viability assay. T47D breast cancer cells were treated with DMSO and 100, 200, and 500 nM of PQ1 for 24 and 48 hours. Cells without treatment were used as controls. Differences in cell morphology were observed between PQ1-treated and control cells under 20X magnification of light microscopy. Morphological changes including cell rounding, shrinkage, and detachment were found in PQ1-treated cells in a dose-dependent manner (Fig. 3.2). Compared with cells treated with PQ1 for 24 hours, cells treated with the same concentration of PQ1 for 48 hours had more significant morphological changes (Fig.3.2). These results suggested that PQ1 induces morphological changes in a dose- and time-dependent manner. To determine if the changes of morphology were associated with cell death, cell viability was examined using dual-fluorescence (AO/PI) viability assay. The results showed that PQ1 decreased cell viability in a dose- and time-dependent manner as well. 100 nM PQ1 did not significantly reduce cell viability with 24-hour of treatment, but significantly reduced cell viability to 85% with 48-hour of treatment (Fig. 3.3). 500 nM PQ1 showed a 37% and 47% reduction of cell viability at 24- and 48-hour of treatment, respectively (Fig. 3.3). DMSO, a PQ1 solvent, showed no changes on cell morphology and viability at any time points (Fig. 3.2, 3.3). All these results indicate that PQ1 induces morphological changes and decreases cell viability in T47D breast cancer cells.

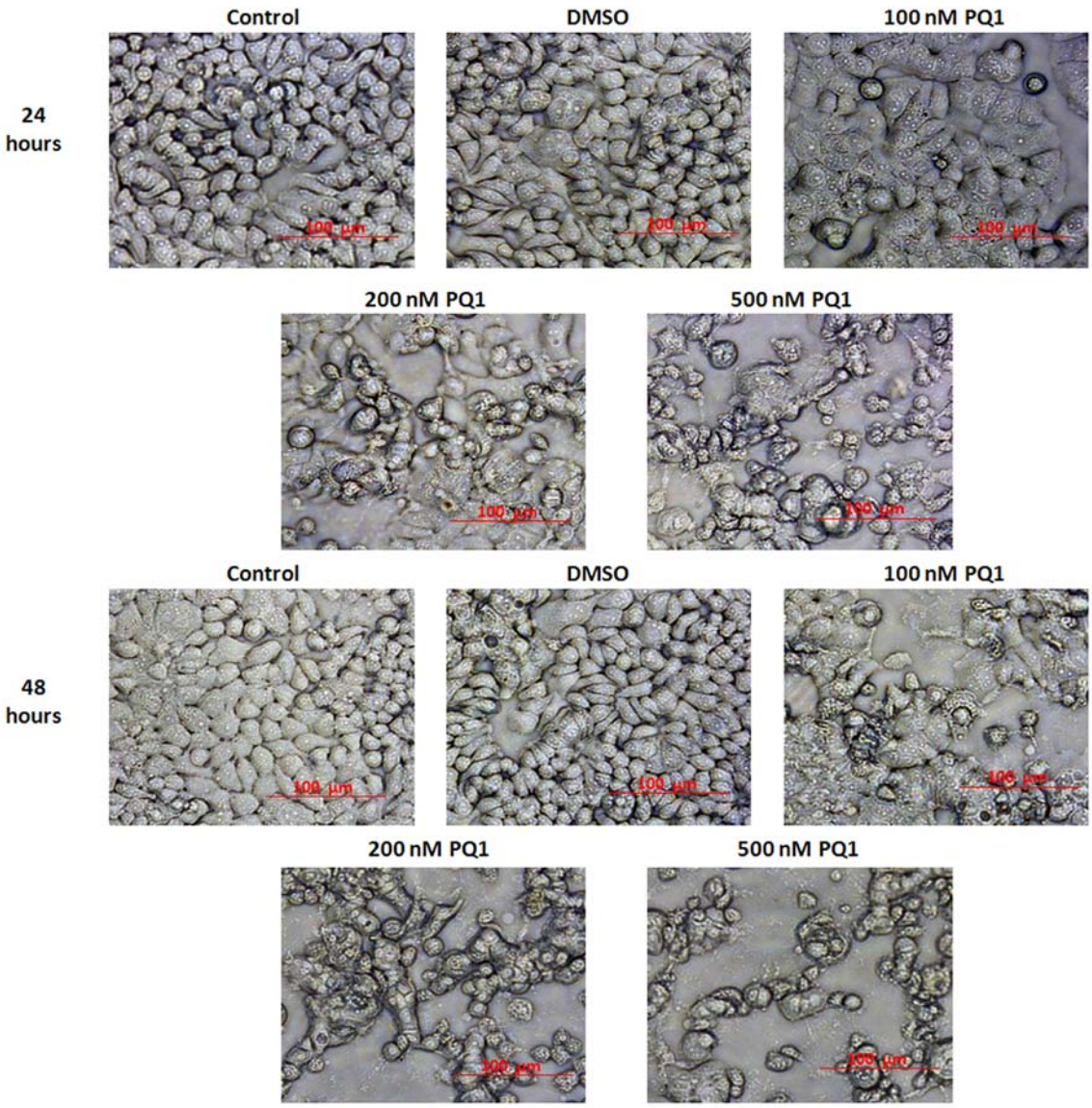


Figure 3.2 Effect of PQ1 on cell morphology in T47D breast cancer cells

T47D cells were treated with DMSO and various concentrations of PQ1 for 24 and 48 hours. Cells without treatments were used as controls. Cell morphology was captured using the light microscope under 20X magnification. The scale bar represents a 100 µm in size.

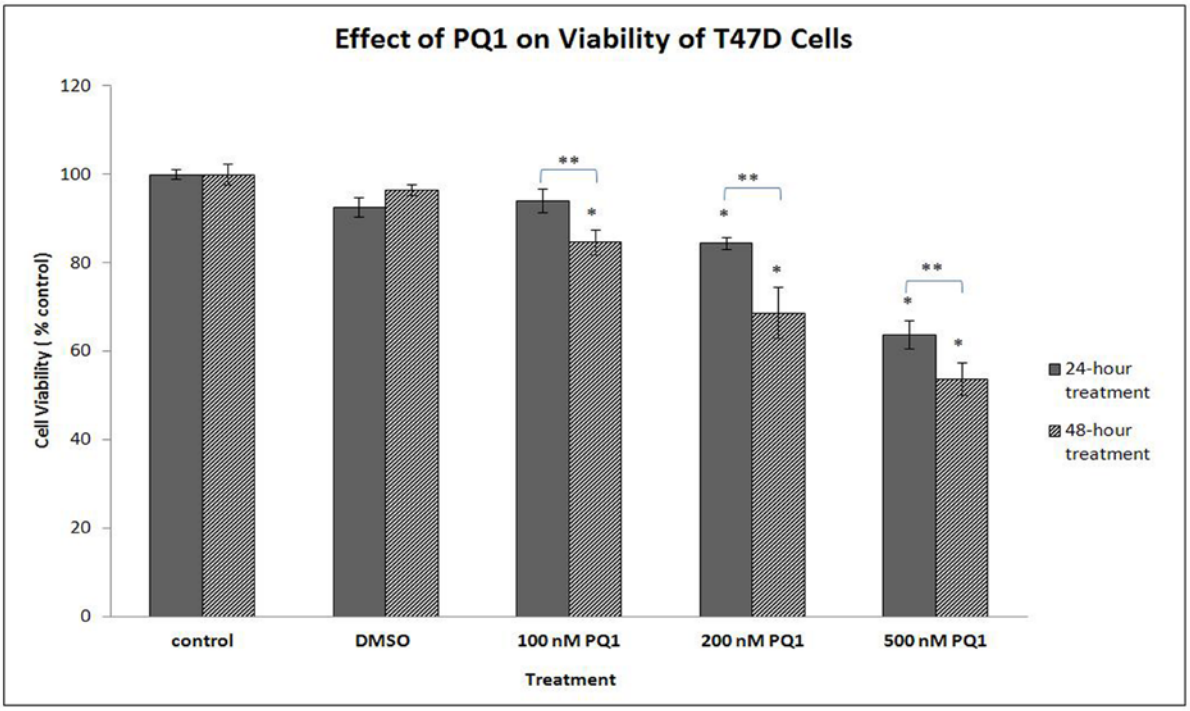


Figure 3.3 Effect of PQ1 on cell viability in T47D breast cancer cells

T47D cells were treated with DMSO and various concentrations of PQ1 for 24 and 48 hours. Cells without treatments were used as controls. Cell viability was determined by the AO/PI staining method. Data were obtained in three independent experiments and are represented as the mean ± S.D. * P-value is <0.05 compared to control. **P-value is <0.05 compared to 24 hours treatment.

3.4.2 PQ1 Induces Apoptosis in T47D Breast Cancer Cells

Morphological features of apoptosis including cell shrinkage and detachment were observed in Figure 3.2. Here, the effects of PQ1 on apoptosis were further determined by flow cytometry. T47D cells were treated with various concentrations of PQ1 for 24 and 48 hours, and cell death was characterized using flow cytometry with propidium iodide (PI) and Alexa Fluor 488 annexin V staining. The Annexin V-FITC positive /PI negative cells located in the lower right quadrant of the histogram represent early apoptotic cells, and the Annexin V-FITC positive/PI positive cells in the upper right quadrant represent late apoptotic cells. Figure 3 shows that 24-hour treatment of 500 nM PQ1 increased the early apoptotic cell population from 0.44% to 9.25% and the late apoptotic cell population from 0.27% to 12.69%, compared with the

control. Same concentration of PQ1 resulted in a higher increase in the percentage of early apoptotic cells (from 0.69% to 11.68%) and late apoptotic cells (from 0.57% to 20.23%) at 48-hour treatment (Fig. 3.4). The results of flow cytometry suggest that PQ1 induces cell death through apoptotic pathways in T47D cells.

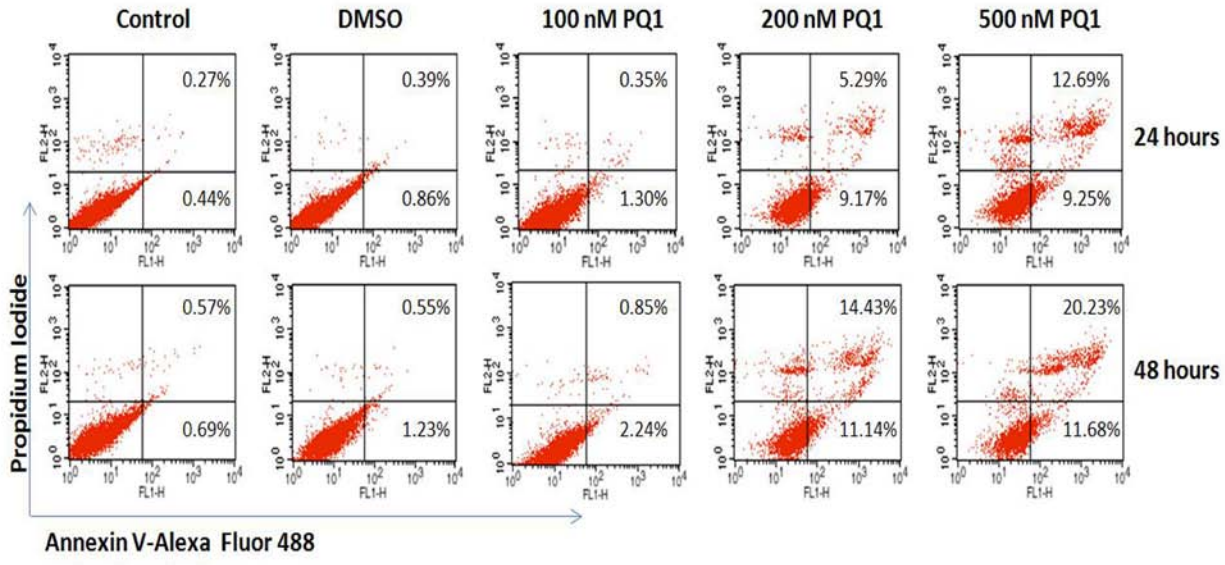


Figure 3.4 Effect of PQ1 on apoptosis in T47D breast cancer cells

T47D cells were treated with DMSO and various concentrations of PQ1 for 24 and 48 hours. Cells without treatments were used as controls. Apoptotic cells were determined by flow cytometry using an Alexa Fluor 488 Annexin V/Dead Cell Apoptosis Kit. Percentages of apoptotic cells were showed in quadrants of the histogram. The results represent one of three independent experiments.

3.4.3 PQ1 Activates Caspase-3 in T47D Breast Cancer Cells

Caspase-3 is an important executioner at the convergence of multiple caspase-dependent apoptotic pathways [15]. Activation of caspase-3 is considered to be the last step of caspase-dependent apoptosis [16, 17]. To evaluate if PQ1 induces caspase-3 activation of apoptotic pathways, expression of active caspase-3 was examined by confocal immunofluorescence microscopy. No detectable staining of cleaved caspase-3 was found in cells without treatment or cells treated with DMSO (Fig. 3.5). Relative to control, a significant increase of cleaved caspase-3 staining was detected in the treated cells with 200 and 500 nM PQ1 for 48 hours, indicating that 200 and 500 nM PQ1 is sufficient to activate caspase-3 (Fig. 3.5).

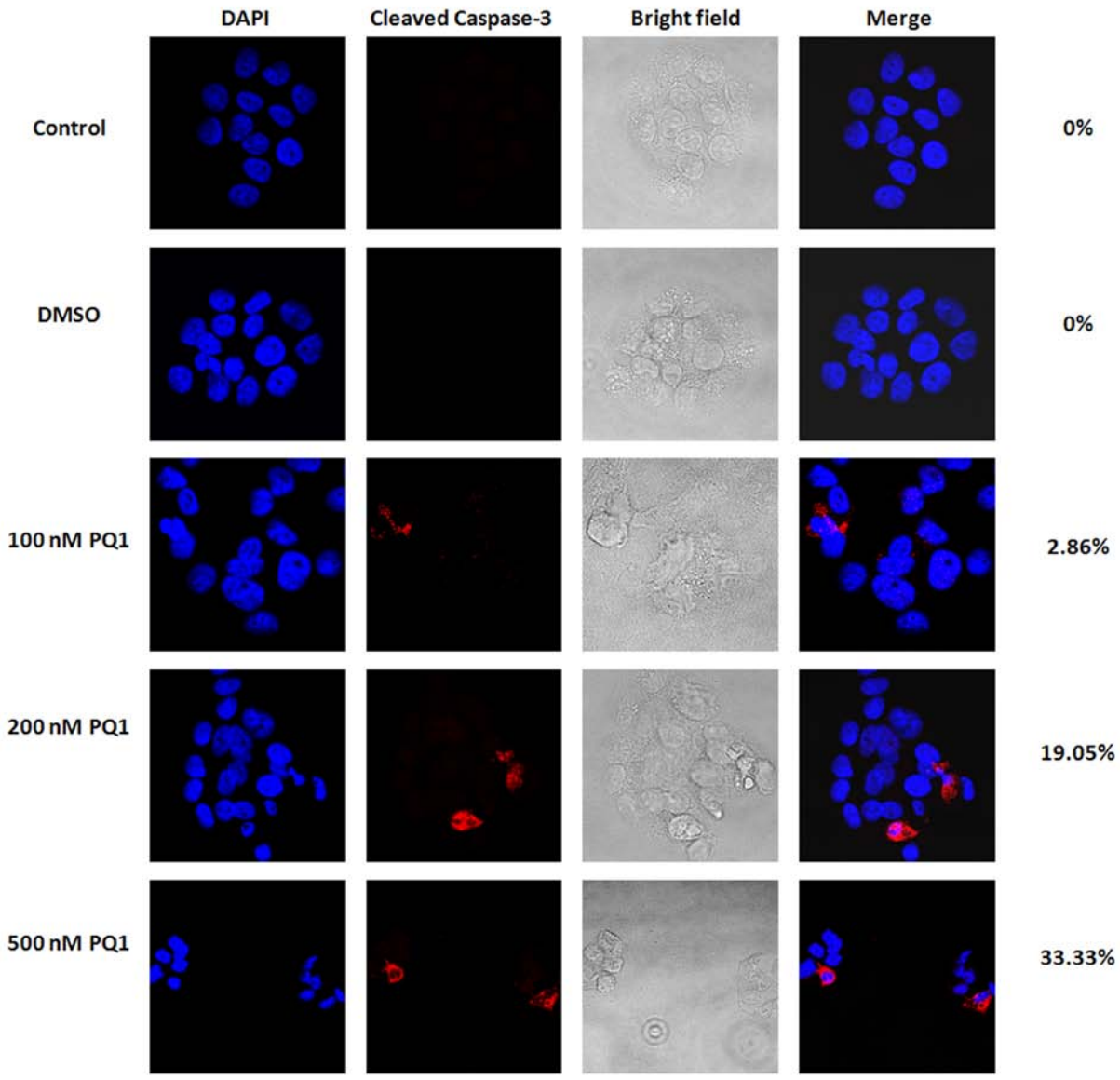


Figure 3.5 Effect of PQ1 on caspase-3 activation in T47D breast cancer cells

T47D cells were treated with DMSO and various concentrations of PQ1 for 48 hours. Cells without treatments were used as controls. Expression of cleaved caspase-3 was determined by confocal microscopic analysis using immunofluorescence staining. Red indicates cleaved caspase-3 and blue indicates nuclei stained by DAPI.

3.4.4 PQ1 Activates Caspase-9 Related Intrinsic Apoptotic Pathway in T47D Breast Cancer Cells

The intrinsic mitochondrial apoptotic pathway is characterized by mitochondrial membrane permeabilization, cytochrome c release, and caspase-9 activation [17]. The anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax are two important members of the Bcl-2 family involved in the mitochondrial apoptotic pathway. It has been reported that the ratio of Bcl-2 to Bax is crucial to the mitochondrial membrane permeabilization and cytochrome c release [18]. Here, expression levels of Bcl-2, Bax, cytochrome c, and active caspase-9 were studied using both Western blot analysis and confocal microscopy to examine the effect of PQ1 on the activation of intrinsic apoptotic pathway. Western blot results showed a gradual increase in Bax protein with an increase of PQ1; interestingly, there was no change in Bcl-2 level (Fig. 3.6a, 3.6b). In contrast to control, 200 and 500 nM of PQ1 significantly increased the level of Bax to 156% and 176%, respectively (Fig. 3.6b). The results were further confirmed by confocal images. PQ1 increased the Bax staining in a dose-dependent manner as well, but again there was no change in Bcl-2 staining (Fig. 3.6d). The effects of PQ1 on the levels of Bax and Bcl-2 subsequently resulted in a decrease of Bcl-2-to-Bax protein ratio. 48-hour treatment with 200 and 500 nM PQ1 decreased the ratio of Bcl-2 to Bax by 33% and 40%, respectively (Fig. 3.6c). These results suggested that PQ1 caused a significant increase of Bax with no change in Bcl-2 expression, leading to a decrease of the Bcl-2/Bax ratio.

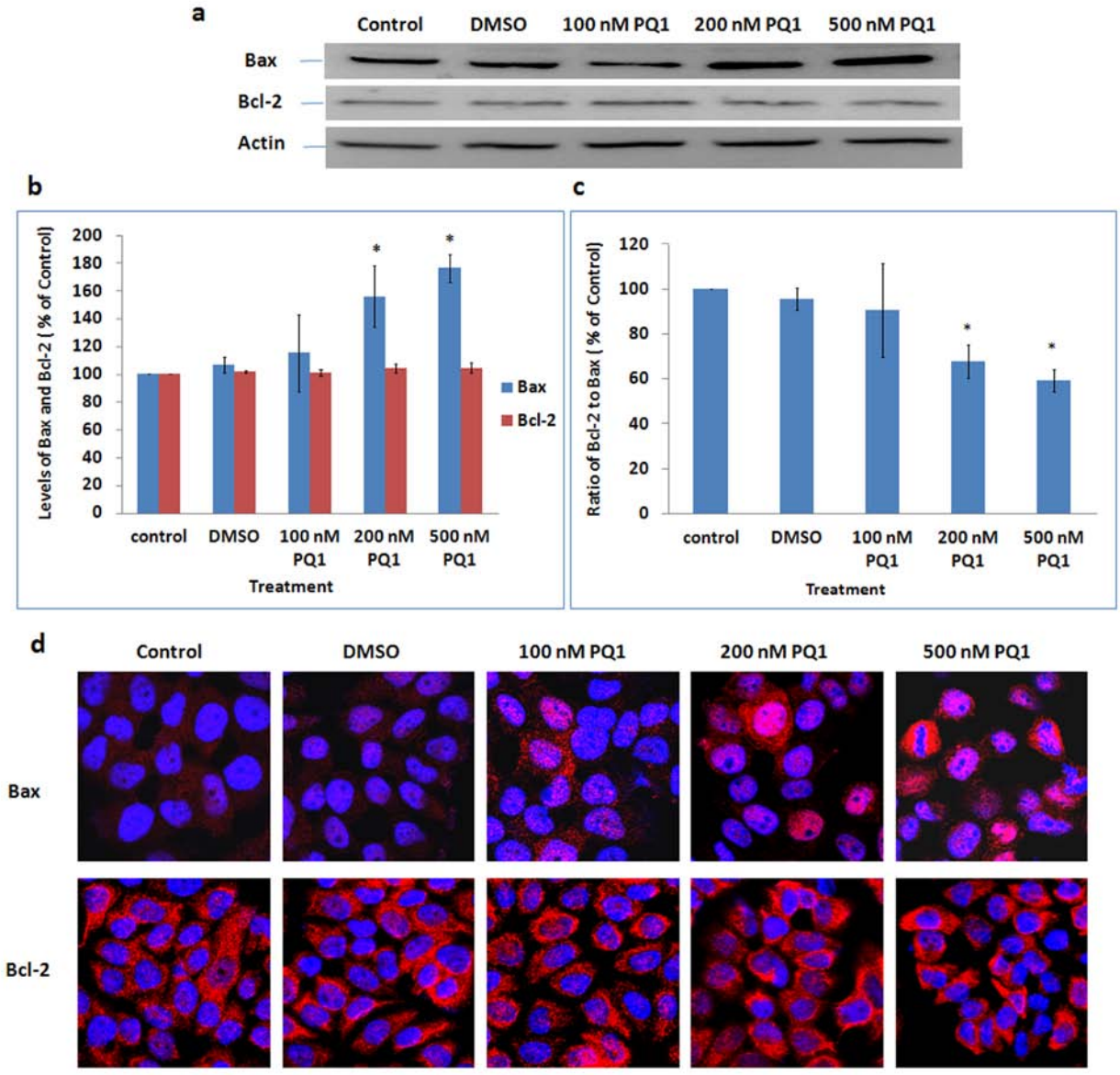
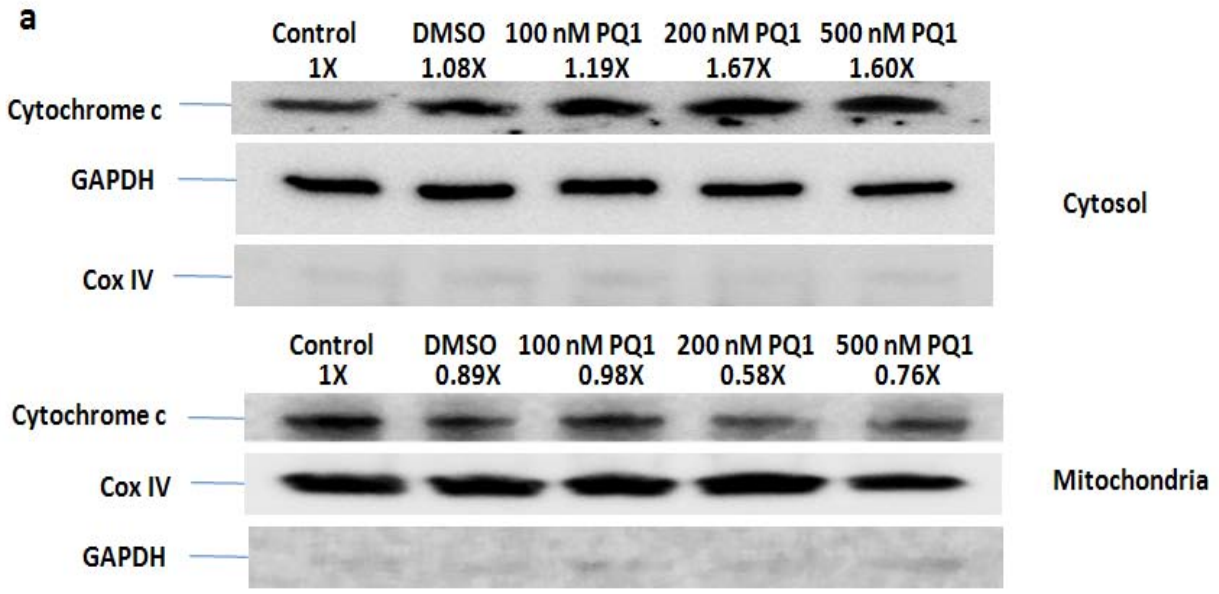


Figure 3.6 Effect of PQ1 on the levels of Bax and Bcl-2 in T47D breast cancer cells

T47D cells were treated with DMSO and various concentrations of PQ1 for 48 hours. Cells without treatments were used as controls. **(a)** Levels of Bax and Bcl-2 were examined by Western blot analysis. Actin was used as a loading control. **(b)** Graphical presentation of three independent experiments shows the pixel intensities of Bax and Bcl-2 normalized to the controls. * P-value is <0.05 compared to control. **(c)** Graphical presentation shows the ratio of Bcl-2 to Bax. Data were obtained in three independent experiments and are represented as the mean ± S.D. * P-value is <0.05 compared to control. **(d)** Immunofluorescence was performed to examine

expression levels of Bax and Bcl-2. Red indicates Bax or Bcl-2 as indicated and blue indicates the nuclei.

To investigate the cytochrome c release, mitochondrial and cytosolic fractions for cytochrome c were isolated after treatment with PQ1 for 48 hours. Western blot analysis showed a decrease of cytochrome c in the mitochondria fraction and an increase of cytochrome c in the cytosol of the PQ1-treated cells, indicating that PQ1 can trigger the release of cytochrome c from the mitochondria into cytosol (Fig. 3.7a). Cytochrome c release was also visualized by confocal immunofluorescence microscopy with double-staining of cytochrome c and mitochondrial marker. In the untreated and DMSO-treated cells, a significant colocalization of cytochrome c and mitochondria was observed (Fig. 3.6b). However, the colocalization signal decreased when cells were treated with 500 nM PQ1 (Fig. 3.7b). The reduction of cytochrome c in the mitochondria is consistent with the translocation of cytochrome c from the mitochondria to the cytosol.



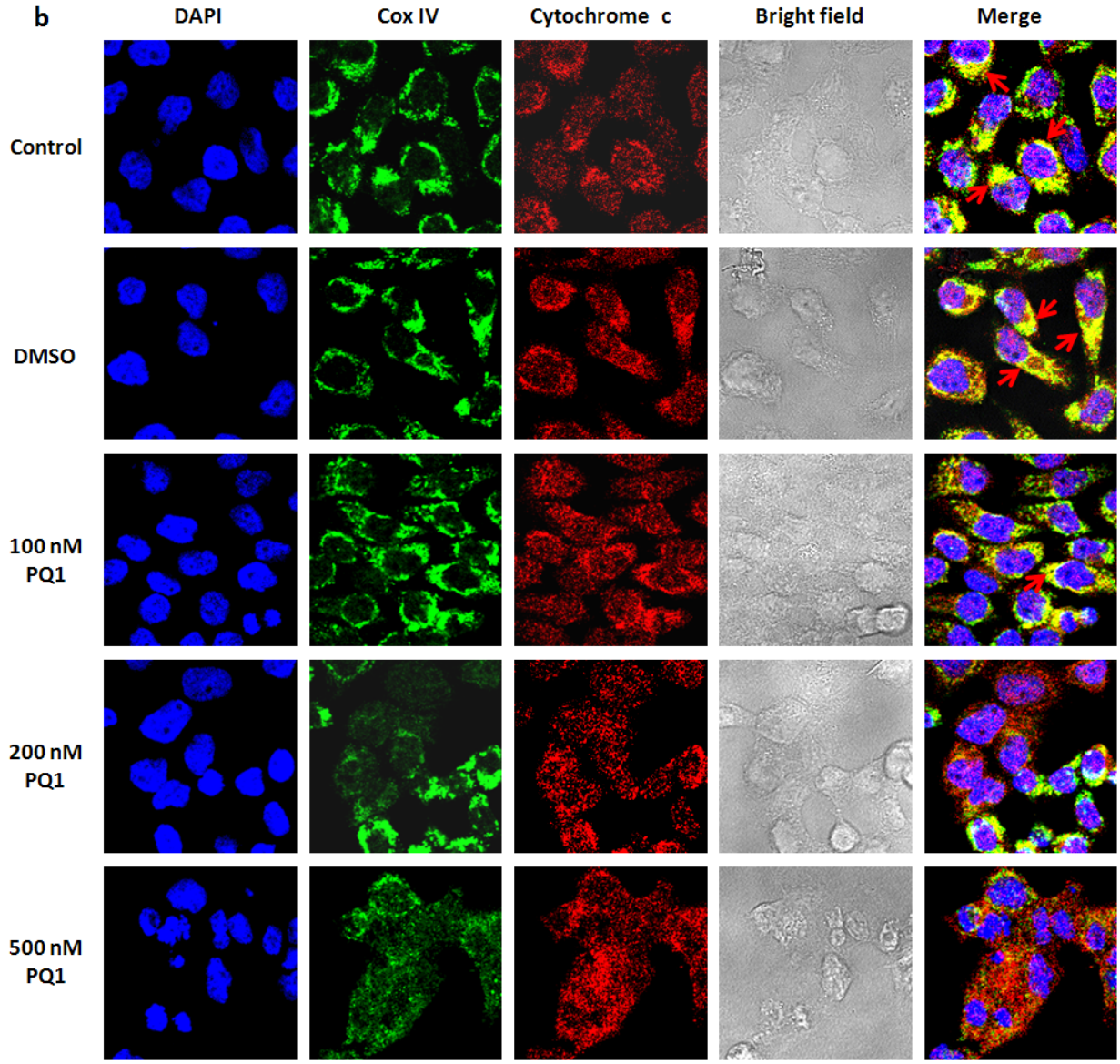


Figure 3.7 Effect of PQ1 on the release of cytochrome c from mitochondira to cytosol in T47D breast cancer cells

T47D cells were treated with DMSO and various concentrations of PQ1 for 48 hours. Cells without treatments were used as controls. **(a)** Mitochondria and cytosol were separated by using mitochondria isolation protocol as described in Materials and Methods. Western blot analysis of cytochrome c was performed. GAPDH and Cox IV were used as loading control in the cytosolic and mitochondrial fractions, respectively. Cox IV in the cytosol and GAPDH in the mitochondria were used as negative controls. Pixel intensities of protein bands were normalized to pixel intensities of loading controls. The results represent one of three independent experiments.

Numbers above the blot show fold changes in expression of cytochrome c normalized to control. **(b)** Colocalization of cytochrome c and mitochondria was determined by confocal microscopy. Red indicates cytochrome c, green indicates the mitochondrial marker Cox IV, and blue indicates nuclei stained by DAPI. Colocalization of cytochrome c and mitochondria is shown in yellow color as indicated by arrows.

Released cytochrome c is a critical activator of caspase-9 in intrinsic apoptotic pathway. When caspase-9 is activated, the procaspase-9 (approximately 45 kDa) is cleaved into a fragment of caspase-9 p35 (approximately 35 kDa) [19, 20]. Therefore, expression level of caspase-9 p35, the active form of caspase-9, was examined by both Western blot analysis and confocal microscopy using anti-caspase-9 p35 antibodies. Immunoblotting results showed that PQ1 can cause a decrease of procaspase-9 and an increase of caspase-9 p35 (Fig. 3.8a, 3.8b). Compared with the control, the level of caspase-9 p35 was increased by 34% in cells treated with 500 nM PQ1 for 48 hours (Fig. 3.8b). The ratio of active caspase-9 to pro-caspase-9 was increased to 250% after treated with 500 nM PQ1 for 48 hours (Fig. 3.8c). Confocal images further supported that the increase of active caspase-9 was due to the presence of PQ1 (Fig. 3.8d). All the results indicate that PQ1 can activate intrinsic apoptotic pathway in T47D cells.

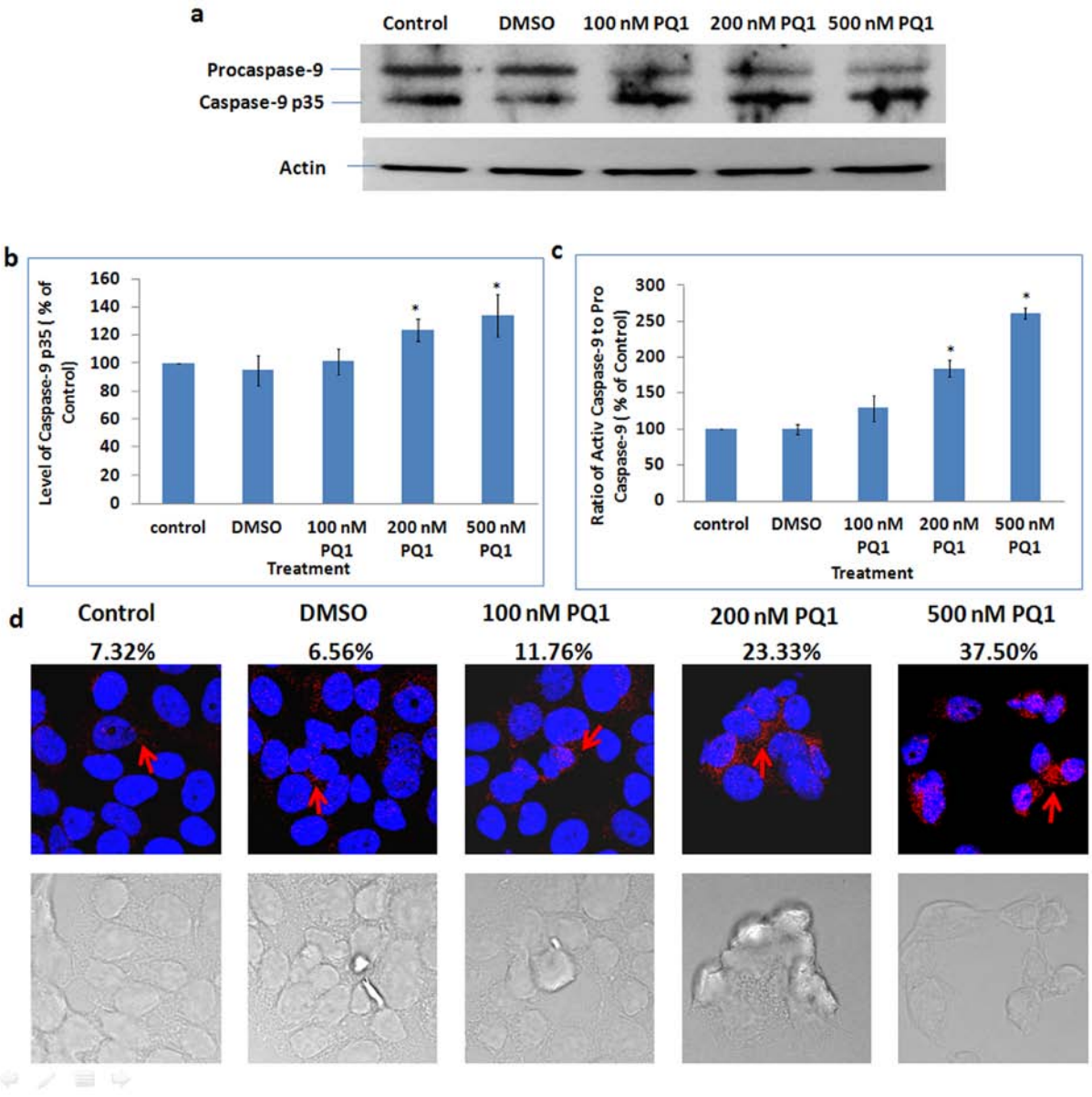


Figure 3.8 Effect of PQ1 on the activation of caspase-9 in T47D breast cancer cells

T47D cells were treated with DMSO and various concentrations of PQ1 for 48 hours. **(a)** Levels of procaspase-9 and caspase-9 p35 were examined by Western blot analysis using anti-caspase-9 p35 (H-170) antibody which detects the p35 subunit and precursor of caspase-9. Actin was used as a loading control. **(b)** Graphical presentation of three independent experiments shows the pixel intensities of caspase-9 p35 normalized to the controls. * P-value is <0.05 compared to control. **(c)** Graphical presentation of three independent experiments shows the ratio of active caspase-9 (caspase-9 p35) to pro-caspase-9. Results are normalized to the control. * P-value is <0.05

compared to control. **(d)** Immunofluorescence was performed using anti-cleaved caspase-9 (Asp315) antibody, a rabbit polyclonal antibody specific to the 35 kDa large fragment of caspase-9 following cleavage at aspartic acid 315. Red indicates caspase-9 p35 and blue indicates the nuclei. Percentages of cells with positive staining were labeled on top of relative images.

3.4.5 PQ1 Activates Caspase-8 in T47D Breast Cancer Cells

Caspase-8 is a key reporter of extrinsic apoptotic pathway. When extrinsic pathway is initiated, procaspase-8 will be activated and cleaved into active form, leading to the activation of caspase-3 [21]. Thus, the activation of capase-8 was examined by comparing the cleaved caspase-8 in the presence and absence of PQ1. The results showed that 200 and 500 nM PQ1 can significantly cause a cleavage of procaspase-8 (55 kDa) into 43 kDa, 41 kDa, and 18 kDa fragments (Fig. 3.9a). Quantification analysis of caspase-8 p18 showed that 48-hour treatment with 500 nM PQ1 increased the level of caspase-8 p18 to 152% relative to control (Fig. 3.9b). The results of immunofluorescent staining confirm that 200 and 500 nM PQ1 can cause an increase of active caspase-8 (Fig. 3.9c). Overall, the effect of PQ1 mediates not only the activation of caspase-9 but also the activation of caspase-8.

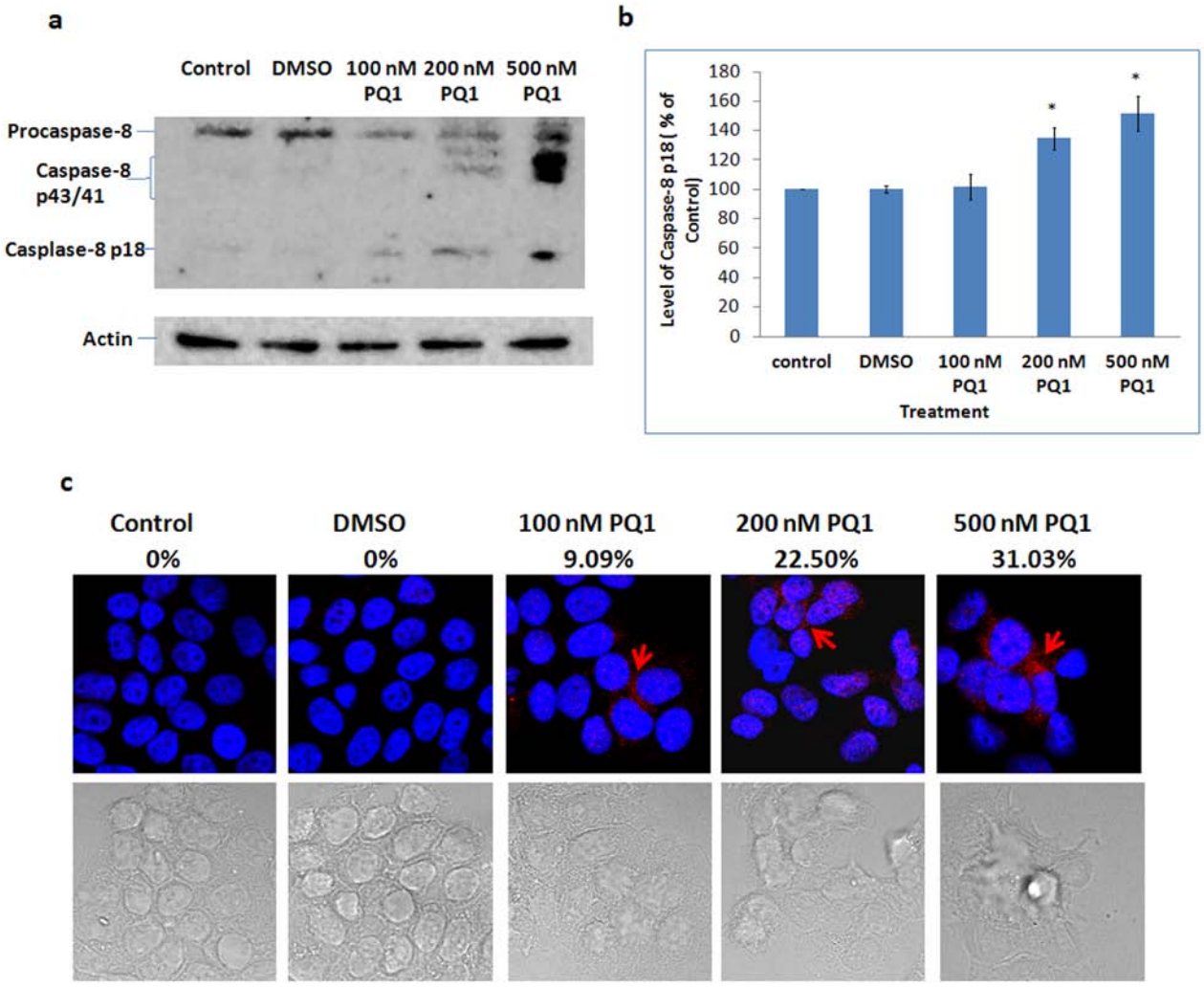


Figure 3.9 Effect of PQ1 on the activation of caspase-8 in T47D breast cancer cells

T47D cells were treated with DMSO and various concentrations of PQ1 for 48 hours. **(a)** Levels of procaspase-8 and cleaved caspase-8 (caspase-8 p43, p41 and p18) were examined by Western blot analysis using anti-caspase-8 p18 (H-134) antibody, a rabbit polyclonal antibody which detects cleaved subunits and precursor of caspase-8. Actin was used as a loading control. **(b)** Graphical presentation of three independent experiments shows the pixel intensities of caspase-8 p18 normalized to the controls. * P-value is <0.05 compared to the control. **(c)** Immunofluorescence was performed using anti-cleaved caaspase-8 (Asp391) antibody, a rabbit monoclonal antibody which detects p18 subunit after cleavage at Asp391 of human caspase-8.

Red indicates caspase-8 p18 and blue indicates the nuclei. Percentages of cells with positive staining were labeled on top of relative images.

3.4.6 Cytotoxicity of PQ1 is Counteracted by Caspase Inhibitors

To examine if activation of caspases is necessary for PQ1 cytotoxicity, T47D cells were pre-treated with 20 μM inhibitor of caspase-3/-8/-9 for 1 hour, and then treated with 500 nM PQ1 for 23 hours. Cell viability results showed that the cytotoxicity of PQ1 was completely inhibited by the pre-treatment of Ac-DMQD-CHO, a caspase-3 inhibitor (Fig. 3.10). The pre-treatment of Ac-IETD-CHO, a caspase-8 inhibitor, and Ac-LEHD-CHO, a caspase-9 inhibitor, partially inhibited the cytotoxicity of PQ1 (Fig. 3.10). Compared with 500nM PQ1 treatment, pre-treatment of Ac-IETD-CHO and Ac-LEHD-CHO increased the cell viability from 64% to 87% and 84%, respectively. The effects of caspase inhibitors indicate that the cytotoxicity of PQ1 is related to the activation of caspase cascade.

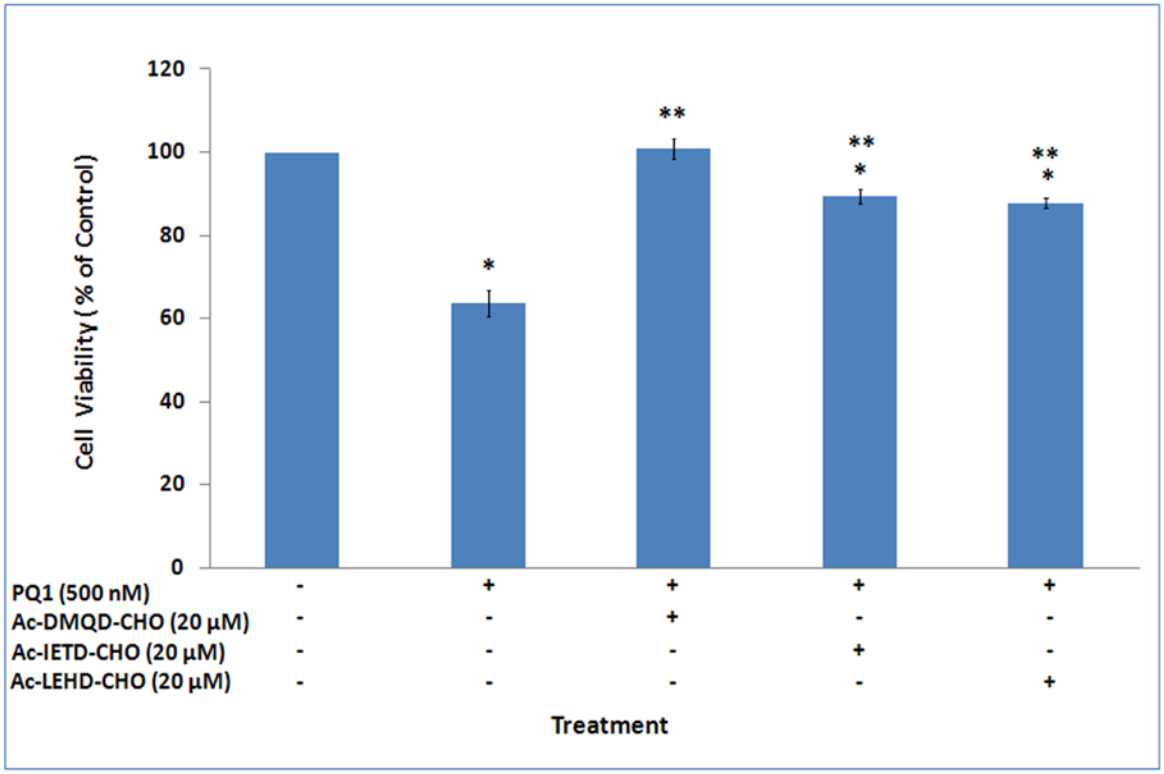


Figure 3.10 Effects of caspase inhibitors on the cytotoxicity of PQ1

T47D cells were pre-treated with 20 μM caspase-3 inhibitor (Ac-DMQD-CHO), caspase-8 inhibitor (Ac-IETD-CHO), or caspase-9 inhibitor (Ac-LEHD-CHO) for 1 hour, and exposed to 500 nM PQ1 for 23 hours. Cells without treatments and cells treated with 500 nM PQ1 for 24 hours were used as controls. Cell viability was determined by trypan blue method. Data were obtained in three independent experiments and are represented as the mean ± S.D. * P-value is <0.05 compared to control. **P-value is <0.05 compared to treatment with 500 nM PQ1 for 24 hours.

3.5 Discussion

Cancer is a complex disease with multiple deregulated signaling pathways, including apoptosis. Targeting apoptotic pathways has emerged as an attractive approach for cancer treatment. So far, numerous quinoline derivatives, both in natural and synthetic products, have been reported to possess anticancer activities through induction of different pathways of apoptosis [1]. By investigating the effect of quinoline anti-malarials on MCF-7 breast cancer cells, Zhou et al. reported that quinidine, a natural alkaloid, and chloroquine, a synthetic alkaloid, trigger apoptosis via a p53-dependent pathway [22]. Studies of another quinoline derivative, QBS (2-amino-*N*-quinoline-8-yl-benzenesulfonamide), revealed that QBS induces apoptosis in Jurkat cells via a caspase-dependent pathway [23]. The quinoline ring by itself showed no evidence of apoptosis induction [22], indicating that this activity is conferred by addition of the side chain substituents. The properties of side chains and the steric structures of the derivatives may be related to apoptotic pathways selection. In this report, effect of PQ1, 6-methoxy-8-[(3-aminopropyl)amino]-4-methyl-5-(3-trifluoromethylphenyloxy)quinoline, on apoptosis was examined in T47D breast cancer cells. The results showed that PQ1 induces apoptosis by initiating caspase cascade. It is not clear that which specific substituent of PQ1 is responsible for the activation of apoptosis. More studies are needed to establish the relationship between structure and function.

The present study demonstrated that 200 and 500 nM PQ1 for 48 hours significantly increased the population of apoptotic cells (Fig. 3.4) and activated caspase-3, -9 and -8 (Fig. 3.5, 3.8 and 3.9), indicating that the effective concentrations of PQ1 in apoptosis induction fall in the nM range.

Caspases are a group of ICE (interleukin 1 β-converting enzyme)-like proteases that play a crucial role in apoptosis mediation [24]. Currently, 14 caspases have been identified in humans [25, 26], and among them caspase-3 is frequently activated and serves as the executioner. Activation of caspase-3 by cleavage of procaspase-3 is considered to be a hallmark of apoptosis [27]. By examining the expression of cleaved caspase-3 after PQ1 treatment, we provide evidence that PQ1 can induce the activation of caspase-3. Caspase-3 is activated both by extrinsic and intrinsic pathways via interacting with two initiator caspases, caspase-8 and caspase-9 [28]. In intrinsic pathway, activation of caspase-9 is regulated by cytochrome c and

two members of Bcl-2 family, Bax and Bcl-2. The pro-apoptotic factor Bax has been reported to form heterodimers with the anti-apoptotic factor Bcl-2, which consequently induce cytochrome c release and accelerate apoptosis [29]. Overexpressed Bax counteracts the activity of Bcl-2 [29]. However, this view has been questioned due to the fact that the dimeric interaction of Bax and Bcl-2 can only be detected in the presence of nonionic detergents, such as Triton X-100 and Nonidet P-40 [30]. Furthermore, Knudson and Korsmeyer reported that although there is an *in vivo* competition between Bax and Bcl-2, they are able to regulate apoptosis independently [31]. Consistent with this report, our results showed that PQ1 can cause an increase in Bax without any changes in Bcl-2, indicating that PQ1 can activate intrinsic apoptotic pathway through Bax-dependent but Bcl-2 independent mechanisms. Activation of caspase-8 is a crucial step in extrinsic apoptotic pathway. 200 and 500 nM PQ1 can cause a cleavage of procaspase-8 into active caspase-8 fragments, suggesting that PQ1 can also mediate the activation of extrinsic pathway reporter. However, the cleaved caspase-8 can activate caspase-3 through two alternative signaling pathways [32, 33]. One pathway is through the activation of caspase-3 directly. Another pathway is through the cleavage of BID, a Bcl-2 interacting protein, and the truncation of BID (tBID) to translocate to the mitochondria where tBID induces mitochondrial damage and releases cytochrome c. The released cytochrome c activates caspase-9 and sequentially activates caspase-3. It is not clear which downstream pathway is dominant in caspase-8 mediated apoptotic pathway. Further studies are needed to elucidate the downstream events after caspase-8 activation.

In conclusion, this study showed that the quinoline derivative, PQ1, exerts its anti-cancer effects in T47D breast cancer cells through the induction of both caspase-8 and caspase-9 mediated pathways of apoptosis. PQ1 can significantly increase bax, cause the release of cytochrome c, activate caspase-9 and caspase-8, and subsequently induce caspase-3-mediated apoptosis.

Acknowledgements

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Chapter 4 - Gap Junction Enhancer Potentiates Cytotoxicity of Cisplatin in Breast Cancer Cells

4.1 Abstract

Cisplatin is one of the most widely used anti-cancer drugs due to its ability to damage DNA and induce apoptosis. However, increasing reports of side effects and drug resistance indicate the limitation of cisplatin in cancer therapeutics. Recent studies showed that inhibition of gap junctions diminishes the cytotoxic effect and contributes to drug resistance. Therefore, identification of molecules that counteract gap junctional inhibition without decreasing the anti-cancer effect of cisplatin could be used in combinational treatment, potentiating cisplatin efficacy and preventing resistance. This study investigates the effects of combinational treatment of cisplatin and PQ1, a gap junction enhancer, in T47D breast cancer cells. Our results showed that combinational treatment of PQ1 and cisplatin increased gap junctional intercellular communication (GJIC) as well as expressions of connexins (Cx26, Cx32 and Cx43), and subsequently decreased cell viability. Ki67, a proliferation marker, was decreased by 75% with combinational treatment. Expressions of pro-apoptotic factors (cleaved caspase-3/-8/-9 and bax) were increased by the combinational treatment with PQ1 and cisplatin; whereas, the pro-survival factor, bcl-2, was decreased by the combinational treatment. Our study demonstrates for the first time that the combinational treatment with gap junction enhancers can counteract cisplatin induced inhibition of gap junctional intercellular communication and reduction of connexin expression, thereby increasing the efficacy of cisplatin in cancer cells.

4.2 Introduction

Cisplatin is a potent agent used in cancer chemotherapy. Since the anti-cancer properties of cisplatin were discovered in 1960s, it has been widely employed for treating various cancers, including testicular, ovarian, bladder, cervical, head and neck, esophageal, lung and breast cancer [1-4]. Numerous studies have provided information to elucidate the molecular mechanism of cisplatin cytotoxicity. It is widely accepted that the anti-tumor action of cisplatin is attributed to the formation of cisplatin-DNA adducts, inducing several signal pathways and subsequently leading to cell cycle arrest, necrosis or/and apoptosis [5]. Recently, other mechanisms without DNA-damaging effect have added to the complexity of cisplatin, including the binding of cisplatin to cellular proteins and other constituents [6]. Although cisplatin is widely used in practice due to its success in the treatment of malignancies; unfortunately, increasing drug resistance and side effects of cisplatin evoke a lot of concerns about the application [7].

Gap junctions (GJ) are intercellular channels connecting adjacent cells to allow small molecules of less than 1.2 kDa in size to transport between cells, thereby keeping homeostasis of cells and tissues [8, 9]. Many molecular processes including proliferation, differentiation, migration and apoptosis, are reported to be affected by this communication [10, 11]. Loss of gap junctional intercellular communication (GJIC) and connexins, the gap junction proteins, is a hallmark of malignancy [12]. Connexins have been viewed as therapeutic targets in cancer treatment due to two important mechanisms: the GJIC-independent mechanism and GJIC-dependent mechanism [13]. By interacting and regulating tumor-suppressing molecules and tumor susceptible genes, connexins exhibit their tumor suppressive functions in a GJIC-independent manner [14]. A growing amount of reports suggest that over-expressing connexins can reduce cancer proliferation and attenuate tumor growth [15]. In addition to this GJIC-independent mechanism, GJ-based therapies mainly rely on the GJIC-dependent bystander effect, a mechanism by which cytotoxic molecules are transferred from target cells to neighboring cells [16]. Restoration and/or activation of GJIC have been applied in gene therapy, radiation therapy and chemotherapy [17-19]. In chemotherapy, up-regulation of GJIC and overexpression of connexins have been used to potentiate drug efficacy and reduce drug resistance [20].

Cisplatin-induced cytotoxicity has been reported to be transduced to neighboring cells through gap junctions. Jensen and Glazer found that the DNA-PK-mediated cytotoxic signal triggered by cisplatin was transmitted between cells via gap junctions [21]. The ability of activated oncogene, src, to induce cisplatin resistance by producing tyrosine phosphorylation of connexin 43 (Cx43) and decreasing GJIC, can be transmitted to adjacent cells by GJIC, even when these cells lack src activity. Moreover, this cisplatin resistant effect on neighboring cells can be counteracted by overexpression of Cx43 [22]. The analgesics, tramadol and flurbiprofen, used in combinational treatment with cisplatin, were shown to depress the cytotoxicity of cisplatin via the inhibition effects on gap junctions [23]. Furthermore, cisplatin was reported to inhibit GJIC by directly inhibiting the channel activity and decreasing expression of connexins [24]. This evidence indicates that inhibition of GJIC and reduction of connexins would decrease cytotoxicity of cisplatin and result in cisplatin resistance. Therefore, development of novel agents or methods to enhance or restore GJIC in combinational treatment with cisplatin is a new strategy to potentiate cisplatin effect and decrease drug resistance.

PQ1, a derivative of quinoline, was reported as a gap junction enhancer [25]. Gakhar et al. reported that 200 nM of PQ1 showed a significant increase in the GJIC in T47D breast cancer cells [25]. Combinational treatment of PQ1 and tamoxifen indicated that PQ1 potentiated the effect of tamoxifen in T47D cells, indicating the synergistic effect of PQ1 in combinational treatment in breast cancers [26].

In this report, the effects of PQ1 on the cytotoxicity of cisplatin in breast cancer cells were examined. Our results showed that PQ1 counteracted the inhibition of GJIC and reduction of connexins caused by cisplatin, subsequently enhancing the cytotoxic effect of cisplatin.

4.3 Materials and Methods

4.3.1 Reagents and Antibodies

PQ1, a quinoline derivative, was obtained as described by Shi at al. [27] and graciously provided by Dr. Duy Hua (Kansas State University). Cis-Diamminedichloro-platinum, trypan blue, Lucifer yellow dye and Rhodamine-dextran dye were all purchased from Sigma (St Louis, MO, USA). Anti-Cx43, Alexa-568-conjugated anti-rabbit IgG, and Alexa-594-conjugated anti-mouse IgG antibodies were obtained from Invitrogen (Camarillo, CA, USA). Anti-Cx32, anti-Cx26, anti-Ki67, anti-cleaved caspase-3, anti-caspase-8 p18, anti-caspase-9 p35, anti-Bax, and anti-Bcl2 antibodies were all purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). The HRP-linked anti-rabbit/mouse antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA).

4.3.2 Cell Line and Cell Culture

The T47D human breast cancer cell line was purchased from American Type Cell Culture (ATCC) (Manassas, MA, USA). The cells were grown in RPMI-1640 (Sigma-Aldrich, St Louis, MO, USA) supplemented with 2 g/L sodium bicarbonate, 1 mM sodium pyruvate, 10 mM hepes, 4.5 g/L glucose, 0.2 units/ml bovine insulin, and 10% fetal bovine serum (Atlanta biological, Lawrenceville, GA, USA). Cells were maintained in T-75 cm^2 flasks at 37 °C with 5% CO_2 and cultured in 6-well plates or T-25 cm^2 flasks for experimental analysis.

4.3.3 Cell Morphology

T47D cells were cultured in six-well plates until 80% confluent state and treated according to the following conditions: untreated, PQ1 (100, 200, and 500 nM) alone for 28 hours, cisplatin (40 µM) alone for 24 hours, and PQ1 (100, 200, and 500 nM) for 4 hours followed by addition of cisplatin for 24 hours. Cell morphology was captured using Nikon 80i light microscope.

4.3.4 Cell Viability Assay

Cell viability was measured using trypan blue excision method. T47D cells were cultured into 6-well plates until 80% confluent state (high density) or 40% confluent state (low density),

corresponding to the conditions in which junctional channel formation was permitted or not, respectively. Cells were treated with PQ1 and cisplatin as discussed for the cell morphology protocol. Cells floated in the media were collected and cells attached to the wells were trypsinized. Two parts of cells were combined together, centrifuged and resuspended. A cell suspension was mixed with trypan blue dye and viable cells were examined by the Cellometer Auto 2000 (Nexcelom Bioscience, Lawrence, MA, USA).

4.3.5 Scrape Load/ Dye Transfer Assay

T47D cells were seeded on coverslips in 6-well plates and cultured until the confluency reached 80% - 100%. Cells were treated with PQ1 (100, 200, and 500 nM) and cisplatin (40 µM) alone for 4 hours or sequential combinational treatment, treating PQ1 for 4 hours followed by cisplatin for 4 hours. After treatments, cells were rinsed three times with PBS. Then, 2.5 µl mixtures of 1% (w/v) Lucifer yellow and 1% (w/v) Rhodamine-dextran were added in the center of the coverslips and a scrape was made on the coverslips. The dye solution was left on the coverslips for 3 minutes, after which coverslips were washed by PBS for three times. The cells were incubated in RPMI medium at 37 °C for 20 minutes, washed by PBS, and then fixed with 2.5% paraformaldehyde for 10 minutes. Cells were mounted and image of dye transfer was captured using Nikon TE2000U fluorescence microscope. The distance of dye transfer from cutting site to the farthest visual uptake of dye was measured using ZEN 2010 software.

4.3.6 Western Blot Analysis

T47D cells were cultured in T-25 cm^2 flasks until 80% confluent state. Cells were treated with PQ1 alone for 28 hours, cisplatin alone for 24 hours, or PQ1 for 4 hours followed by addition of cisplatin for 24 hours. Cells without any treatments or DMSO as vehicle were used as controls. After treatments, cells were washed with PBS for three times and harvested in lysis buffer (Cell Signaling Technology, Danver, MA, USA). Cell lysates were sonicated using Vibra-Cell sonicator (Sonics & Materials Inc, Danbury, CT, USA) and then centrifuged at 13,000 rpm for 30 minutes at 4 °C. After centrifugation, supernatants were collected as whole cell extracts. Thirty ug of samples were separated by 4-20% gradient SDS-PAGE for 35 minutes at 200 V, and transferred to nitrocellulose membranes (Midwest Scientific, Saint Louis, MO, USA). Membranes were blocked with 5% milk for 30 minutes and immunoblotted against protein of interest. Immunoreactions using chemiluminescence were visualized by FluorChem E Imaging

Instrument (ProteinSimple, Santa Clara, CA, USA). Intensities of the bands were digitized using Un-Scan-It software.

4.3.7 Immunofluorescence and Confocal Microscopy

T47D cells cultured on coverslips in 6-well plates were treated with PQ1 and cisplatin alone or in combination as described in Western blot analysis protocol. After treatment, cells were rinsed with PBS and fixed with 2% paraformaldehyde for 20 minutes at room temperature. Fixed cells were washed 3 times with PBS and then permeabilized with 0.1% Triton X-100 for 8 minutes. Cells were washed 3 times with PBS again, and blocked with 2.5% BSA in PBS for 1 hour at room temperature. After blocking, cells were incubated with primary antibodies overnight at 4 °C. Samples were incubated with Alexa-conjugated secondary antibodies for 1 hour at room temperature. DAPI was used to stain nuclei. The slides were mounted by prolong-antifade reagent (Invitrogen, Camarillo, CA, USA) and sealed. Image was captured using a confocal microscope (Carl Zeiss LSM 700 META, Narashige, MN, USA).

4.3.8 Statistical Analysis

Data were statistically analyzed by using student's t-test. Data presented were expressed as mean ± S.D. of at least three independent experiments. Significance was considered at $p < 0.05$.

4.4 Results

4.4.1 Combinational Treatment of PQ1 and Cisplatin Has a Synergistic Effect on Cell Morphology and Proliferation in T47D Cells

Cisplatin and PQ1 separately have been reported to inhibit cell proliferation and induce cell death in breast cancer cells via different mode of action [25, 28]. To address whether cisplatin and PQ1 can work synergistically to attenuate cell proliferation, cell morphology and proliferation were first examined with the combinational treatment of PQ1 and cisplatin. T47D breast cancer cells were treated with PQ1 and cisplatin alone and in combination as PQ1 for 4 hours followed by addition of cisplatin for 24 hours. Cells without any treatment were used as controls. Morphological evaluation showed that both PQ1 and cisplatin changed cell morphology and decreased the number of adherent cells (Fig. 4.1). Combinational treatment of PQ1 and cisplatin induced more significant morphological changes compared to PQ1 and cisplatin alone. Changes including irregular shape, shrinkage, rounding, and detachment were found in PQ1-pretreated cells in a dose-dependent manner (Fig. 4.1). Combinational treatment of T47D cells with 40 μM cisplatin and 200 or 500 nM PQ1 caused a significant change in the morphology with less than 50% of cells attached to the bottom of flasks (Fig. 4.1). The proliferation of cells was examined by the staining of Ki67, a nuclear protein as a proliferation marker. 24 hours treatment of 40 μM cisplatin did not significantly decrease expression of Ki67, indicating that 24 hours treatment of cisplatin is not sufficient for the inhibition of cell proliferation (Fig. 4.2A, 4.2B). However, Ki67 staining was decreased in the presence of PQ1 in a dose-dependent manner. The combinational treatment of 500 nM PQ1 and 40 μM cisplatin has a significant decrease of 23.6% of Ki67 stained cells compared to 92.5% with cisplatin alone and 47.1% with PQ1 alone (Fig. 4.2B), indicating a synergistic effect of the combinational treatment on anti-proliferation. DMSO, a PQ1 vehicle, was used as solvent control, showing no changes on cell morphology and proliferation induced by the solvent (Fig. 4.1, 4.2A, 4.2B). These results suggest that combinational treatment of PQ1 and cisplatin has a synergistic effect on the changes of cell morphology and the inhibition of cell proliferation.

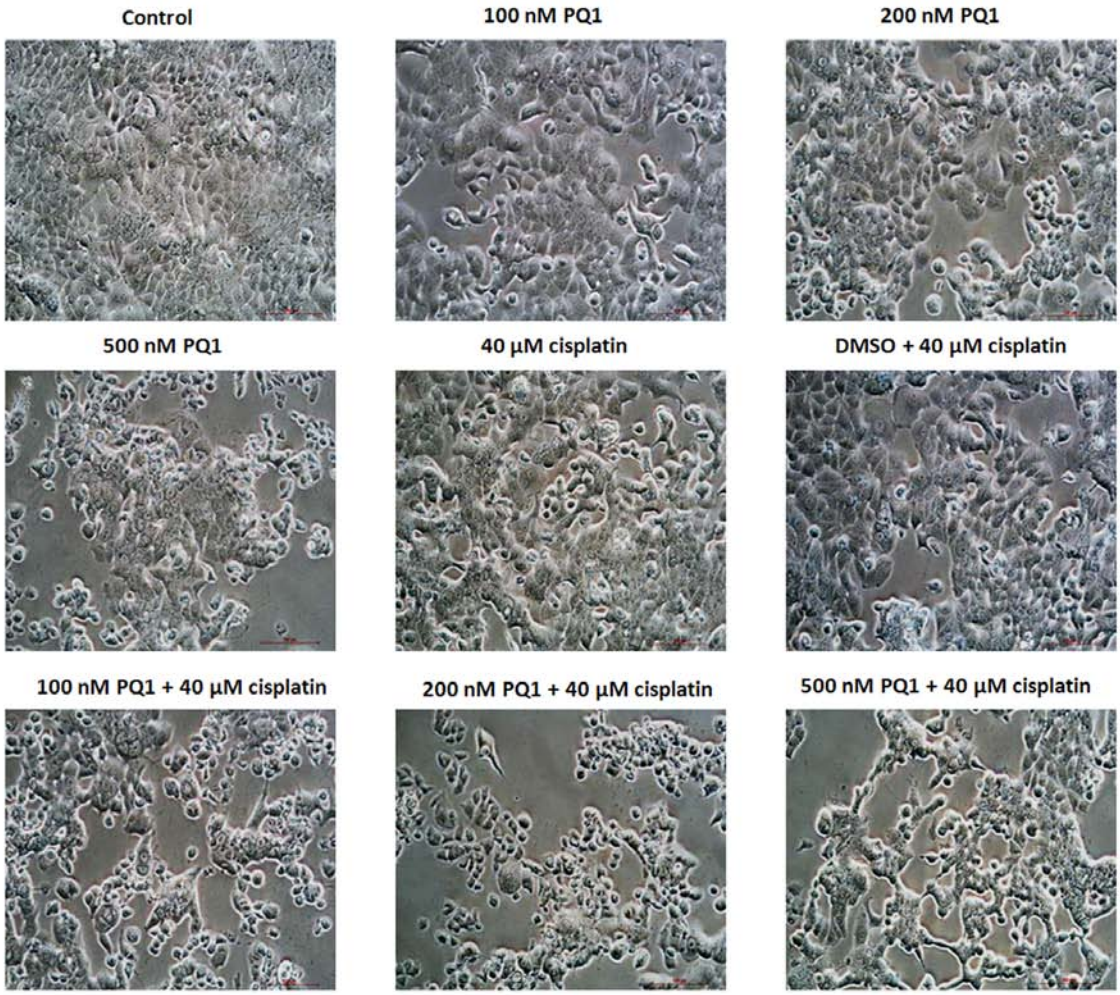
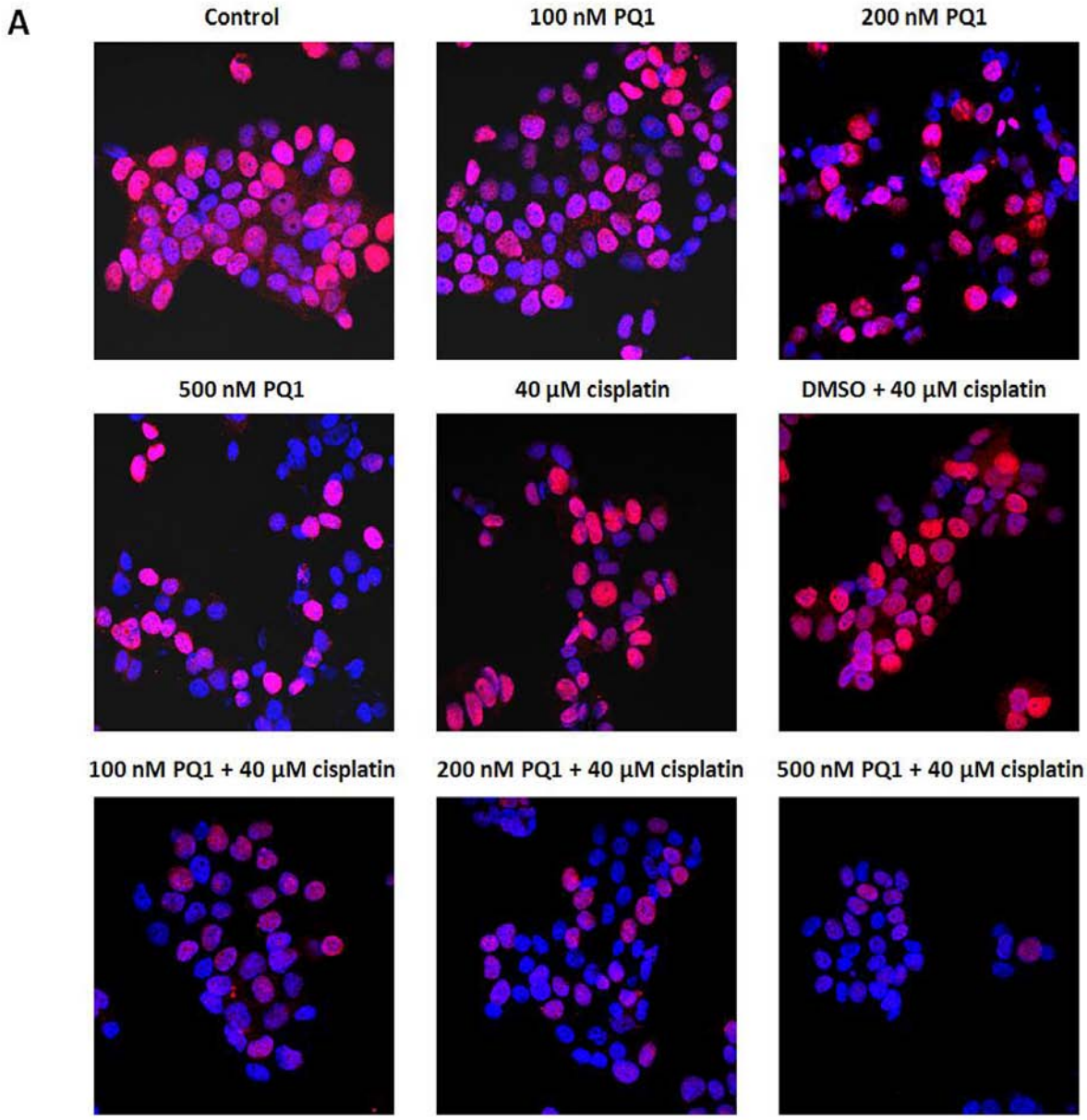


Figure 4.1 Synergistic effect of the combinational treatment of PQ1 and cisplatin on cell morphology

T47D cells were treated with PQ1 and cisplatin alone and in combination as indicated. Cells without treatment were used as controls. DMSO, a PQ1 vehicle, was used as solvent control. Cell morphology was captured using the light microscope under 20X magnification. The scale bar is 100 µm in size.


A
Control
100 nM PQ1
200 nM PQ1
500 nM PQ1
40 µM cisplatin
DMSO + 40 µM cisplatin
100 nM PQ1 + 40 µM cisplatin
200 nM PQ1 + 40 µM cisplatin
500 nM PQ1 + 40 µM cisplatin

B

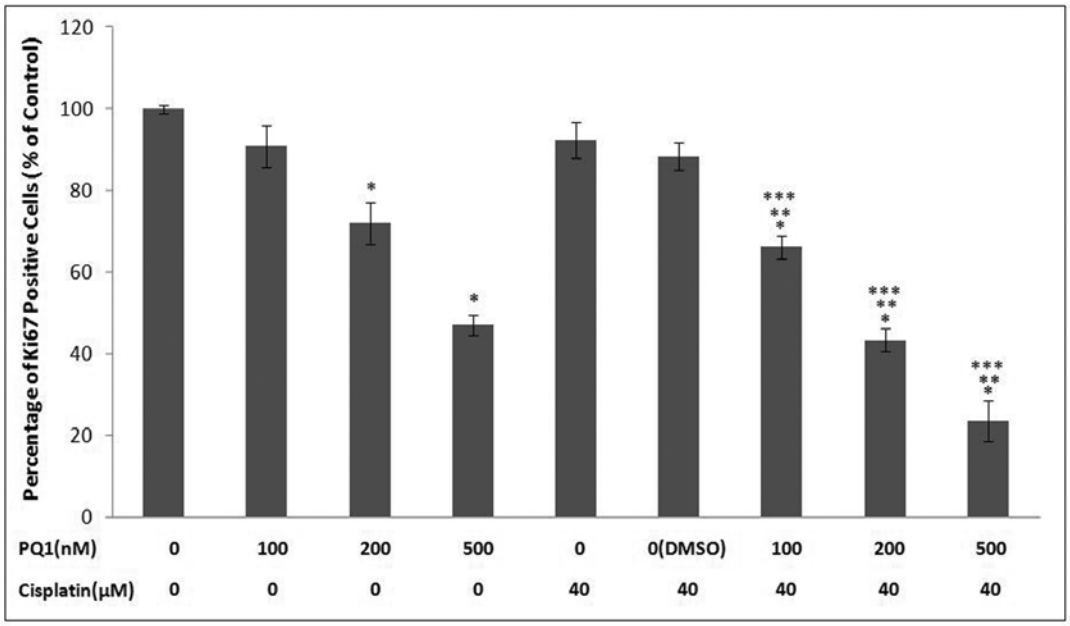


Figure 4.2 Synergistic effect of the combinational treatment of PQ1 and cisplatin on cell proliferation

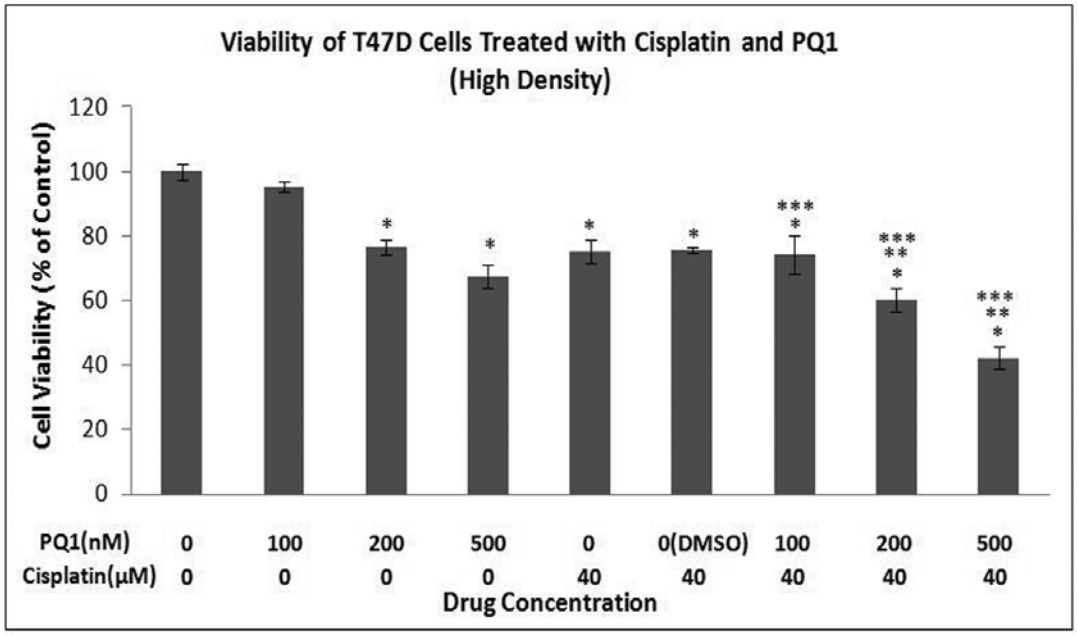
T47D cells were treated with PQ1 and cisplatin alone and in combination. **(A)** Ki67 staining was used to examine cell proliferation under confocal microscope. Red indicates Ki67 and blue indicates nuclei stained by DAPI. **(B)** Percentages of Ki67 in treated cells were calculated and the results of each treatment were normalized to its controls. Data were obtained in three independent experiments and are represented as the mean ± S.E. * P-value is <0.05 compared to control. **P-value is <0.05 compared to cisplatin treatment. *** P-value is <0.05 compared to PQ1 treatment.

4.4.2 PQ1 Increases Cytotoxicity of Cisplatin and the Enhancement Depends on Cell Density

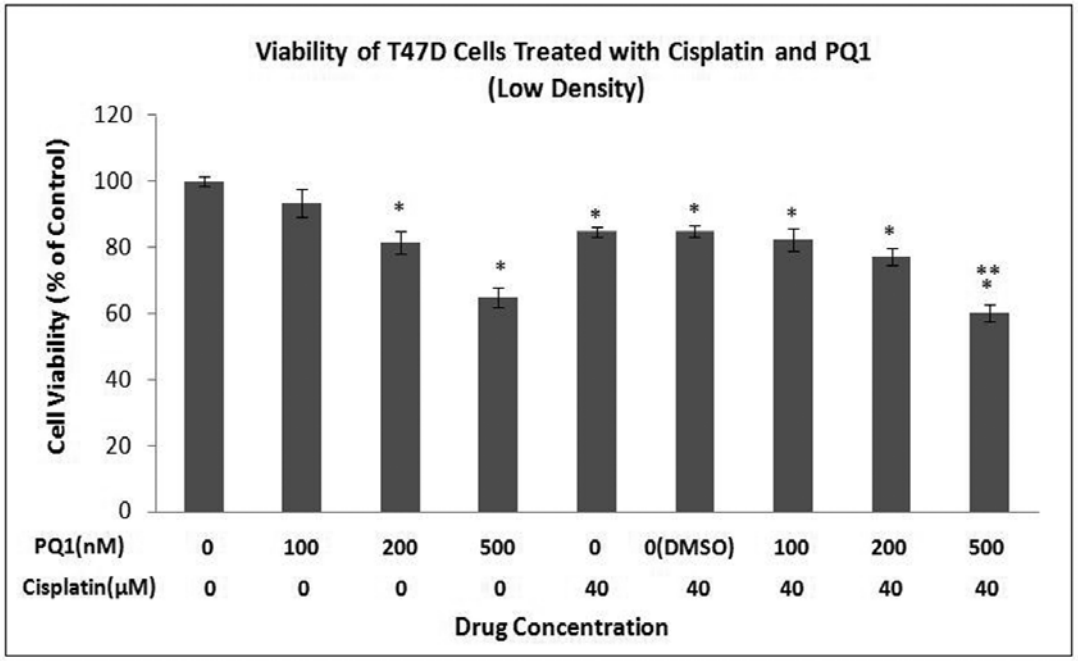
The cytotoxicity of cisplatin in part is due to the bystander effect via GJIC at high cell density [24]. Cisplatin-induced cytotoxicity is transduced to neighbor cells through gap junction and accordingly induces more cell death under high cell density condition [24]. Since PQ1 has been reported as a gap junction enhancer [25], the effect of PQ1 on GJIC raised the possibility

that the cytotoxicity of cisplatin could be potentiated by PQ1 via GJIC mediation. Here, cytotoxicity of combinational treatment was examined under low and high cell density conditions. At low density, cells were well dispersed as single cells without touching the neighboring cells, in which a condition with few gap junctions could be formed. However, at high density, cells were confluent enough to contact with adjacent cells, allowing the formation of gap junctions. Consistent with previous reports [24], the density-dependent toxicity of cisplatin was also observed in this study. 24 hours treatment of cisplatin decreased cell viability to 85% at low density and 75% at high density, indicating that the cytotoxicity of cisplatin is GJIC-dependent (Fig. 4.3A, 4.3B). PQ1 enhanced the cytotoxicity of cisplatin at low and high density in a concentration-dependent manner, but the increase of toxic effects at high density is more significant (Fig. 4.3A, 4.3B, 4.3C). Compared with cisplatin treatment alone, a 33% decrease (75% to 42%) of cell viability was observed in the combinational treatment of cisplatin and 500 nM PQ1 under high density condition; whereas, same combinational treatment only caused a 24% decrease (85% to 61%) under low density condition (Fig. 4.3A, 4.3B, 4.3C). Relative to cisplatin treatment alone, quantification of differences in survival, caused by the combinational treatment at different PQ1 concentrations between low and high densities, were showed in Fig. 4.3C. PQ1 had a constant effect on cell toxicity regardless on cell density (Fig. 4.3A, 4.3B). Therefore, the fact that PQ1 had a greater effect on the cytotoxicity of cisplatin at high density suggested an enhanced toxic effect of cisplatin via up-regulated GJIC mediated by PQ1.

A


Viability of T47D Cells Treated with Cisplatin and PQ1
(High Density)
Cell Viability (% of Control)
120
100
80
60
40
20
0
PQ1(nM) 0 100 200 500 0 0(DMSO) 100 200 500
Cisplatin(μM) 0 0 0 0 40 40 40 40 40
Drug Concentration

B


Viability of T47D Cells Treated with Cisplatin and PQ1
(Low Density)
Cell Viability (% of Control)
120
100
80
60
40
20
0
PQ1(nM) 0 100 200 500 0 0(DMSO) 100 200 500
Cisplatin(μM) 0 0 0 0 40 40 40 40 40
Drug Concentration

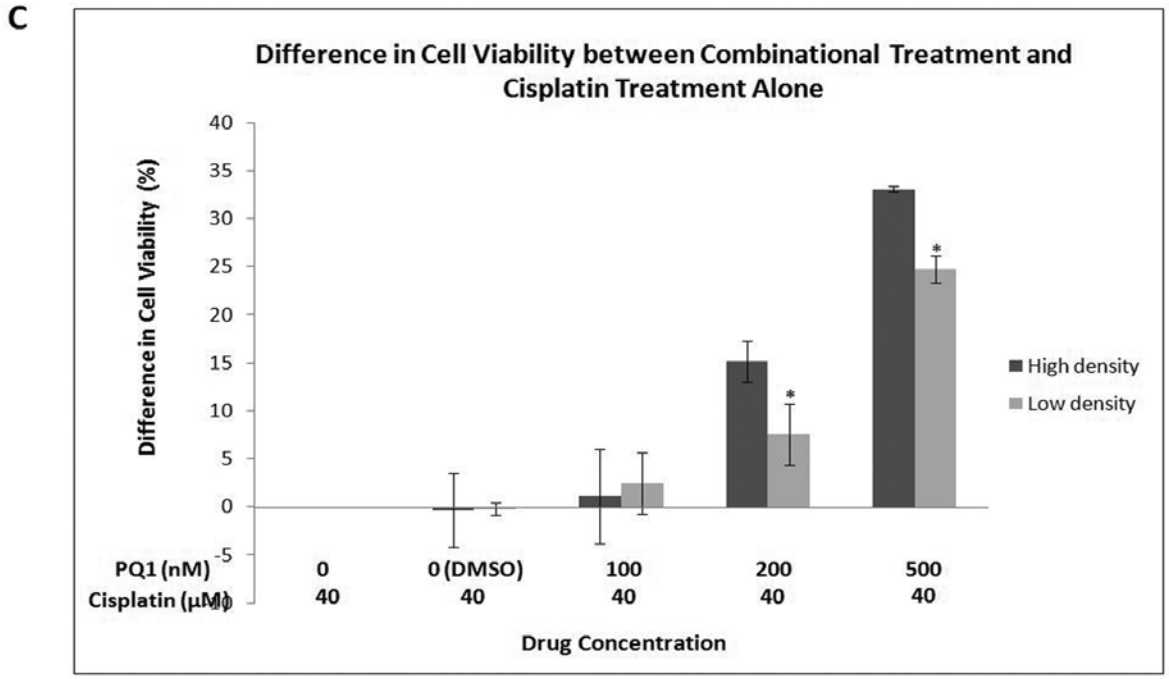


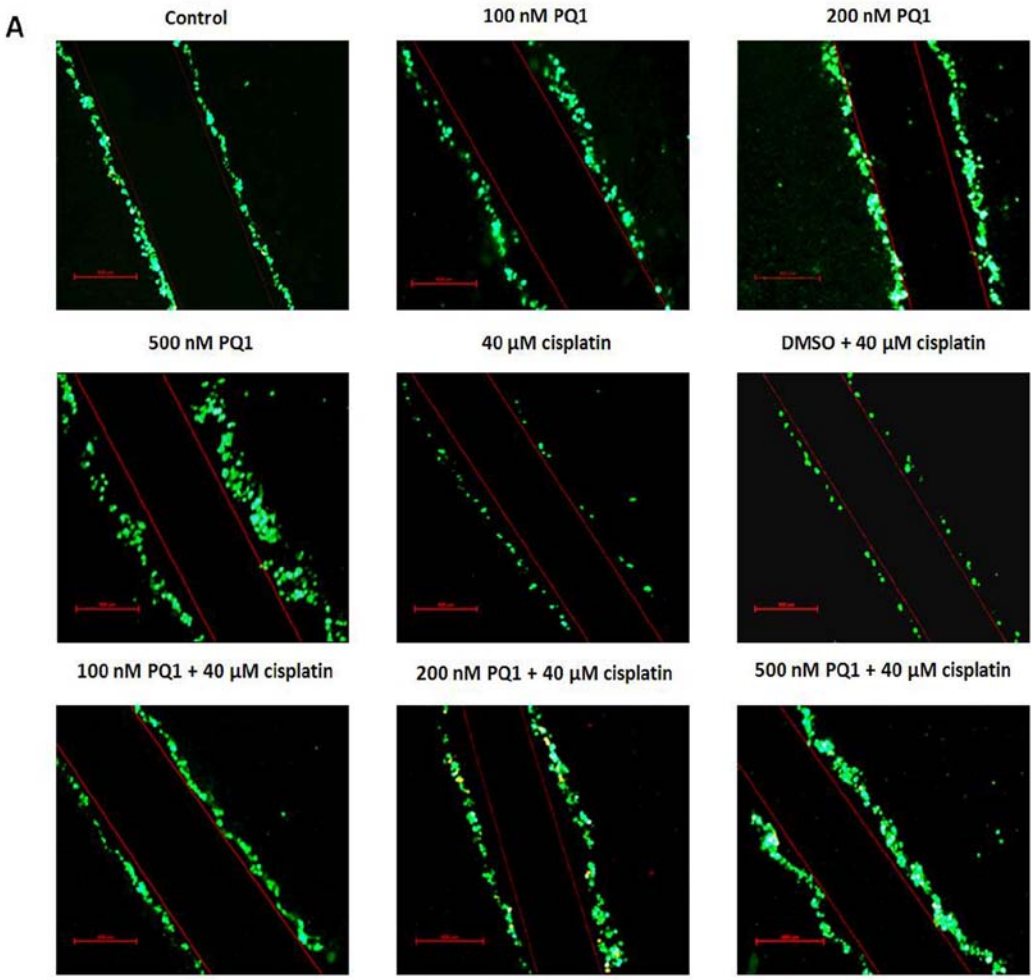
Figure 4.3 Effect of PQ1 on cytotoxicity of cisplatin

T47D cells were treated with PQ1 and cisplatin alone or in combination as indicated. Cell viability was measured under high density **(A)** and low density **(B)**. Graphical presentation of three independent experiments is presented with statistical significance. * P-value is <0.05 compared to control. **P-value is <0.05 compared to cisplatin treatment. *** P-value is <0.05 compared to PQ1 treatment. **(C)** Quantification of differences in survival, caused by PQ1 at different concentrations in the combinational treatment between low density and high density. The viabilities of cells treated with cisplatin alone at high and low density were used as controls. Data were obtained in three independent experiments and are represented as the mean ± S.E. * P-value is <0.05 compared to treatment at high density.

4.4.3 PQ1 Counteracts Cisplatin Inhibition of GJIC and Reduction of Connexins

Effects of PQ1 on GJIC mediation in the combinational treatment was further investigated by examining the activity of the gap junction channels and the expression of connexins. From previous findings, 24 hours treatment with PQ1 and cisplatin can cause cell death, which subsequently reduces the cell density and gap junctions (Fig. 4.1, 4.3A, 4.3B).

Therefore, 4 hours incubation was used to avoid the substantial cell death in the SL/DT experiment. T47D cells were treated with PQ1 and cisplatin alone or in combination, and then scrap load/dye transfer (SL/DT) was performed. As a gap junction enhancer, PQ1 significantly increased GJIC. 500 nM PQ1 increased the distance of dye transfer by 3.7 times compared to control (Fig. 4.4B). In contrast, cisplatin treatment alone decreased 60% of the dye transfer from cell to cell compared to control, indicating that cisplatin can cause an inhibition of GJIC (Fig. 4.4B). When cells were incubated with PQ1 prior to cisplatin treatment, a decrease of dye transfer induced by cisplatin was rescued (Fig. 4.4A, 4.4B). Combinational treatment of 500 nM PQ1 and cisplatin caused a 7.4-fold increase in the distance of dye transfer compared with cisplatin treatment alone, as well as a 2.6-fold increase of dye transfer compared to control (Fig. 4.4B). This implies that not only does 500 nM PQ1 restore cisplatin-inhibited GJIC, but also increases the overall GJIC activity in T47D cells. The dye transfer increased with an increase in PQ1, suggesting that PQ1-mediated GJIC is concentration dependent (Fig. 4.4A, 4.4B).


A
Control
100 nM PQ1
200 nM PQ1
500 nM PQ1
40 µM cisplatin
DMSO + 40 µM cisplatin
100 nM PQ1 + 40 µM cisplatin
200 nM PQ1 + 40 µM cisplatin
500 nM PQ1 + 40 µM cisplatin

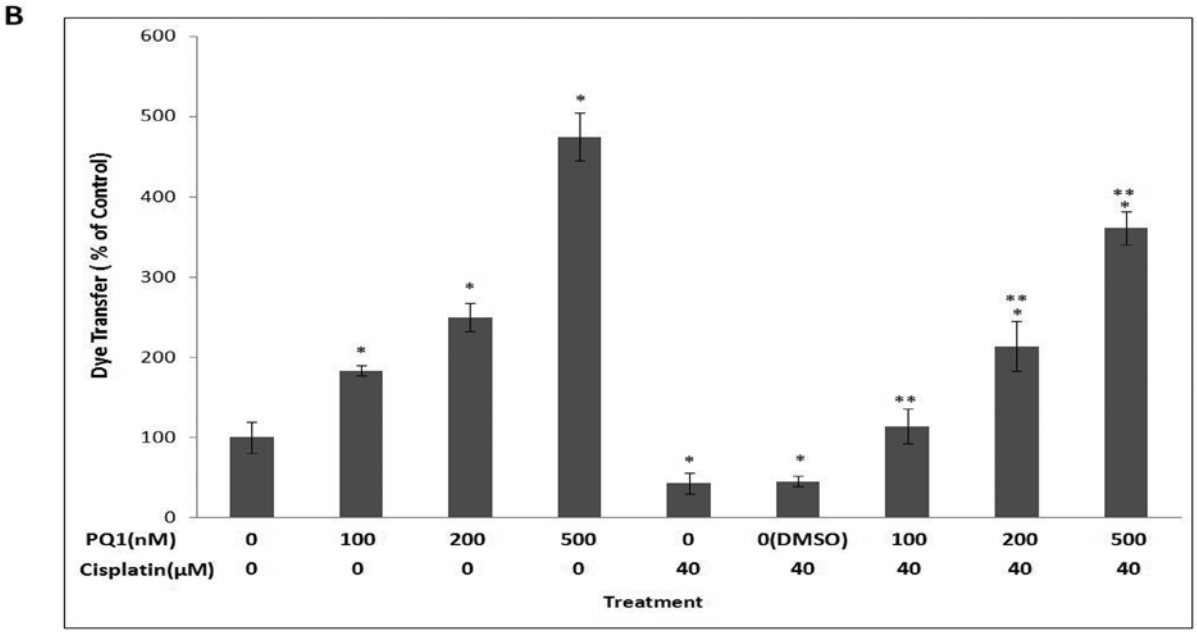

B
Dye Transfer (% of Control)
600
500
400
300
200
100
0
PQ1(nM) 0 100 200 500 0 0(DMSO) 100 200 500
Cisplatin(µM) 0 0 0 0 40 40 40 40 40
Treatment

Figure 4.4 Effect of PQ1 on cisplatin inhibition of GJIC

T47D cells were treated with PQ1 and cisplatin alone and in combination as indicated. Cells without treatment were used as controls. **(A)** Scrape load/dye transfer assay was performed. Green indicates lucifer yellow and red indicates rhodamine-dextran. Gap junction activity is examined by measuring the distance of Lucifer yellow dye transfer. Image of dye transfer was captured using a fluorescence microscope under 4X magnification. The scale bar is 100 µm in size **(B)** The distance of dye transfer from cutting edge to the farthest cells with the dye uptake was measured using Zen 2010 software. The distance of dye transfer for treated cells was normalized to the distance of dye transfer of its control group and the graphical presentation of three independent experiments was showed with statistical significance. * P-value is <0.05 compared to control. ** P-value is <0.05 compared to cisplatin treatment.

Gap junctional proteins such as connexin 26 (Cx26), connexin 32 (Cx32), and connexin 43 (Cx43) are reported to express in human breast cancer cells [29]. Here, levels of these connexins were measured by Western blotting. The results showed that 500 nM PQ1 alone increased the levels of Cx26, Cx32 and Cx43 by 44%, 55% and 18%, whereas 40 µM cisplatin alone showed a decrease in Cx26, Cx32 and Cx43 by 47%, 54%, and 42%, respectively. These indicate that the effect of PQ1 and cisplatin treatment is on the level of connexin expression (Fig. 4.5B). When cells were treated with both PQ1 and cisplatin, the downregulation of connexin by cisplatin was reversed by PQ1 (Fig. 4.5A, 4.5B). Relative to cisplatin alone, combinational treatment of 500 nM PQ1 and cisplatin significantly increased Cx26, Cx32, and Cx43 by 151%, 189%, and 74%, respectively (Fig. 4.5B). These results provide evidence that PQ1 can rescue cisplatin-induced connexin downregulation and thus subsequently increase the overall GJIC activity in T47D cells.

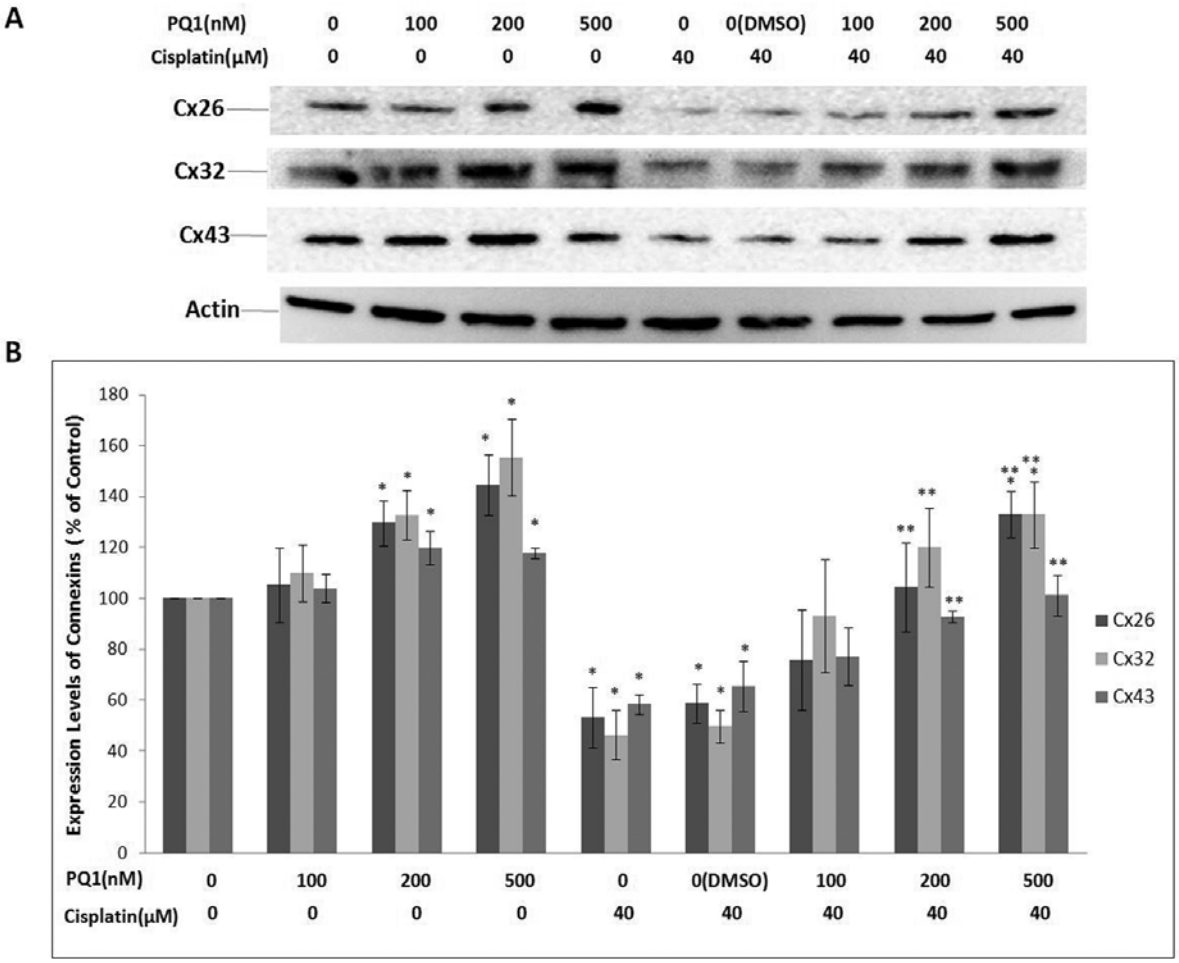


Figure 4.5 Effect of PQ1 on cisplatin reduction of connexins

T47D cells were treated with PQ1 and cisplatin alone and in combination as described. **(A)** Expression of connexin 26 (Cx26), connexin 32 (Cx32), and connexin 43 (Cx43) were examined by Western blot analysis. Actin was used as loading control. **(B)** Graphical presentation of three independent experiments shows levels of connexins normalized to control. Pixel intensities of protein bands were normalized to pixel intensities of actin, and the results of treated cells are normalized to the results of the controls. * P-value is <0.05 compared to control. ** P-value is <0.05 compared to cisplatin treatment.

4.4.4 Combinational Treatment of PQ1 and Cisplatin Enhances Apoptosis

Cisplatin is involved in multiple mechanisms after the formation of cisplatin-DNA adducts to induce cell death [6]. One mechanism is the induction of apoptosis, a programmed cell death [30]. The intrinsic and extrinsic pathways of apoptosis are reported to be activated in

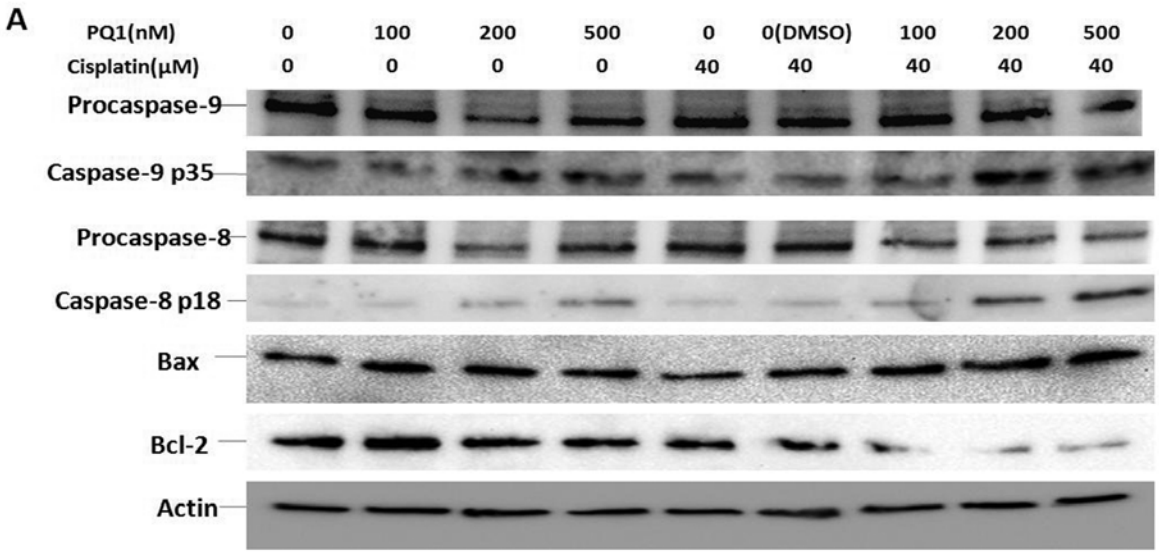
response to cisplatin in different cell lines. In human osteosarcoma, cisplatin activates caspase-8, the initiator of extrinsic pathway, and subsequently activates caspase-3 to cause cell death [31]. However, in many other cell lines (like human SCC-25 squamous carcinoma and cisplatin-sensitive human testicular cancer cell lines), cisplatin was found to activate the intrinsic pathway of apoptosis by releasing cytochrome c and sequentially activating caspase-9 (the intrinsic initiator) and caspase-3 [32, 33]. In this study, both intrinsic and extrinsic pathways of apoptosis were examined to evaluate the effect of combinational treatment on apoptosis.

T47D cells were treated with PQ1 and cisplatin individually or in combination as described in Materials and Methods. Activation of caspase-8 and -9 were determined by Western blotting using antibodies specific to cleaved caspase-9 subunit p35 (caspase-9 p35) and cleaved caspase-8 subunit p18 (caspase-8 p18). Our results showed that 24 hours treatment of 40 μM cisplatin did not significantly change the level of caspase-9 p35, but slightly increased the level of caspase-8 p18 by 13%, indicating that cisplatin induced apoptosis through extrinsic pathway within 24 hours (Fig. 4.6A, 4.6B). However, 500 nM PQ1 increased the levels of both cleaved caspase-8 and -9, indicating that the activation of both pathways can be induced by PQ1 (Fig. 4.6A, 4.6B). Interestingly, the combinational treatment of 500 nM PQ1 and 40 μM cisplatin had a synergistic effect on both caspase-8 and -9 compared to the treatment of PQ1 or cisplatin alone. The relative percentage of caspase-8 p18 expression for the combinational treatment of 500 nM PQ1 and 40 μM cisplatin is 185% compared to 113% for cisplatin and 138% for PQ1 (Fig. 4.6B). Similarly, the combinational treatment also significantly increased the relative percentage of caspase-9 p35 expression to 149% compared to 94% for cisplatin and 124% for PQ1 (Fig. 4.6B).

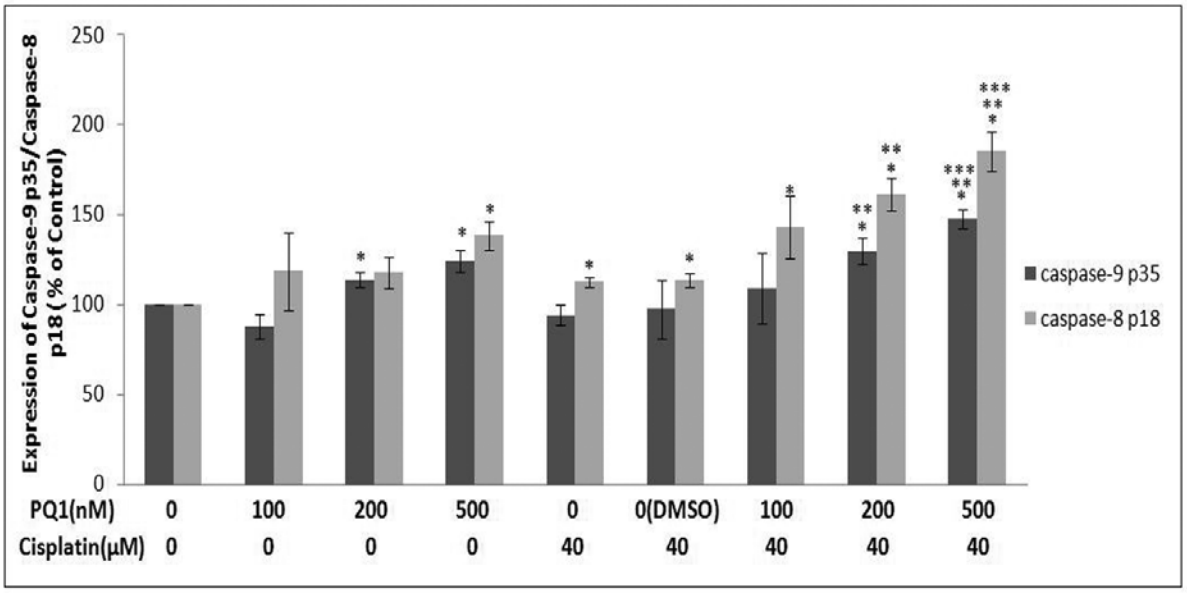
To further investigate the intrinsic pathway, expression of two important effectors: Bcl-2, an anti-apoptotic protein, and Bax, a pro-apoptotic protein, were examined. The ratio of Bcl-2 to Bax is crucial to the release of cytochrome c and subsequently determines if the cell will enter the execution phase [34]. Cisplatin is reported to increase the levels of Bax and keep the expression of Bcl-2 unchanged in cisplatin-sensitive ovarian cells [35, 36]. However, in some cisplatin-resistant ovarian cells, cisplatin-treated cells overexpress Bcl-2 and the high level of Bcl-2 protects cells from apoptosis by suppressing Bax [37]. Our results showed that 24 hours treatment of 40 μM cisplatin did not significantly change the level of Bax and Bcl-2, compared to control (Fig. 4.6B). 200 and 500 nM PQ1 increased the level of Bax, but had no significant

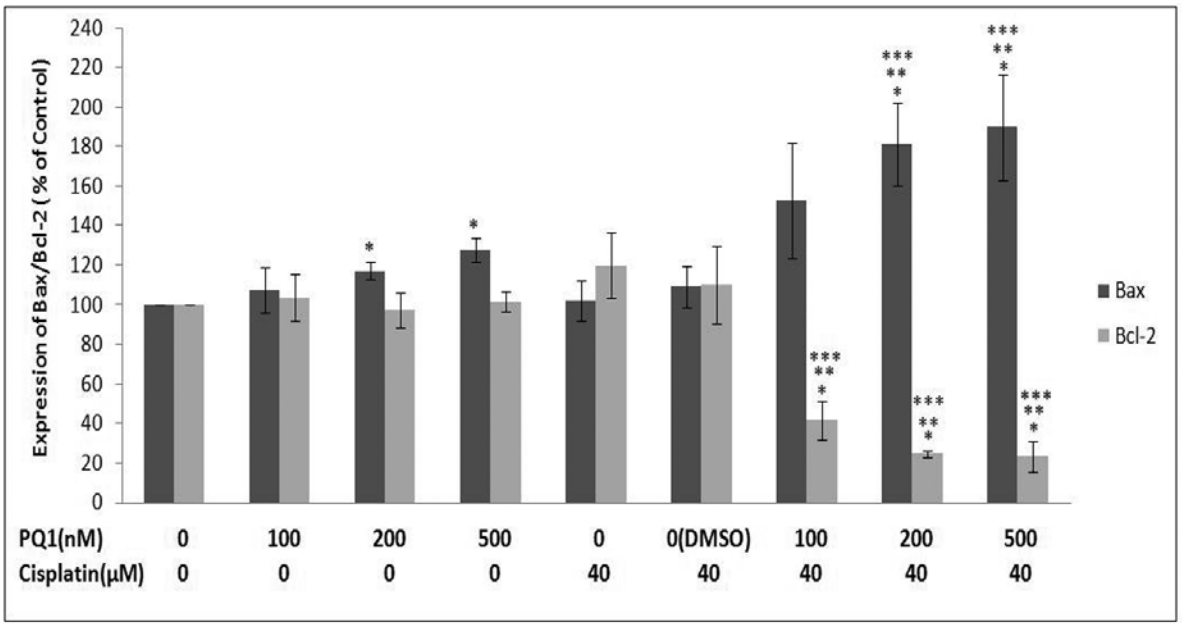
effect on Bcl-2. Interestingly, the combinational treatment with PQ1 and cisplatin synergistically increased Bax and decreased Bcl-2. Combinational treatment with 500 nM PQ1 and 40 μM cisplatin increased the level of Bax to 189.8% compared to 101.9% for cisplatin alone and 127.7% for PQ1 alone. However, the combinational treatment decreased the level of Bcl-2 to 23.3% compared to 119.8% for cisplatin alone and 101.6% for PQ1 alone (Fig. 4.6B). The results of Bax and Bcl-2 are consistent with the expression of caspase-9 p35, indicating that an activation of intrinsic pathway can be induced by the combinational treatment of PQ1 and cisplatin.

Caspase-3 is an executioner at the convergence of multiple apoptotic signaling pathways, and activation of caspase-3 is considered to be the last step of apoptosis. The apoptotic effect of combinational treatment was further investigated by examining the expression of cleaved caspase-3 using confocal microscopy. The staining of cleaved caspase-3 was faint with 40 μM cisplatin for 24 hours treatment (Fig. 4.6C). After combinational treatment of 200 or 500 nM PQ1 and 40 μM cisplatin, a significant increase of cleaved caspase-3 staining was detected (Fig. 4.6C). All the results of apoptotic factors suggest that the combinational treatment with PQ1 and cisplatin greatly enhanced apoptosis by activating both intrinsic and extrinsic pathways.



B


Expression of Caspase-9 p35/Caspase-8 p18 (% of Control)
250
200
150
100
50
0
caspase-9 p35
caspase-8 p18
PQ1(nM) 0 100 200 500 0 0(DMSO) 100 200 500
Cisplatin(μM) 0 0 0 0 40 40 40 40 40


Expression of Bax/Bcl-2 (% of Control)
240
220
200
180
160
140
120
100
80
60
40
20
0
Bax
Bcl-2
PQ1(nM) 0 100 200 500 0 0(DMSO) 100 200 500
Cisplatin(μM) 0 0 0 0 40 40 40 40 40

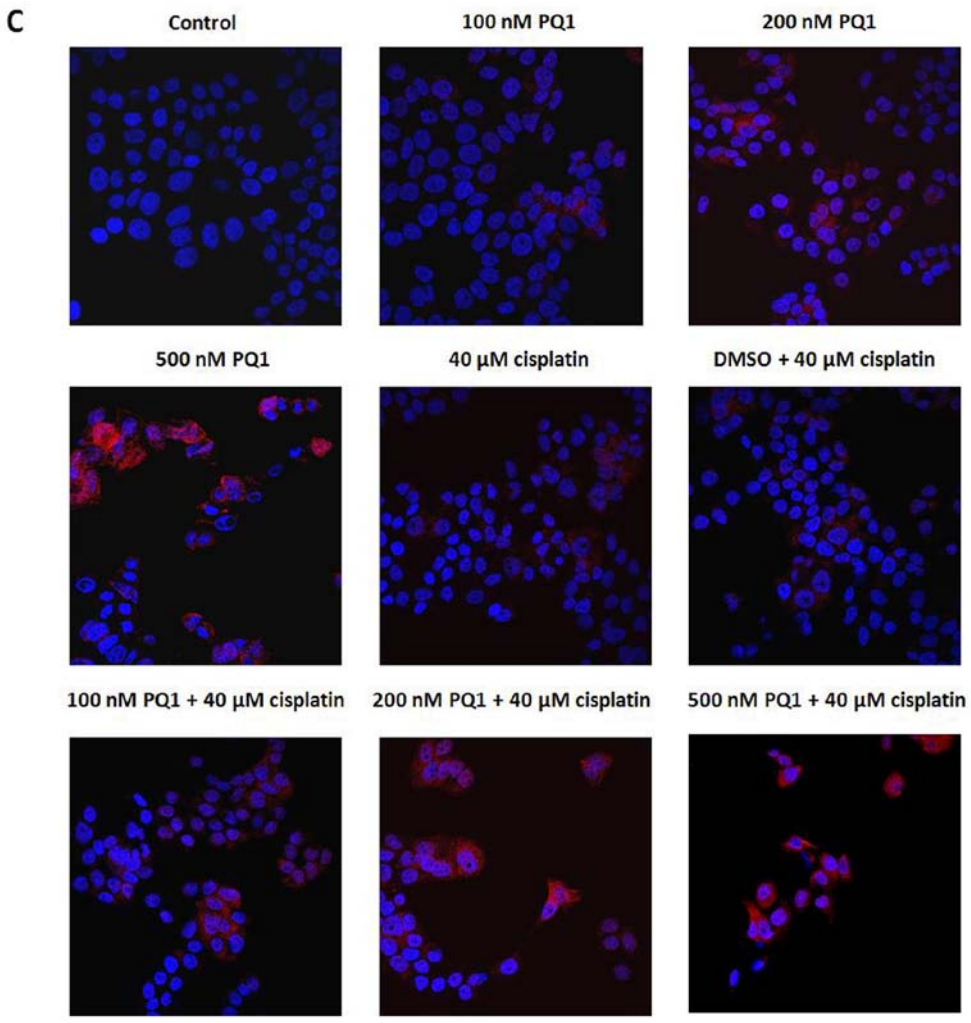


Figure 4.6 Effect of the combinational treatment of PQ1 and cisplatin onapoptosis

T47D cells were treated with PQ1 and cisplatin alone and in combination as indicated. Cells without treatment were used as controls. **(A)** Expression of procaspase-9, caspase-9 p35, procaspase-8, caspase-8 p18, Bax and Bcl-2 were examined by Western blot analysis. Actin was used as loading control. **(B)** Graphical presentation of three independent experiments shows levels of apoptotic factors normalized to control. Pixel intensities of protein bands were normalized to pixel intensities of actin, and the results of treated cells are normalized to the results of the controls. * P-value is <0.05 compared to control. **P-value is <0.05 compared to cisplatin treatment. *** P-value is <0.05 compared to PQ1 treatment. **(C)** Immunofluorescence was performed to examine expression levels of cleaved caspase-3. Red is cleaved caspase-3 and blue indicates the nuclei.

4.5 Discussion

Drug resistance and detrimental side effects are two major problems in platinum-based chemotherapy in cancer treatment [38]. Many mechanisms have been reported to contribute to cisplatin resistance, including DNA repair, signaling pathway regulation, and tumor microenvironment modulation [7]. In addition to these widely-accepted mechanisms which have been studied for many years, recent studies found that cisplatin-induced resistance is also associated with deficiency in cell-cell communication, the GJIC [7]. Deficiency of GJIC in cancer cells and inhibition of GJIC by cisplatin depress the cytotoxicity of cisplatin by preventing the cytotoxic molecules or signals from spreading throughout a tumor mass. Therefore, strategies to regulate gap junctions are needed to circumvent or decrease cisplatin resistance.

PQ1, a derivative of quinoline, has been reported to enhance GJIC, inhibit cell and tumor growth, and increase potential of combinational treatment with tamoxifen in T47D breast cancer cells [25, 26]. Studies about the effects of PQ1 on normal tissues further showed that PQ1 administration can be achieved with low toxicity to normal organs [39]. All these results indicate that PQ1 is a promising agent in GJ-based cancer therapy. The present study investigated the influence of PQ1 on the cytotoxicity of cisplatin in T47D breast cancer cells. The results showed that combinational treatment of PQ1 and cisplatin counteracted the cisplatin-induced inhibition of GJIC and decrease of connexins, and subsequently enhanced cytotoxic effects.

Formation and degradation of gap junctions are dynamic processes with a half-life of connexin not exceeding 5 hours [40]. Therefore, short-term pretreatment is used in current experiments, examining the effect of communication activity before the degradation of connexins. Current study demonstrated that 4 hours treatment is sufficient for PQ1-increasing GJIC in T47D cells. To optimize the activation of GJIC by PQ1 as well as minimize the inhibition of GJIC by cisplatin, PQ1 was dosed 4 hours prior to cisplatin in the combinational treatment.

The GJIC-mediated bystander effect has been demonstrated to play an important role in transferring toxic effects. For example, the application of bystander effect in gene therapy showed that after enhancing connexin 43 (Cx43) and GJIC by 8-bromo-cyclic-AMP treatment the toxic effect was strengthened by herpes simplex virus thymidine kinase/gancyclovir (HSV-

TK/GCV) system [17]. Consistent with previous reports [24], our studies showed that the toxicity of cisplatin was greater at high density when there is opportunity for gap junctional contacts between the cells (Fig. 4.3A, 4.3B), indicating that the cytotoxicity of cisplatin is mediated by GJIC. Therefore, inhibition of gap junctional activity and reduction of connexin expressions by cisplatin (Fig. 4.4, 4.5) may be in part the cause of low cisplatin cytotoxicity in T47D cells. The present data showed that 4 hours pretreatment of PQ1 counteracted inhibition of GJIC induced by cisplatin (Fig. 4.4). The direct effect of PQ1 on GJIC suggested a GJIC-dependent mechanism for the effect of PQ1 on cisplatin cytotoxicity. Moreover, the bystander effect of PQ1 on cisplatin reveals that PQ1 has a direct involvement in cisplatin toxicity in high cell density cultures (Fig. 4.3C).

In addition to GJIC-dependent mechanism, PQ1 also adds tumor-suppressing component to the combinational treatment by GJIC-independent mechanisms. PQ1 inhibited cell proliferation, decreased cell viability and induced apoptosis (Fig. 4.2, 4.3, 4.6). The findings also showed that the cytotoxicity of PQ1 is cell density independent, supporting that PQ1 can mediate cytotoxic effect via GJIC-independent mechanism. In primary breast tumors, Cx26 has been reported to affect cellular process by GJIC-independent functions [41]. In literature, genistein and quercetin increase Cx43 and suppress breast cancer cell proliferation in a GJIC-independent way [42]. All these studies demonstrate that connexins can function as tumor suppressors via GJIC-independent mechanism. Therefore, the GJIC-independent cytotoxicity of PQ1 may be related to the overexpression of connexins by PQ1. In the combinational treatment, Cx26, Cx32, and Cx43 protein levels were increased in the presence of PQ1 (Fig. 4.5). 500 nM of PQ1 not only rescued cisplatin-induced decreases of Cx26 and Cx32, but also increased Cx26 and Cx32 to a level higher than control (no treatment) (Fig. 4.5). Cell viability results showed that PQ1 increased toxicity of cisplatin at low cell density when there is no gap junction formation (Fig. 4.3B). Current findings established a GJIC-independent mechanism for the effect of PQ1 on cisplatin-mediated response.

This report showed that combinational treatment of PQ1 and cisplatin had a synergistic effect on apoptosis by activating both the intrinsic and extrinsic apoptotic pathways (Fig. 4.6A, 4.6B). Both the bystander effect and GJIC-independent mechanism may be responsible for the apoptosis induction. The cisplatin-DNA adducts have been reported to activate caspases and induce apoptosis [5]. Therefore, one hypothesis related to bystander effect is that PQ1 restores

gap junction channels in the combinational treatment, allowing increasing amounts of cisplatin-induced cytotoxic signals to enter the neighboring cells, which accordingly trigger the apoptosis by a GJIC-dependent mechanism. The other hypothesis related to GJIC-independent mechanism depends on connexins. Expressions of Cx26 and Cx43 have been reported to be correlated with the expression level of the proapoptotic factor Bax, suggesting that connexins may participate in apoptotic pathways [43]. Therefore, the second hypothesis is that apoptosis induced by combinational treatment may attribute to the overexpression of connexins in the presence of PQ1.

Cisplatin resistance has been widely reported in many cancer treatments, including testicular, ovarian, and cervical cancers. Studies of gap junctions revealed that GJIC and connexin expressions are dramatically reduced in these cancer cells. In ovarian cancer cell lines, expressions of Cx26 and Cx43 are reduced [44]. Chemosensitivity studies found that loss of Cx43 proteins may be associated with sensitivity to anticancer drugs [44]. In dysplastic ectocerivix, a premalignant lesion which can turn cancerous, Cx26, Cx30 and Cx43 display a loss of expression [45]. The characteristic loss of connexins in these cancers as well as the results of this study implies that the application of the combinational treatment of PQ1 and cisplatin can be expanded to the therapy of various cisplatin-resistant cancers.

The present study showed that combinational treatment of PQ1 and cisplatin activates the activity of gap junction channels, increases the expression of connexins, and potentiates the cytotoxicity of cisplatin by inducing apoptosis. PQ1 is a promising molecule for combinational therapy aimed at potentiating cisplatin efficacy, decreasing cisplatin resistance and reducing side effects.

Acknowledgement

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References

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Chapter 5 - The Effect of the PQ1 Anti-breast Cancer Agent on Normal Tissues

5.1 Abstract

Gap junctions are intercellular channels connecting adjacent cells, allowing cells to transport small molecules. Loss of gap junctional intercellular communication (GJIC) is one of the important hallmarks of cancer. Restoration of GJIC is related to the reduction of tumorigenesis and increase of drug sensitivity. Previous reports showed that PQ1, a quinoline derivative, increases GJIC in T47D breast cancer cells, and subsequently attenuates xenograft breast tumor growth. Combinational treatment of PQ1 and tamoxifen can lower the effective dose of tamoxifen in cancer cells. In this study, effects of PQ1 were examined in normal C57BL/6J mice, evaluating the distribution, toxicity and adverse effects. Distribution of PQ1 was quantified by HPLC and mass spectrometry. Expressions of survivin, caspase-8, cleaved caspase-3, aryl hydrocarbon receptor (AhR), and gap junction protein, connexin 43 (Cx43), were measured using Western blot analysis. Our results showed that PQ1 absorbed and distributed to vital organs within one hour and the level of PQ1 diminished after 24 hours. Furthermore, PQ1 increased the expression of survivin, while decreased the expression of caspase-8 and caspase-3 activity. Interestingly, expression of AhR increased in the presence of PQ1, suggesting that PQ1 may be involved in AhR-mediated response. Previously, PQ1 caused an increase in Cx43 expression in breast cancer cells; however, PQ1 induced a decrease of Cx43 in normal tissues. Hemotoxylin and eosin staining of the tissues showed no histological change between treated and untreated organs. Our studies indicate that PQ1 administration by oral gavage can be achieved with low toxicity to normal vital organs.

5.2 Introduction

Gap junctional intercellular communication (GJIC) plays an important role in controlling cell growth, regulating cell differentiation, and maintaining homeostasis in normal cells and tissues [1, 2]. Gap junction is a hydrophilic channel which is formed by transmembrane proteins, connexins [3]. Six connexins oligomerize into a hexameric structure known as connexon. Connexon at the plasma membrane may stand alone as a hemichannel or may dock with another connexon of an adjacent cell to form a gap junction [4]. The gap junction channel allows cells to exchange small molecules of less than 1.2 kDa in size including small metabolites, electrical signals, and secondary messengers including cAMP, Ca^{2+}, K^{+}, etc [5]. This maintenance of communication keeps cells at homeostasis. Literature shows that mutations in connexin genes or deficiency in GJIC are related to various human diseases, such as deafness, peripheral neuropathy, skin disorders, cataracts, and even cancers [6, 7].

Diminished connexin expression and deficiency in GJIC are considered to be two characteristics of tumorigenesis [8, 9]. Although it is still controversial about the function of connexins in invasion, intravasation, extravasation and metastasis, it has been widely accepted that connexins are tumor suppressors due to both the GJIC-dependent and GJIC-independent mechanisms [10-14]. Restoration or/and activation of GJIC in cancer cells are suggested to have the ability to reduce cancer cell proliferation and tumor growth [15, 16]. In addition to directly suppressive function, upregulation of GJIC in cancer cells is also important to increase efficacy of anticancer drugs. Re-establishment of GJIC is helpful in the delivery of drug or pro-drug throughout a tumor, and subsequently mediates bystander effect, a mechanism by which cytotoxic molecules are transported from a treated cell to a neighboring cell [13]. This mechanism has demonstrated to be an effective way to potentiate drug effect. The application of bystander effect in gene therapy showed that after enhancing connexin 43 (Cx43) and GJIC by 8-bromo-cyclic-AMP treatment, gene therapy effect was strengthened by herpes simplex virus thymidine kinase/gancyclovir (HSV-TK/GCV) system [17]. Besides gene therapy, bystander effect is also responsible for improving radiation therapy and chemotherapy [18, 19]. Therefore, development of novel agents or methods to enhance or restore GJIC in cancer cells is a new research strategy in cancer treatment.

PQ1 is a quinoline derivative which has been reported as a gap junction enhancer in T47D breast cancer cells. Previously, PQ1 increased GJIC in T47D cells, whereas it had no effect on GJIC in normal human mammary epithelial cells (HMECs) [20]. One μM of PQ1 decreased cell viability to 50% in T47D cells and attenuated 70% of xenograft tumor in nude mice [20]. Combinational treatment of PQ1 and tamoxifen showed that PQ1 potentiated the effect of tamoxifen in T47D cells [21]. All these studies imply therapeutic potential of PQ1 in breast cancer treatment. However, data of PQ1 on normal tissues are needed prior to preclinical trial of PQ1.

In this study, effect of PQ1 was evaluated in healthy C57BL/6J mice. Drug distribution to vital organs was determined and effect of PQ1 on apoptosis was analyzed. We also examined the expression of aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor that regulates transcription and activity of several important drug-metabolizing enzymes. Further analysis using histological observation of PQ1-treated tissues showed no alteration in structure change. Our results showed that the distribution of PQ1 via oral administration in mice can be assessed and low toxicity in vital organs was found.

5.3 Material and Methods

5.3.1 PQ1

PQ1. A quinoline derivative, PQ1, was obtained as described by Shi et al. [22].

5.3.2 Animals

Female C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, Maine). All mice were housed together in a temperature controlled environment (72°F) with a 12-hour light-dark cycle and unlimited access to standard mouse chow and water. Five-week-old mice, with an average weight of 24 grams, were used. Twenty-five mg/kg PQ1 was administered by oral gavage to each animal. Animal care and use protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Kansas State University, following NIH guidelines.

5.3.3 Extraction of PQ1 from Organs

Organs were diced into small pieces and diluted with 4 ml of deionized water and 10 ml of a solution of 9:1 ratio of ethyl acetate and 1-propanol. Tissue mixture was sonicated for 40 minutes, and the organic layer was separated from a separatory funnel. The aqueous layer was extracted twice with 10 ml of a 9:1 mixture of ethyl acetate and 1-propanol. The organic layers were combined, washed with 5 ml of brine, dried over anhydrous $MgSO_4$, and concentrated to dryness on a rotary evaporator. The residue was diluted with 1 ml of 1-propanol, filtered through a 0.2 µm filter disc (PTFE 0.2 µm, Fisherbrand), and analyzed using high-performance liquid chromatography (HPLC) and mass spectrometry as described below.

5.3.4 Quantification of PQ1 in Tissue Extracts using HPLC

HPLC analysis was carried out on a Varian Prostar 210 with a UV-Vis detector and a reverse phase column (250 x 21.20 mm, 10 micron, Phenomenex Inc.). A flow rate of 4 ml/min and detection wavelength of 254 nm were used. A gradient elution of solvent A, containing deionized water and 0.01% of trifluoroacetic acid, and solvent B, containing acetonitrile and 0.01% of trifluoroacetic acid, was applied for the analysis. 1,2,4,5-Benzenetetracarboxylic acid (BTA) was used as an internal standard to quantify the amount of PQ1 in the tissue extracts.

Solutions of 100 µl of various mixtures of authentic PQ1 and BTA were injected into a HPLC instrument, the peak areas corresponding to PQ1 and BTA were integrated from the HPLC chromatogram, and the ratios of the peaks were obtained. Results of the ratios of HPLC peak areas and ratios from PQ1 and BTA concentrations were plotted, and a linear correlation line was obtained from the graph. Hence using this correlation diagram, the ratio of HPLC peak areas of PQ1 and BTA from tissue extract and the added known amount of BTA to the tissue extract, the amount of PQ1 in the tissue extract was determined. Moreover, the peak that has the same retention time as that of PQ1 from the injection of the tissue extract was collected, and its mass was determined using a mass spectrometer. The mass spectrum acquired from collected peak of PQ1 from the tissue extract was identical to that of the authentic PQ1 mass spectrum. Hence, the molecular identity of PQ1 in the tissue extract was verified by mass spectrometry.

5.3.5 Mass Spectroscopy

An Applied Biosystem API 2000 LS/MS/MS mass spectrometer was used in the analysis. The eluent corresponding to PQ1 peak from the HPLC was collected and injected into the mass spectrometer. A mass of 406 corresponding to M+1 of PQ1 was found in the mass spectra, and the fragmentation pattern of this M+1 mass is identical to that of authentic PQ1.

5.3.6 Western Blot Analysis

Organs from treated or untreated mice were collected and homogenized with lysis buffer (Cell Signaling Technology, Inc, Danver, MA) using Vibra-Cell sonicator (Sonics & Materials Inc, Danbury, CT). The mixture was centrifuged at 13,000 rpm (15,700 x g using an Eppendorf centrifuge 5415R with rotor F-45-24-11) for 30 minutes at 4°C, and the supernatant was collected. Total protein concentration was determined by a Bio-Rad protein assay kit. Forty µg of protein extract were separated by 4 - 20% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) for 35 minutes at 200 Volts and proteins separated were transferred to nitrocellulose membrane. The membrane was immunoblotted against protein of interest. The goat anti-survivin antibody and mouse anti-caspase-8 antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit anti-cleaved caspase-3 and rabbit anti-connexin 43 antibodies were obtained from Cell Signaling Technology (Danvers, MA). The rabbit anti-AhR and rabbit anti-actin antibodies were purchased from Sigma-Aldrich (St. Louis, MO). Immunoreactions using chemiluminescence were visualized by FluoChem E Imaging Instrument

(Cell Biosciences, Inc, Santa Clara, CA). Intensities of the bands were digitized using Un-Scan-It software.

5.3.7 Hematoxylin and Eosin (H&E) Staining

H&E staining was performed on paraffin-embedded tissues by following standard protocol. Five µm sections were dewaxed and rehydrated in xylene and decreasing ethanol concentrations to water. Sections were stained with hematoxylin and eosin and mounted for microscopic imaging.

5.3.8 Statistical Analysis

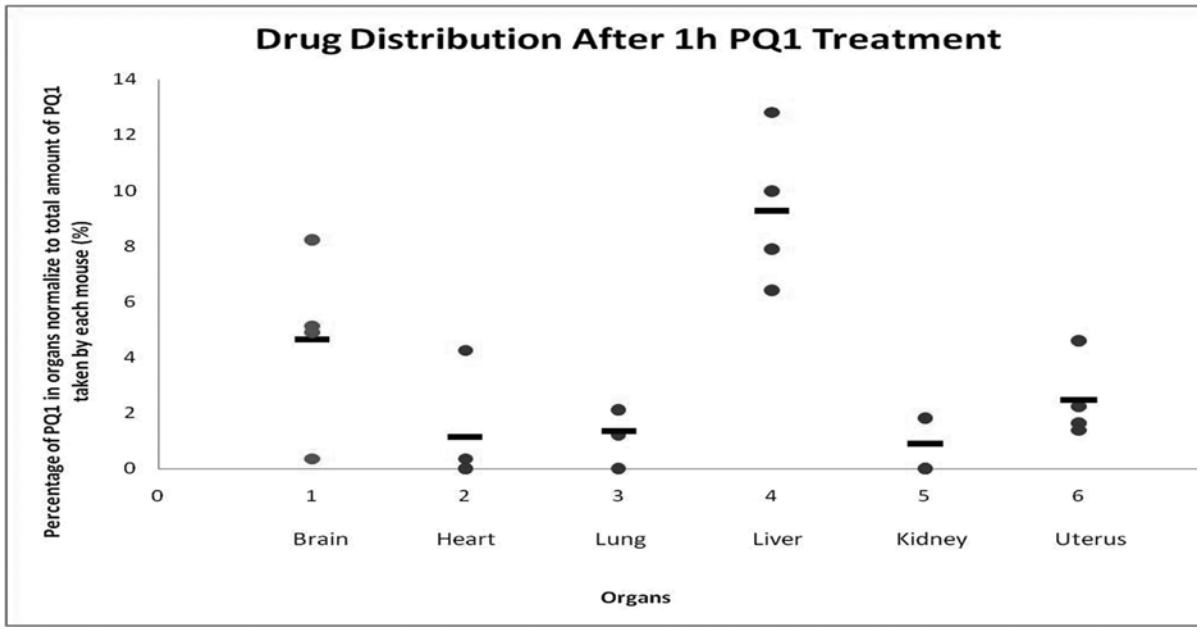
Pixel intensities of protein bands were normalized to pixel intensities of loading control protein, actin or GAPDH. All protein expression data presented were expressed as mean ± S.D. of at least three independent experiments from different animals. Significant differences were analyzed by comparing the data between treated animals and control (untreated) animals. Significance was considered at $p < 0.05$ using student's t-test.

5.4 Results

5.4.1 Distribution of PQ1

Examination of distribution is important for the development of PQ1 as an anti-cancer drug. Twenty-five mg/kg of PQ1 were administered to five-week-old female C57BL/6J mice by oral gavage. The total amount of PQ1 administered to each animal was defined as 100%. One-hour post-treatment the majority of PQ1 was detected in liver and brain at levels of 10% and 5% of the total amount administered, respectively. PQ1 was at a low detectable level in the heart with 1%, lung with 1.5%, kidney with 1%, and uterus with 2.5% (Fig. 5.1A). Interestingly, PQ1 distribution changed after 12 hours of administration. The percentage of PQ1 in liver decreased from 10% to 5%, and percentage of PQ1 in brain dropped from 5% to 2%. On the contrary, PQ1 in kidney increased from 1% to 3%, indicating a shift of PQ1 from liver to kidney had occurred. Amounts of PQ1 in heart, lung and uterus remained consistent at 12 hours of administration (Fig. 5.1B). After 24-hour treatment, no PQ1 was found in brain and heart. Percentage of PQ1 decreased to 3% in liver and 1% in kidney. The average percentage of PQ1 in uterus stayed at 3%. PQ1 in lung had a slight increase from 1.5% to 2.6% at 24-hour time point (Fig. 5.1C).

A



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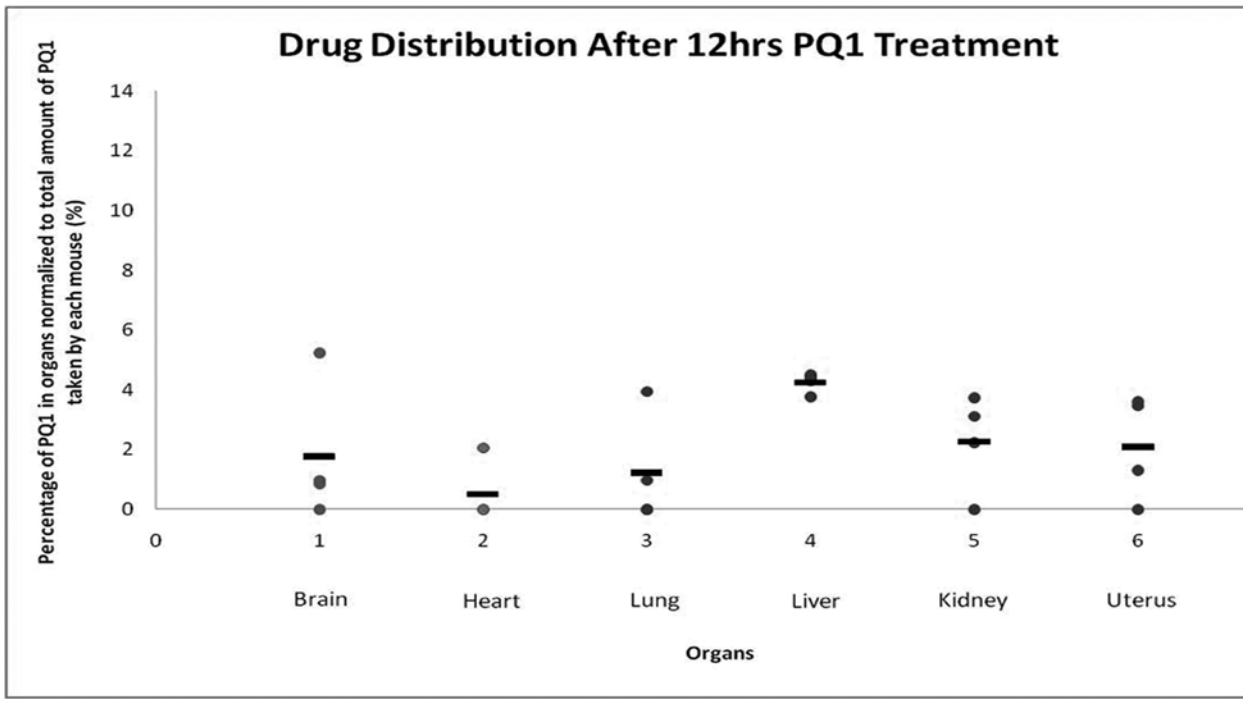

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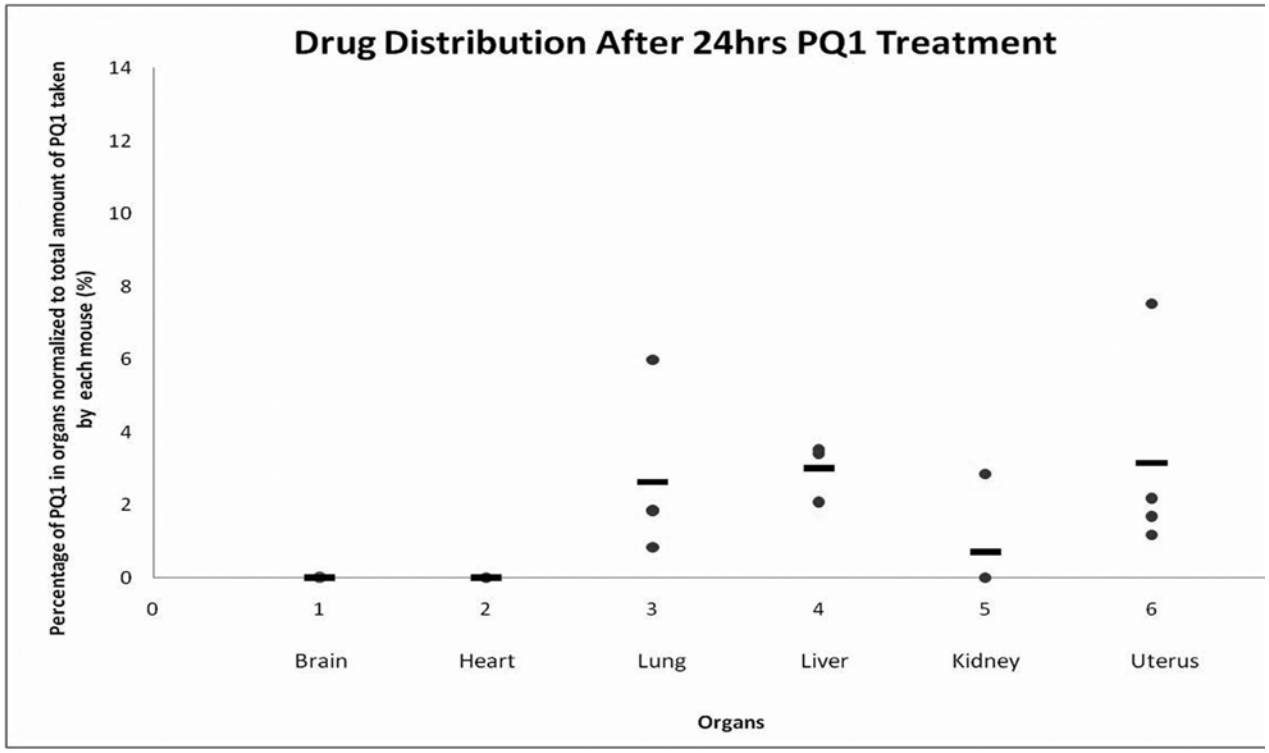


Figure 5.1 PQ1 distribution in mice

Mice, treated with 25 mg/kg of PQ1, were sacrificed at 1hr (A), 12hrs (B), and 24hrs (C). The total amount of PQ1 administered to each animal was defined as 100%. Percentages of PQ1, normalized to total amounts of PQ1 in brain, heart, lung, liver, kidney, and uterus, were presented. Data of each experiment were obtained from four mice. Data points represent the percentage of PQ1 in an organ of each mouse, and the dash lines show the average of PQ1 in four mice.

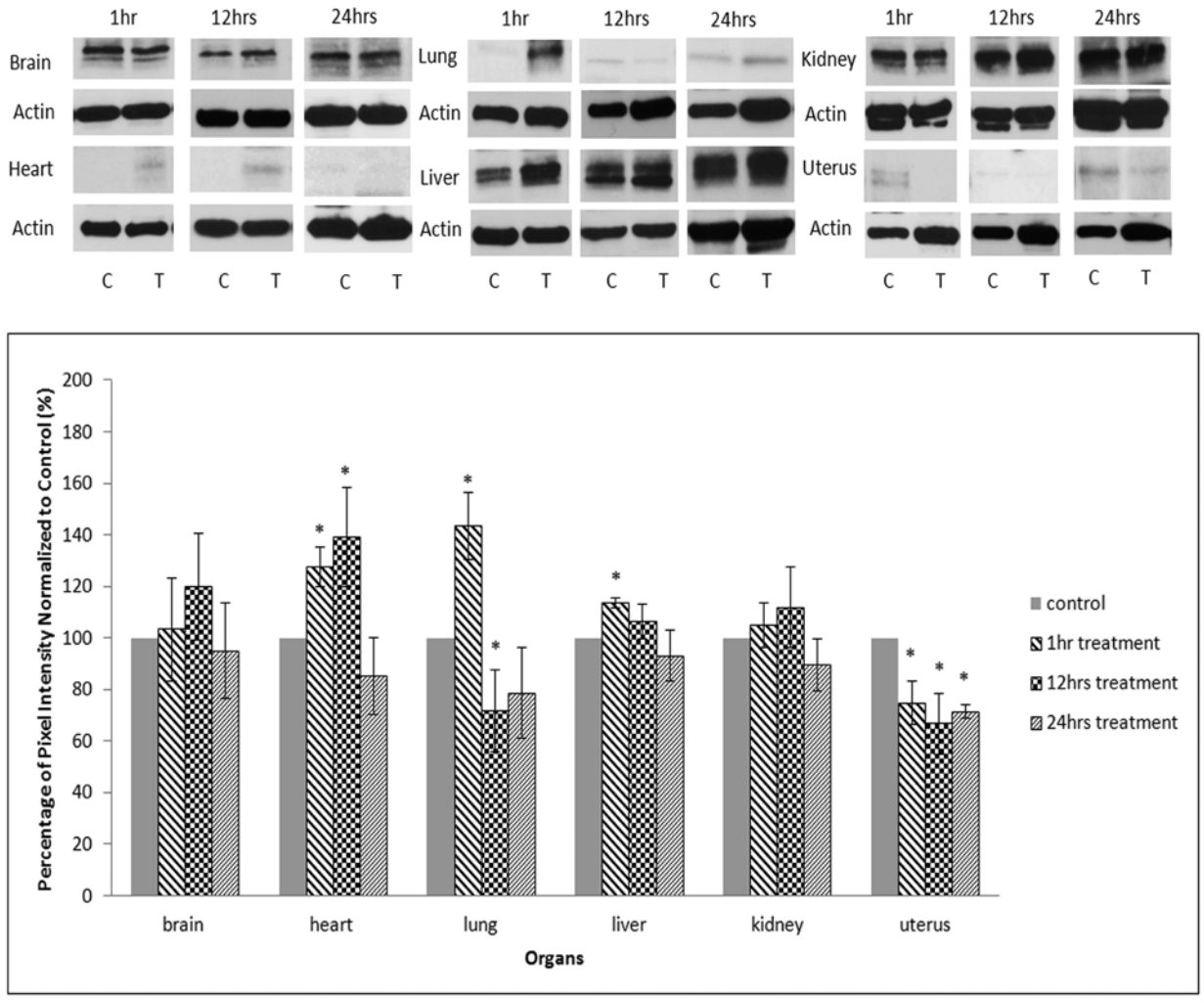
5.4.2 Effect of PQ1 on Apoptosis in Normal Tissues

Apoptosis is a programmed cell death, an important event in homeostasis of healthy organs [23, 24]. Drugs, affecting apoptosis in healthy organs, are concerned, because they may cause apoptosis-related side effects [25]. Cell proliferation or cell death often depends on the balance of pro- and anti-apoptotic factors. Thus, expressions of survivin, an anti-apoptotic factor, and caspases, pro-apoptotic proteins, were evaluated. Two specific members of caspase family were examined in the presence of PQ1: cleaved caspase-3 is the checkpoint protein of both intrinsic and extrinsic apoptotic pathways and caspase-8 is the key reporter of extrinsic apoptotic pathway [26].

The results showed that the level of survivin increased in PQ1-treated organs, while both cleaved caspase-3 and caspase-8 decreased in these organs (Fig. 5.2A, 5.2B, 5.2C). The level of survivin increased by 14% in liver, 28% in heart, and 44% in lung at 1 hour after PQ1 administration, compared to controls. These effects are consistent with the detected level of PQ1. Interestingly, the level of survivin in these organs was reduced to the same level as the controls at 24-hour time point. In brain and kidney, there were no detectable changes in survivin expression at any time point. Uterus was the only exception in which survivin decreased more than 25% after PQ1 treatment (Fig. 5.2A). As for caspase 8 expression, brain, heart, lung, liver, and uterus of the treated animals had a slight decrease in expression ranging from 12% to 37% compared to untreated animals; however, there was no significant change in the kidney (Fig. 5.2B). Cleaved caspase-3 was only detected in the uterus, liver, and lung of untreated animals; thus, the change in cleaved caspase-3 upon PQ1 treatment was measured in these three organs. A significant decrease ranging from 37% to 45% of cleaved caspase-3 at 12-hour post-treatment was observed, compared to the control (Fig. 5.2C). Results of caspases and survivin suggest that

PQ1 inhibits pro-apoptotic factors and promotes anti-apoptotic proteins in different normal organs.

A



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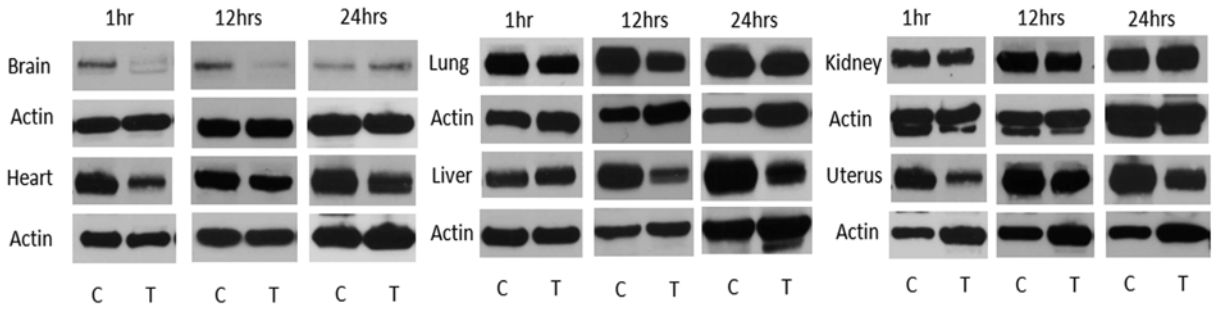
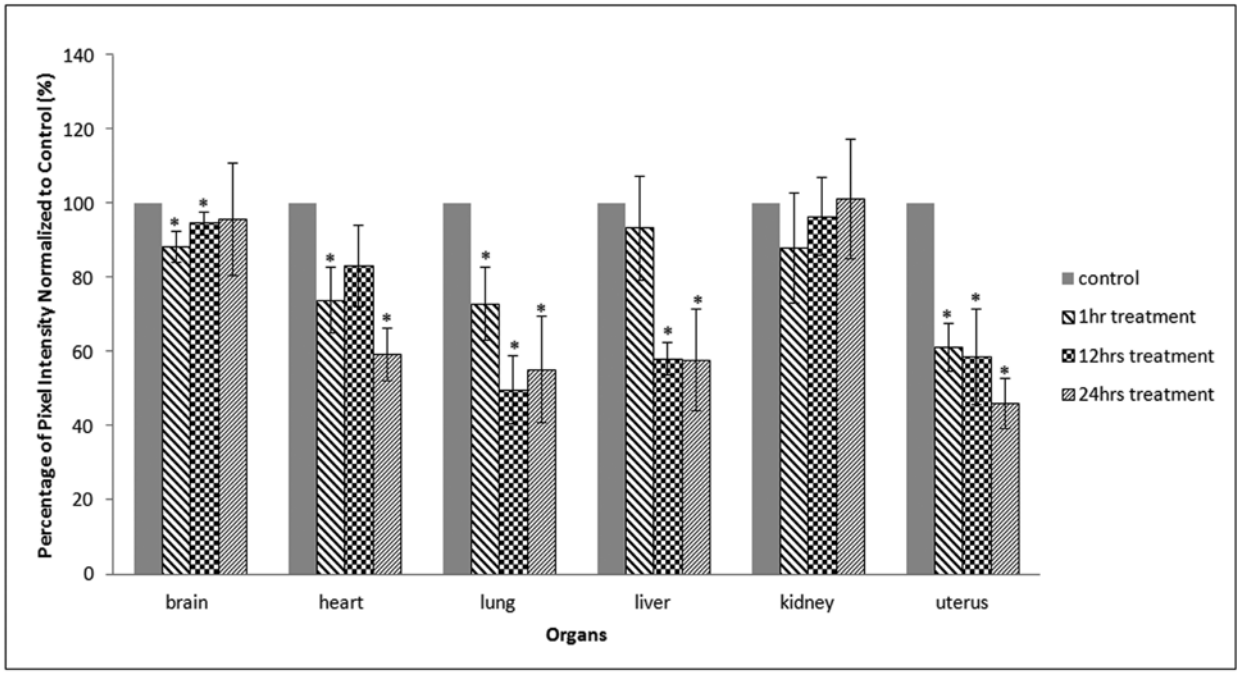
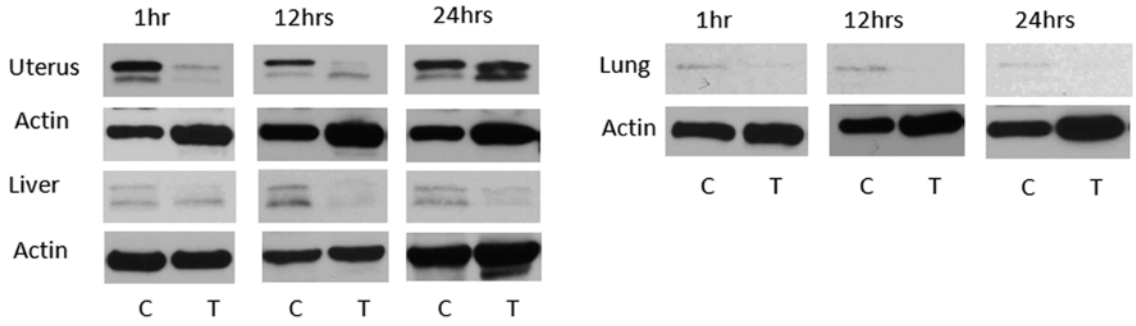




C **The Levels of Cleaved Caspase-3 in Different Organs**



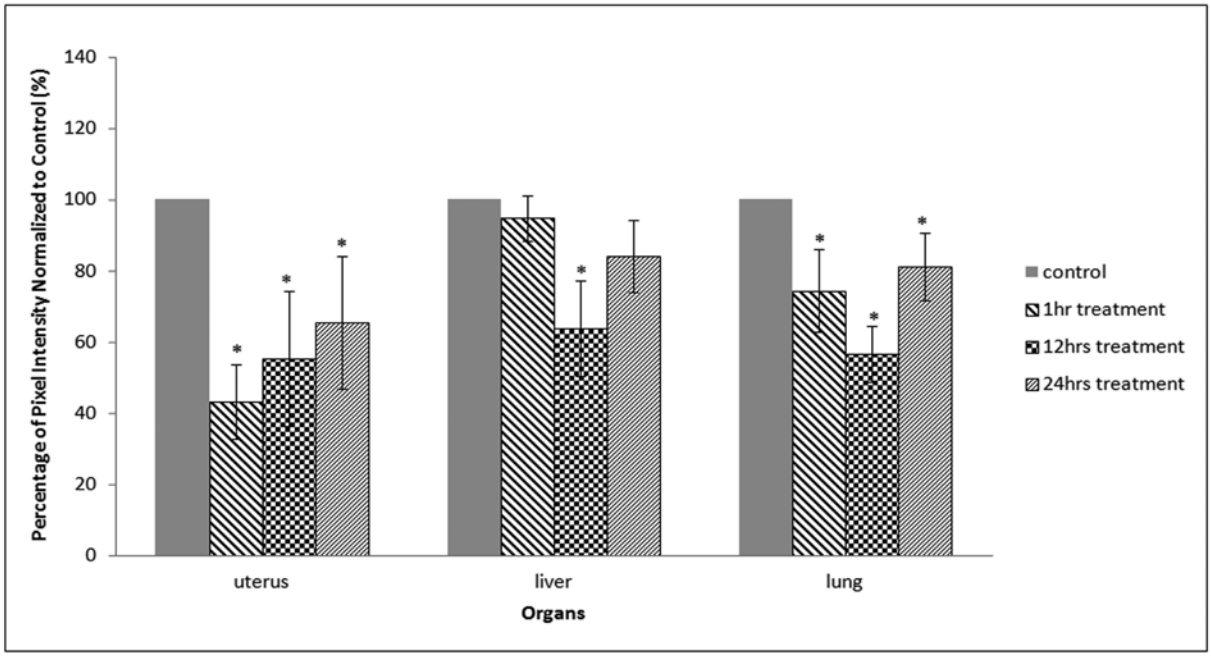


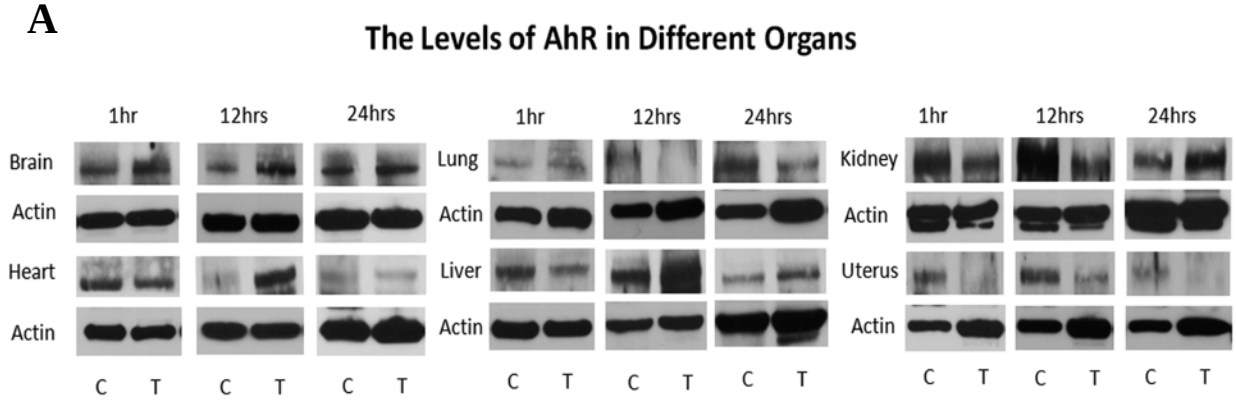
Figure 5.2 Effect of PQ1 on apoptosis in normal tissues.

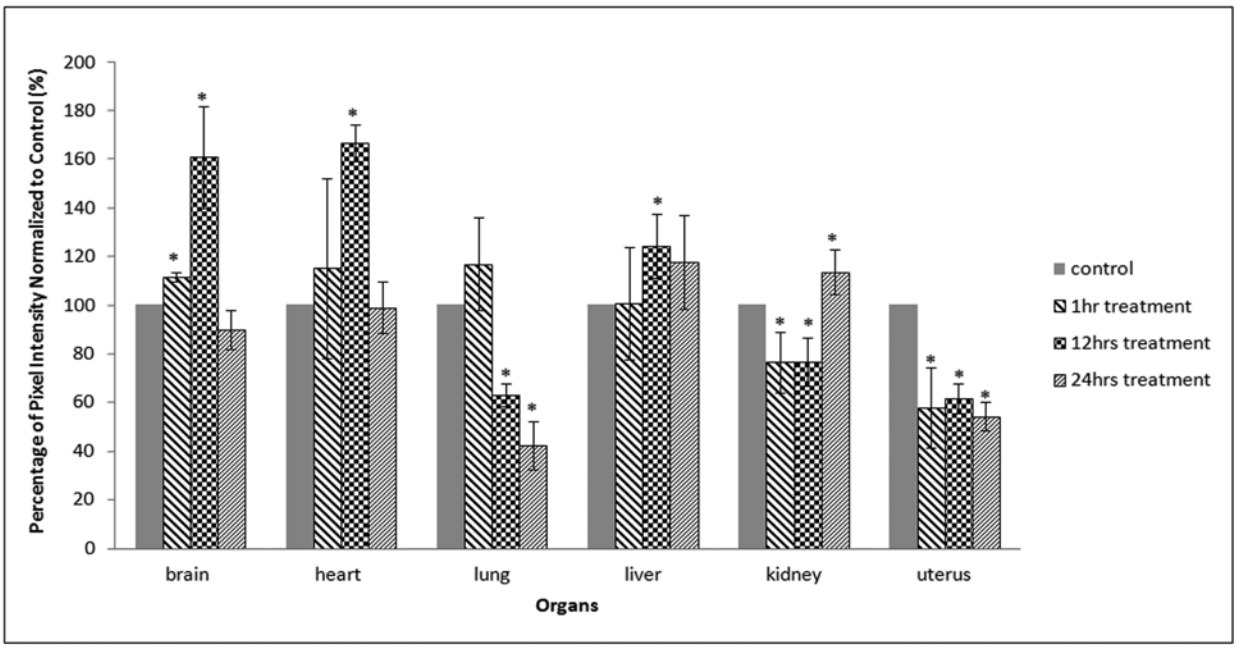
Vital organs from PQ1-treated and untreated animals were subjected to Western blot analysis, examining the effect of 1hr, 12hrs, and 24hrs treatments of PQ1 on the levels of survivin (A), caspase-8 (B), and cleaved caspase-3 (C). Immunblotting images and graphical data are presented. “C” indicates the control animals without treatment and “T” indicates PQ1-treated animals. Two fragments of activated caspase-3 were detected in uterus and liver. The upper band is 19 kDa fragment and the lower band is 17 kDa fragment. In the bar graph, pixel intensities of protein bands were normalized to pixel intensities of loading control protein, actin, and the results of treated animals are normalized to the results of control animals. Graphical presentation of three experiments are presented with ±SD and statistical significance, $*p<0.05$.

5.4.3 Effect of PQ1 on AhR Levels in Normal Tissues

Aryl hydrocarbon receptor (AhR) is a transcriptional factor involved in the metabolic pathway of aromatic hydrocarbon compounds [27]. The main adaptive response of AhR is the binding of AhR and hydrocarbon compounds, inducing metabolizing enzymes that are involved in its metabolic pathway [27]. Aromatic hydrocarbon compounds have been demonstrated to trigger AhR-mediated pathway for its metabolism; thus, the effect of PQ1, an aromatic hydrocarbon compound, on AhR was examined.

The results showed that the level of AhR in brain, heart, and liver increased significantly at 12-hour post-PQ1 treatment, 161%, 167%, and 124% compared to controls, respectively; however, there was a delay in detecting AhR in the kidney. A 114% AhR was detected in the kidney at 24-hour point (Fig. 5.3A). From the drug/tissue distribution data, the amounts of PQ1 peaked at 1 hour in brain, heart and liver, but peaked at 12-hour point in kidney (Fig. 5.1A, 5.1B). These suggest that there is a time-delay response in AhR in these organs. Interestingly, the level of AhR fluctuated from 117% at 1-hour of dosing to 63% at 12-hour of dosing. Furthermore, only 57%, 61%, and 54% of AhR were detected in the treated uterus at 1-, 12-, and 24-hour time points, respectively, compared to controls (Fig. 5.3A). An early onset of AhR downregulation after PQ1 administration implies that PQ1 might be involved in a different mode of action in the uterus. At 1 hour of PQ1 administration, level of AhR proportionally changed along with the amount of PQ1 in liver, indicating a direct dependent function of AhR to PQ1 in liver (Fig. 5.3B). The data demonstrated that PQ1 can trigger the change in expression of AhR in brain, heart, liver, and kidney.





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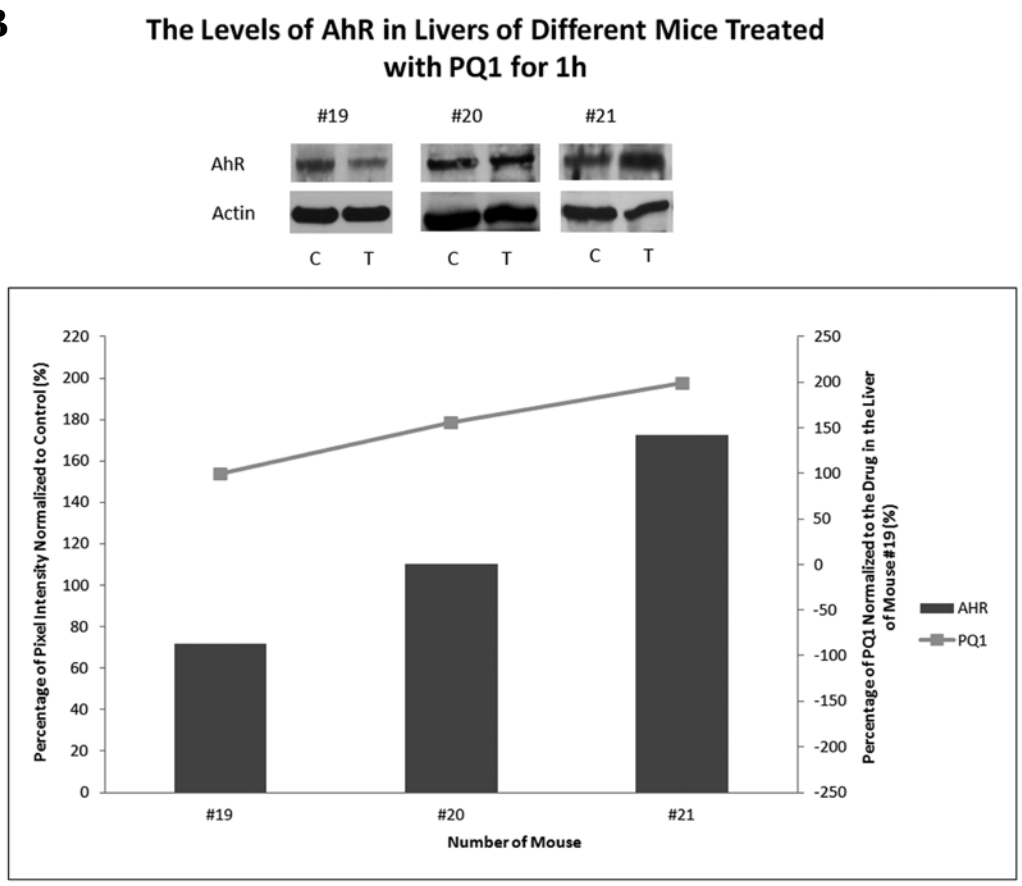


Figure 5.3 Effect of PQ1 on AhR levels in normal tissues.

(A) Western blot analysis, examining the effect of 1hr, 12hrs, and 24hrs treatments of PQ1 on the level of AhR, was performed. Mice without PQ1 treatment were used as control. Immonoblotting images and graphical data are presented. “C” indicates the control animals without treatment and “T” indicates PQ1-treated animals. In the bar graph, pixel intensities of protein bands were normalized to pixel intensities of loading control protein, actin. Graphical presentation of three experiments are presented with ±SD and statistical significance, $*p<0.05$. (B) The level of AhR changes proportionally along with the amounts of PQ1 in liver after 1-hour treatment. Immnoblotting images are also shown above the graph. A line indicates percentage of PQ1 normalized to the amount of PQ1 in the liver of a corresponding animal. AhR level normalized to control group are shown by the bar graph. All the data have been normalized with the body weight of each mouse as well.

5.4.4 Effect of PQ1 on Connexin in Normal Tissues

Since PQ1 has been shown to enhance GJIC [20] and increase Cx43 expressions (data not shown) in breast cancer cells, expressions of Cx43 in PQ1-treated and -untreated organs was measured. Cx43 was detected in heart, brain, and lung in the absence of PQ1 treatment; however, the level of Cx43 diminished in all PQ1-treated organs. A statistically significant decrease of 31% compared to control was found at 24-hour point in the heart. A constant level of Cx43 in the lung was observed at all-time points. Interestingly, level of Cx43 in brain gradually declined over time (Fig. 5.4). These results suggest that the function of PQ1 in normal cells may involve in different mode of action as compared to previously observed in cancer cells.

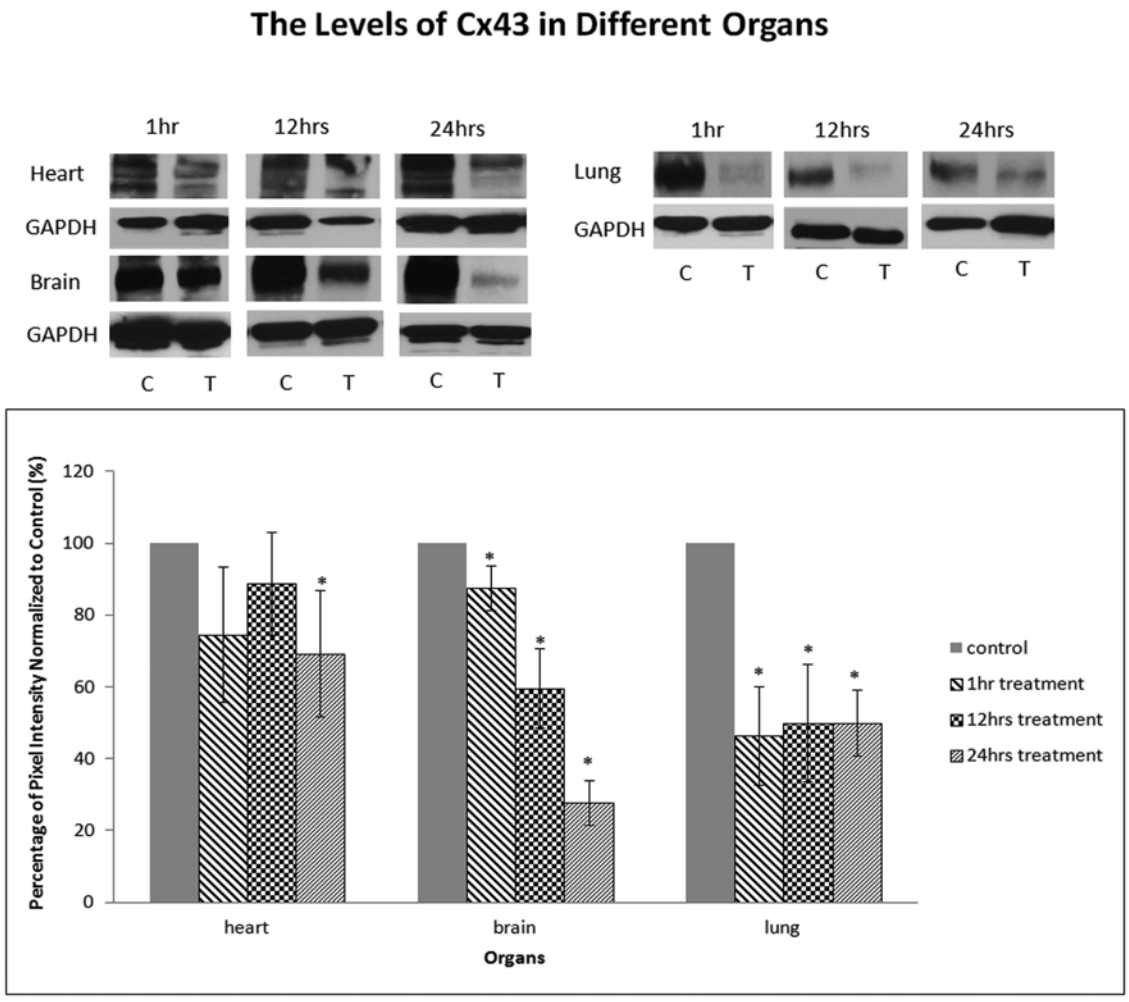


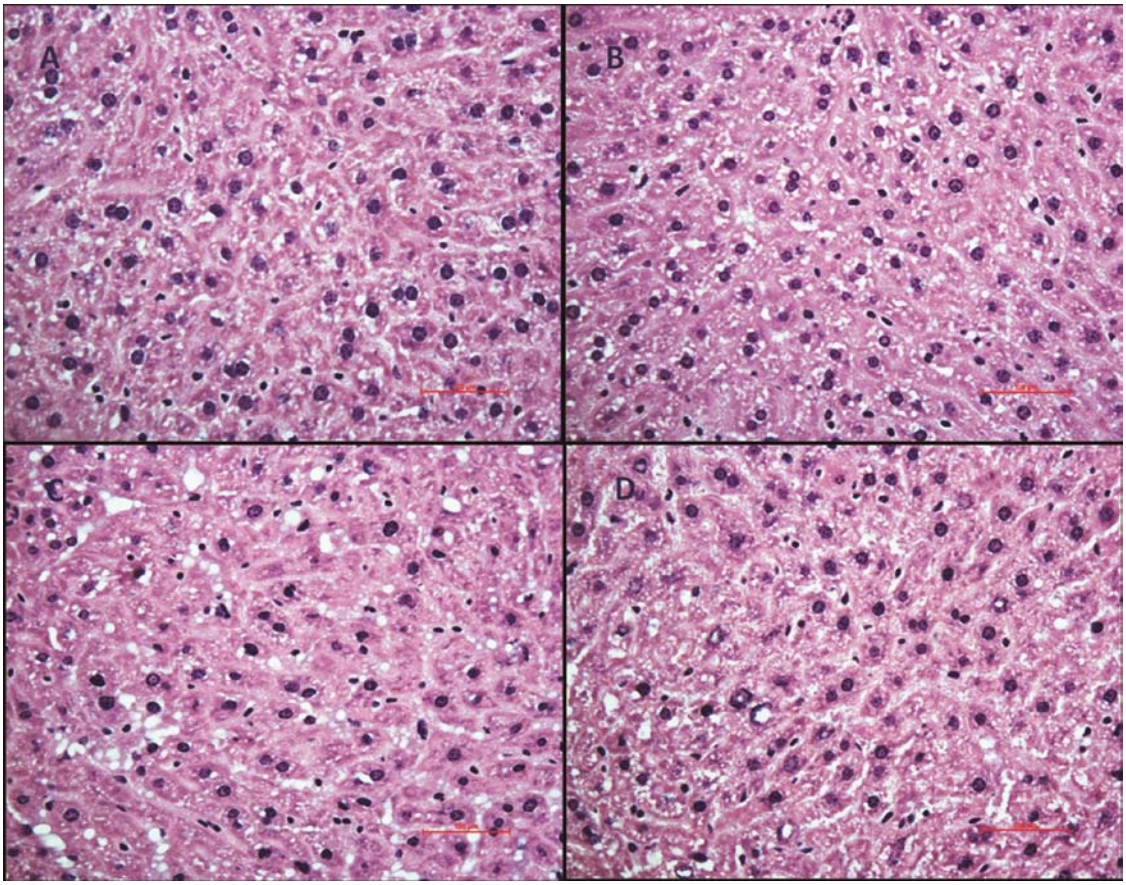
Figure 5.4 Effect of PQ1 on connexin 43 expression in normal tissues.

Brain, heart and lung from treated and untreated mice were subjected to Western blot analysis, examining the effect of 1hr, 12hrs, and 24hrs treatments of PQ1 on the level of connexin 43. Mice without PQ1 treatment were used as control. Both immunoblotting images and graphical data are presented. Both the phosphorylated Cx43 and unphosphorylated Cx43 were detected in heart. The upper band indicates the phosphorylated Cx43, and the lower band indicates the unphosphorylated Cx43. Pixel intensities of protein bands were normalized to pixel intensities of loading control protein, GAPDH, in the bar graph. Graphical presentation of three experiments are presented with ±SD and statistical significance, $*p<0.05$.

5.4.5 Histological Analysis of Normal Tissues

Liver is an important organ in drug metabolism. Hematoxylin and eosin (H&E) staining of PQ1-treated organs was performed. All twenty-four mice were assessed grossly or microscopically for histological changes. Histological results showed that PQ1-treated liver remained unchanged compared to control, which indicate no observable toxicity of PQ1 to liver at the treated dosage and time (Fig. 5.5A). Other tissues including heart, adrenal gland, kidney, and reproductive tract were also examined and no histological change was observed (Fig. 5.5B). Twenty-one of the histologically PQ1-treated mice had no evidence of hemorrhage or inflammatory cells. These mice had no histological evidence of lesion compared to control mice without PQ1 treatment at any time point.

A



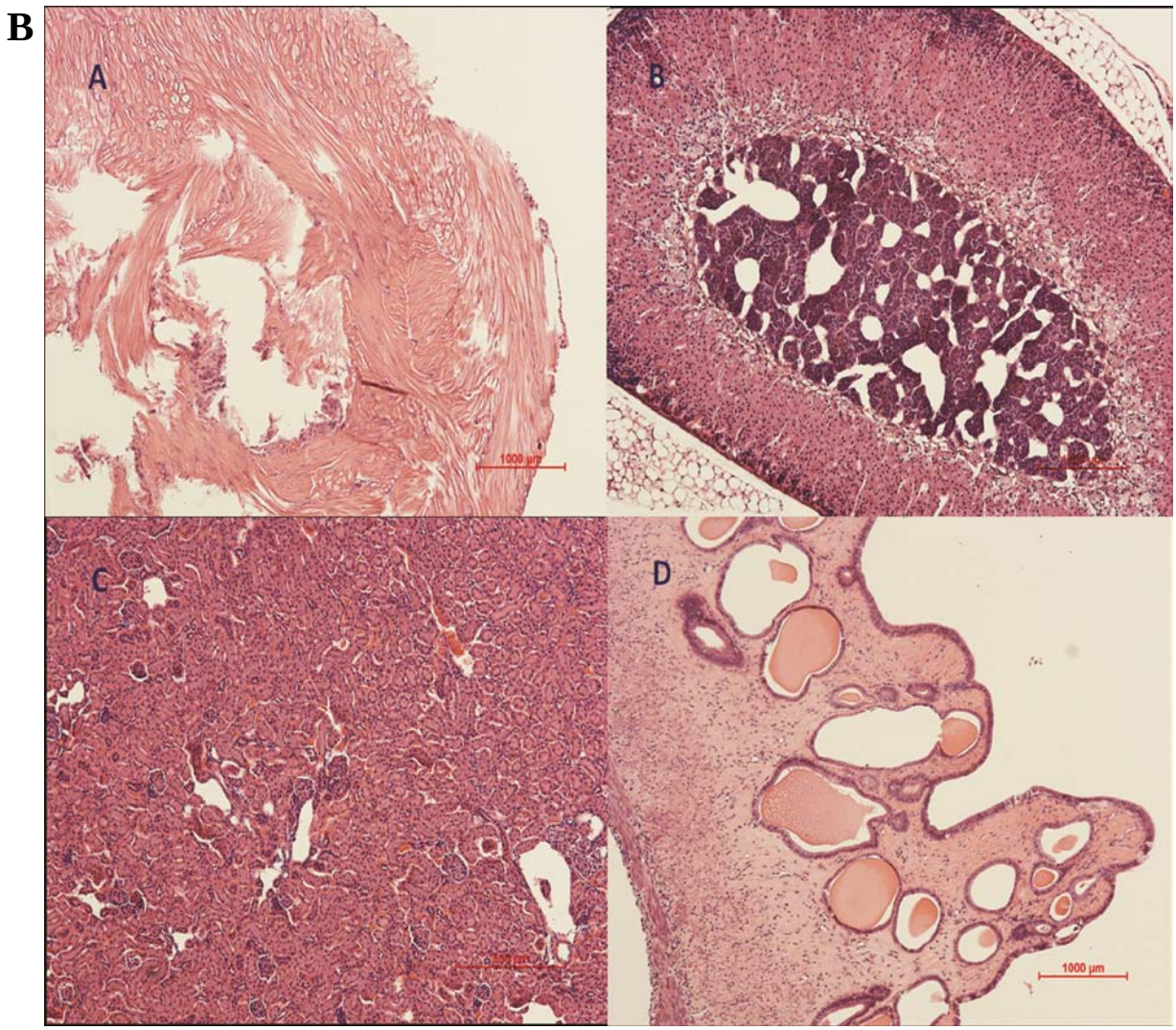


Figure 5.5 A H&E staining of whole organs.

(A) PQ1-treated tissues were examined by H & E staining. A. Liver sections from untreated animal (a) and PQ1-treated animals at 1hr (b), 12hrs (c), and 24hrs (d) are presented. Toxicity of PQ1-treated liver was examined by H & E staining using 40X magnification. Histological results showed that PQ1-treated liver had no change compared to control. (B) Histology of PQ1-treated animals for heart (a), adrenal gland (b), and reproductive tract (d) were observed under 4X magnification, and kidney (c) was observed under 10X magnification. The results show no histological alteration in the treated animals compared to control.

5.5 Discussion

Cancer is a complicated disease with multiple deregulation pathways, necessitating cancer treatment with multiple and combinational approaches [28]. The deficiency of GJIC in cancer cells adds to the complexity of cancer treatment in which the lack of drug transfer to the surrounding area creates challenges to cancer therapy [14]. Some anticancer drugs are reported to inhibit GJIC and reduce connexin expression [29, 30]. Hence, restoration of GJIC in cancer cells is a focal point in combinational treatment by potentiating the effect of anticancer drugs. In addition to combinational treatment, overexpression of connexin and activation of GJIC also play a suppressive role to tumors [13]. Therefore, the development of molecules and agents increasing the connexin expression and GJIC can be a useful therapeutic strategy in cancer therapy.

Quinolines are known for their anticancer effects by targeting tumor hypoxia and modulating multidrug resistance [31, 32]. Previous reports showed that PQ1, a quinoline derivative, enhances GJIC, inhibits cell and tumor growth, and increases potential of the combinational treatment with tamoxifen in T47D breast cancer cells [20, 21]. Therefore, the current study provides data of drug/tissue distribution and examines key factors of apoptotic pathways in normal mice.

Oral gavage, a desirable and safe route of administration, is used in this study. Uptake of any drug is depending on the rate of blood flow; thus, the level of PQ1 was evaluated in five vital organs (brain, heart, lung, kidney, and liver) that have high rate of blood flow. PQ1 was measured in each vital organ after oral administration. The effective dosage of PQ1 falls in nM range in cells and xenograft tumors [20]. To investigate the toxicity in normal organs, a higher concentration of PQ1 was administered at 25 mg/kg body weight, which is equivalent to 47.7 μM. The concentrations of PQ1 in organs examined were more than 20-fold higher than the effective dosage. PQ1 was detected in all tested organs after 1-hour treatment and diminished at 24-hour post-treatment, suggesting that PQ1 can be eliminated or excreted after 24 hours (Fig. 5.1). The highest concentrations of PQ1 were found in the liver and kidney at different time points (Fig. 5.1A and 5.1B). A high percentage of PQ1 was detected in the brain at 1 hour. This detectable level may be due to the processing of tissue in which PQ1 in the blood vessels could

not be excluded during the whole tissue extract (Fig. 5.1A). Our results show that PQ1 can be absorbed, distributed to vital organs, and metabolized in C57B/6J mice.

A serious side effect of therapeutic drugs is a potential activation of apoptosis pathway in normal cells. For example, diarrhea, a common side effect of chemotherapy, is partly caused by induced apoptosis in normal cells of the small intestinal epithelium [25]. It has also been reported that both chemotherapeutic drugs and irradiation can induce apoptosis in normal thymocytes [33, 34]. In this report, the presence of PQ1 via oral gavage caused a decrease in cleaved caspase-3 and an increase in survivin of normal tissues, indicating the inactivation of apoptosis (Fig. 5.2A, 5.2C). Further study of extrinsic apoptotic pathway showed a decrease of caspase-8 after treatment of PQ1, which further elucidates that PQ1 cannot activate the extrinsic pathway of apoptosis in normal tissues (Fig. 5.2B). The effect on apoptosis in normal organs indicates a minor, apoptosis-related side effect caused by PQ1. Interestingly, PQ1 increased caspase-3 cleavage [20] and the level of caspase-8 protein in T47D cells. The opposing aspect of PQ1 on apoptosis in cancer cells compared to normal tissues implies that PQ1 may have a different mechanism in cancer cells. The difference between cancer and normal cells is also shown by the function of PQ1 on connexin expression. PQ1 enhances GJIC [20] and increases connexin expression in T47D breast cancer cells; however, it decreases the expression of Cx43 in a normal heart, brain, and lung (Fig. 5.4). PQ1 mechanism of opposing effects in normal and cancer cells is not clear. Further studies are needed to clarify the causes of anti-tumor effects.

AhR, a ligand-dependent transcription factor involved in the transcription of many important drug-metabolizing enzymes [35], is widely expressed in rodent and human tissues [36]. Increase of AhR protein level in PQ1-treated mice was observed in vital organs, indicating the possible involvement of PQ1 in the activation of ligand-dependent transcription of AhR pathway (Fig. 5.3A). The proportional relation between AhR expression and detected level of PQ1 in liver at 1 hour showed a direct impact of PQ1 on AhR expression. However, AhR was decreased by PQ1 treatment in the lung compared to control. Previous report demonstrated that increase of AhR was found in the early stage of lung adenocarcinoma [37], suggesting that low level of AhR in PQ1-treated lung is due to tissue specificity. Furthermore, increase of AhR in PQ1-treated organs implies that PQ1 is involved in AhR-mediated pathway. Further analysis of gene regulation and enzyme activities in AhR-mediated pathways is needed to elucidate the metabolism of PQ1.

Gap junction has been studied for more than forty years. Until recently, the involvement of gap junction in cancer has been reported and widely discussed. Although several molecules have been developed to modulate different levels of gap junctional proteins and GJIC [13], none of these molecules has reached clinical trials for the treatment of cancer. Our present findings support the notion that PQ1 is a promising anti-breast cancer candidate and may serve as a lead compound for drug development.

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Chapter 6 - General Discussions

Cancer is a disease characterized by deregulated cell proliferation and suppressed cell apoptosis. To develop strategy for the prevention and treatment of cancers, it is necessary to understand the mechanisms of carcinogenesis. Carcinogenesis is a multi-step, multi-mechanism process, which can be divided into at least three steps: initiation, promotion, and progression [1, 2]. In principle, prevention and treatment of cancer can occur at each step. Avoiding known risk factors can prevent cancer initiation but can never reduce the initiation step to zero. Recent studies shed some light on the treatment of metastatic cells by uncovering the biological mechanisms of cancer cells; however, it is still a big challenge to inhibit the progression step. Compared to the initiation and progression steps, promotion step is a reversible and rate limiting step of carcinogenesis. Therefore, developing methods to block this step makes the most sense [3].

Considering GJIC has been linked in normal cells to regulation of growth control, differentiation, and apoptosis, it is not surprising that disruption of GJIC is related to carcinogenesis. Mounting research indicates that GJIC is involved in every step of carcinogenesis, especially in cancer promotion [4]. A wide variety of tumor promoters, such as drugs, environmental pollutants, dietary chemicals, heavy metals, natural plant and animal toxins, solvents, metabolites, growth factors, hormones, and neurotransmitters, has been shown to inhibit GJIC *in vitro* and/or *in vivo* [5]. On the contrary, several anti-tumor promoting natural and synthetic chemicals have been shown to restore GJIC with the loss of tumorigenicity [5]. In addition to the channel activity of GJIC, connexin proteins, especially Cx43, is considered as a tumor suppressor in the promotion step. Loss of connexin expression has been observed in most, if not all, non-metastatic cancer cells. Over-expression of connexins has been reported to inhibit cancer cell proliferation and re-gain the growth control, even though the connexins are failed to form gap junctions [6]. Therefore, developing methods or molecules to restore the connexin expression and/or the GJIC in the cancer promotion stage is a strategy in cancer treatment.

PQ1 has been considered as a gap junction enhancer as well as an anti-cancer agent in T47D breast cancer cells and xenograft tumors. In the current study, the anti-cancer effect of PQ1 was examined in T47D breast cancer cells. T47D is a non-metastatic human breast cancer cell line [7]. PQ1 decreased cell viability in a time- and dose-dependent manner, indicating the

cytotoxicity of PQ1 on T47D cells. Mechanism studies showed that PQ1 exerted its anti-cancer effect by inducing apoptosis through caspase cascade. In the presence of PQ1, both caspase-8, an extrinsic apoptotic pathway reporter, and caspase-9, an intrinsic apoptotic pathway reporter, were activated, which subsequently activated caspase-3.

It is not clear that how PQ1 induce the caspase cascade. Krutovskikh et al. have reported that GJIC propagates cell death in rat bladder carcinoma BC31 cells by spreading cell-killing signals (such as Ca^{2+}) from apoptotic cells to healthy surrounding cells [8]. Because PQ1 is a gap junction enhancer which increases GJIC in T47D cells [9], initial hypothesis is that the apoptosis induced by PQ1 is related to GJIC. However, later studies provided some evidence that PQ1-mediated apoptosis is through GJIC-independent pathway. The critical evidence of this effect was demonstrated via cell density method. The toxic effect of PQ1 was examined at both high cell density and low cell density. At high cell density, there are cell-cell contacts for the formation of gap junctions, while at low cell density, few gap junctions could be formed due to the lack of cell-cell contacts. Results showed that the cytotoxicity of PQ1 on T47D cells was not cell density dependent. Cell viabilities decreased by PQ1 have no significant difference between high and low cell density, indicating that the cytotoxicity of PQ1 is GJIC-independent. However, the possibility cannot be ruled out that the caspase cascade induced by PQ1 is related to connexin via a GJIC-independent mechanism. Expressions of connexins have been reported to be related to expressions of apoptotic markers. Using immunohistochemical staining, Kanczuga-Koda et al. found the positive correlation between Cx26 and Bax expression, as well as Cx26 and Bcl-XL expression in colorectal cancer [10]. The same group also reported a positive correlation between Cx26 and Bak expression, as well as between Cx43 and Bak, but not between connexins and Bcl-2 in breast cancer [11]. The associations between connexin expression and apoptotic marker expression suggest that connexins might be a target for modulations of apoptosis. PQ1 has been shown to increase expressions of Cx26, Cx32, and Cx43 in T47D cells [12]. Studies of intrinsic apoptotic pathway showed that PQ1 increased expression of Bax, but had no effects on the expression of Bcl-2. Based on all the studies, one explanation of PQ1 induced apoptosis is that PQ1 over-expresses connexins which modulate the caspase cascade via a channel activity independent mechanism.

Another explanation is related to the chemical structure of PQ1. PQ1 is a quinoline derivative. Many quinoline derivatives have been reported as DNA intercalators with the abilities

to induce apoptosis in cancer cells. For example, the quinoline derivative, 8-methoxy pyrimido[4',5':4,5]-,50:4,5]thieno (2,3-b)quinoline-4(3H)-one (MPTQ), has been reported to induce the activation of caspase-3 through both intrinsic and extrinsic pathways [13]. MPTQ has a small alkyl methoxy group and the substituted oxygen in the aromatic ring that may facilitate its entry into the cells [13]. After entering the cells, MPTQ has been shown to intercalate into double-stranded DNA, as well as induce intracellular reactive oxygen species (ROS), which subsequently affect major physiological functions [13]. Similar to MPTQ, PQ1 has a small alkyl methoxy group in the aromatic ring. Therefore, the second explanation of PQ1 induced apoptosis is that after entering the cells with the help of methoxy group, PQ1 intercalates into DNA or interacts with regulatory proteins, which may cause DNA-strand breaks, cytokinesis disruptions, and apoptosis. More studies are needed to figure out which explanation is correct.

In addition to the anti-cancer effect, the interest with PQ1 may be its function on gap junctions, which is distinct from other quinoline derivatives with anti-cancer effects. PQ1 acts as a gap junction enhancer by increasing GJIC in T47D cells [9]. The main strategy in GJIC-based cancer therapies relied on bystander effect. Therefore, combinational effect of PQ1 and cisplatin was examined in T47D cells to evaluate the bystander effect induced by PQ1. Cisplatin is a common used anti-cancer drug. Cisplatin-induced cytotoxicity has been reported to be transduced to neighboring cells through GJIC [14]. Recent studies showed that cisplatin inhibits GJIC by direct decrease of channel activity and by reduction of connexin expressions, thereby counteracting its cytotoxic efficacy [15]. Therefore, one of the strategies to potentiate cisplatin effect is increase of GJIC. Studies showed that combinational treatment with PQ1 and cisplatin counteracted cisplatin reduction of GJIC and connexin expressions, thus increasing cytotoxic effects on T47D cells [12]. Consisting with previous reports, the cytotoxicity of combinational treatment was greater at high cell density with cell-cell contacts of gap junction formation, indicating that GJIC is an important factor involved in the enhancement of cytotoxic effects [12]. Further studies would be required to identify the toxic molecules or signals that transmitted from target cells to neighboring cells via GJIC in the combinational treatment. Overall, the combinational study suggests that PQ1 as a gap junction enhancer can be used to potentiate efficacy of anti-cancer drugs.

Prior to the transition from basic research of drug development to translation medicine, a major concern of anti-cancer drugs is the side-effect, an adverse effect resulted from damage to

normal cells. In the previous study, minimal toxicity of PQ1 on normal human mammary epithelial cells (HMECs) was observed within the effective doses of anti-cancer effects and GJIC-mediated responses [9]. Compared to controls, treatment of 200 nM PQ1 has 67% cell viability in T47D cells, but has 103% cell viability in HMECs [9]. Based on this study, the effects of PQ1 on normal organs were evaluated in healthy C57BL/6J mice via oral administration. Results showed that PQ1 can be absorbed, distributed to vital organs, and diminished after 24 hours [16]. In most, if not all, tested organs, PQ1 increased the expression level of anti-apoptotic marker, survivin, while decreased the expression levels of pro-apoptotic markers, cleaved caspase-3 and caspase-8 [16]. In the organs with Cx43, the expression of Cx43 was decreased in the presence of PQ1 [16]. No histological changes were observed after PQ1 treatment [16]. All these results suggest that PQ1 administration by oral gavage can be achieved with low toxicity to normal vital organs. It is very interesting that PQ1 has opposite effects on apoptosis in normal organs and cancer cells. The causes of PQ1 opposing effects are not clear. Because PQ1 up-regulates Cx43 in cancer cells while down-regulates Cx43 in normal organs, a possibility is that PQ1 acts as a modulator of connexin expression and subsequently mediates apoptosis. More studies are needed to identify the primary molecular targets or signaling pathways that are involved in the PQ1-induced regulation of connexin expressions.

Overall, studies in this dissertation have provided information that PQ1 is an effective tool to enhance GJIC in combinational chemotherapy, as well as a promising anti-cancer candidate for drug development due to its high cytotoxicity on cancer cells and low toxic effects on normal organs.

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