EFFECTS OF QUINOLINES ON SW480 COLORECTAL CANCER CELLS: GAP JUNCTION DEPENDENT AND INDEPENDENT PATHWAYS

by

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Abstract

Colorectal cancer is one of the most common cancers in the United States with an early detection rate of only 39%. Colorectal cancer cells along with other cancer cells exhibit many deficiencies in cell-to-cell communication, particularly gap junctional intercellular communication (GJIC). GJIC has been reported to diminish as cancer cells progress. Gap junctions are intercellular channels composed of connexin proteins, which mediate the direct passage of small molecules from one cell to the next. They are involved in the regulation of the cell cycle, cell differentiation, and cell signaling. Since the regulation of gap junctions is lost in colorectal cancer cells, the goal of this study is to determine the effect of GJIC restoration in colorectal cancer cells. Overexpression of connexin 43 (Cx43) in SW480 colorectal cancer cells causes a 6-fold increase of gap junction activity compared to control un-transfected cells. This suggests that overexpressing Cx43 can restore GJIC. Furthermore, small molecule directly targeting gap junction channel was used to increase GJIC. Gap junction enhancers, PQs, at 200 nM showed a 4-fold increase of gap junction activity in SW480 cells. Using Western blot analysis, Cx43 isoform expression was seen to shift from P0 to P1 and P2 isoforms after treatment with PQ1 200 nM for 1 hour. Overall, the results show that overexpression of connexin and small molecules such as gap junction enhancers, PQs, can directly increase gap junction activity. The findings provide an important implication in which restoration of gap junction activity can be targeted for drug development.

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Chapter 1 - Introduction

Colorectal Cancer

Statistics

Colorectal cancer is the third most common cancer and the third leading cause of cancer related death in the United States[1,2]. The life time risk of developing colorectal cancer is 5%[2]. In 2013, approximately 136,830 people were diagnosed with colorectal cancer. Approximately 50,310 deaths in the past year were due to colorectal cancer[3]. The incidence of colorectal cancer is higher in men than in women[2]. The majority of cases and deaths due to colorectal cancer occur in people 65 years and older[3].

Risk Factors

Many risk factors have been found for colorectal cancer. A personal history of colorectal polyps, colorectal cancer, or inflammatory bowel disease increases the risk of colorectal cancer. In 1 out of 5 people that develop colorectal cancer, a familial connection to the disease is found. Lifestyle also influences the risk of colorectal cancer. A diet high in red meat and / or processed meats increases the risk of colorectal cancer as well as cooking meats at high temperatures. The consumption of vegetables, fruits and whole grains decreases the risk of colorectal cancer. Physical inactivity increases the risk and physical activity decreases the risk of colorectal cancer. Obesity and smoking increase both the risk of developing and dying of colorectal cancer. Heavy alcohol use also increases the risk of colorectal cancer[1].

Colorectal Cancer Stages

The colon wall is made up of five tissue layers (Figure 1.1). The first layer from the lumen is the mucosa, next is the submucosa, third is the muscularis propria, then the subserosa

and last is the serosa as shown in Figure 1.1. The rectum has all of the layers except for the subserosa and serosa. There are five stages of colorectal cancer stage 0 to stage IV. Even though overall occurrence rates are combined for both colon and rectal (colorectal) cancer, the treatment and survival rates differ between the two cancer types[4]. Survival rates are shown in Table 1.1. Cancer progression is shown in Figure 1.1.

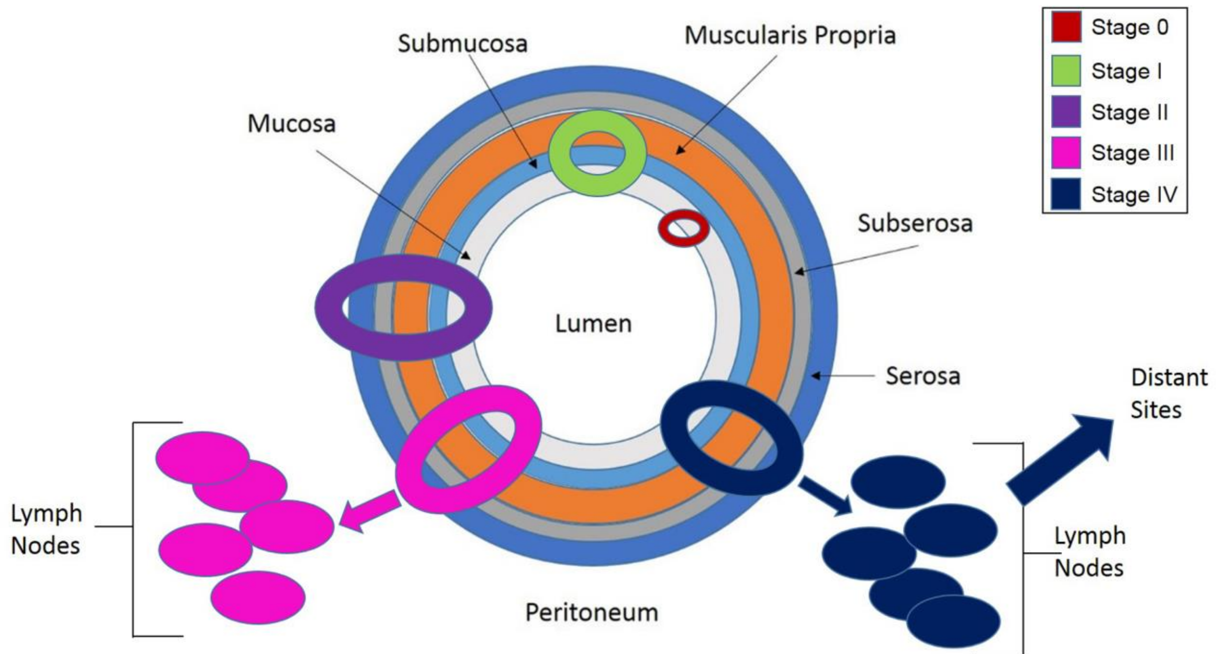

Figure 1.1 Cancer progression through layers of the colon.
Image was adapted from Edge et al., 2010[4].

In stage 0 colorectal cancer, the cancer has not grown past the mucosa layer of the colon. Polyps make up this stage and they may contain invasive cells. So to ensure that the polyp does not become invasive, a polypectomy is used to remove the polyp[4].

In stage I colorectal cancer, the cancer has progressed into the submucosa and possibly into the muscularis propria, but not to lymph nodes or distant sites. The 5-year observed survival rate is 74% for both colon and rectal cancer. The treatment for this stage is surgery to remove the part of the colon that is afflicted. In some rectal cancer cases, chemotherapy after surgery is given[4].

In stage II colorectal cancer, the cancer has spread to the outer layer of the colon or rectum and, possibly, to nearby organs but not to lymph nodes or distant sites. The 5-year observed survival rate is 67% in early stage II colon cancer and down to 37% in late stage II colon cancer. For this stage, surgery may be the only treatment needed. However, in some cases surgery may be followed by chemotherapy. In early stage II rectal cancer, survival rate is 65% and late stage II survival rate is 32%. The treatment is surgery with chemotherapy and/or radiation treatment for 6 months[4].

In stage III colorectal cancer, the cancer has spread to nearby lymph nodes, but not too distant sites in the body. The 5-year survival rate for cancer in the colon is 73% in early stage III and 28% in late stage III. The treatment is surgery, followed by radiation and/or chemotherapy. In the rectum, early stage III cancer is associated with 74% survival rate, and late stage III has a survival rate of 33%. Treatment for this stage is radiation therapy along with chemotherapy followed by surgery. After surgery, chemotherapy is given for about 6 months[4].

In stage IV colorectal cancer, the cancer has spread from the colon or rectum to distant organs and tissues throughout the body. The survival rate for this stage of colon cancer is 6%. The treatment is chemotherapy followed by surgery and then more chemotherapy. For rectal cancer, the 5-year survival rate for stage IV is 6%. The treatment varies, in some cases surgery is performed, and then chemotherapy. In other cases, it is chemotherapy, surgery, and then chemotherapy and radiation, or chemotherapy and radiation, followed by surgery, followed by chemotherapy[4].

	5-year Observed Survival Rate	
Stage	Colon	Rectum
I	74%	74%
IIA	67%	65%
IIB	59%	52%
IIC	37%	32%
IIIA	73%	74%
IIIB	46%	45%
IIIC	28%	33%
IV	6%	6%

Table 1.1 5-year survival rate.

Table was adapted from Edge et al., 2010[4].

Hallmarks of Cancer

As colorectal and other cancers form and progress, characteristics common to all cancers are seen. These characteristics are known as hallmarks. Cancers do not gain these characteristics all at once; they are gained over time as cancer progresses. There are 6 well known hallmarks in cancer formation. The hallmarks are; ability to self-proliferate, the ability to ignore signals to stop proliferating, the ability to ignore cell death signals, immortalization, recruitment of blood vessels, and the ability to invade and metastasize[5,6].

The ability to self-proliferate, can occur by mutations, such as 35% of colon tumors having a mutation in the Kirsten rat sarcoma viral oncogene homolog gene (KRAS) that causes it to be active. KRAS activation will lead to the propagation of growth factors and, therefore, will lead to proliferation of the cell[5,7].

A second hallmark is the ability to ignore signals to stop proliferating. An example is the loss of heterozygosity in the Adenomatous polyposis coli (APC) gene in colorectal cancer. APC is a tumor suppressor gene, which regulates cell growth. With homozygosity or heterozygosity of normal APC gene, the APC protein can help control cell growth and suppress cancer. However, after mutation of both genes, APC (loss of heterozygosity) can no longer function, allowing the cell to grow uncontrollably. The loss of both APC genes is found in 80% of colon cancers[3,5,7] .

The third hallmark is the evasion of apoptosis (cell death signals). For instance, tumor protein p53 (p53), a tumor suppressor gene, can activate the apoptotic pathway and lead to cell death by binding to DNA and causing the cell to produce the cyclin-dependent kinase inhibitor 1 protein (p21)[7]. p21 complexes with cyclin-dependent kinase 2 (cdk2) to prevent the cell from going to the next stage of cell division[8]. The loss of heterozygosity in the p53 gene occurs in a little less than 50% of colon cancers[5,7].

A fourth hallmark is limitless replicative potential, also known as immortalization. In normal cells, every time cell division occurs the telomere shortens until it gets too short, and then the cell can no longer replicate. Telomerase is the enzyme that maintains telomeres and, even with this enzyme, 50 – 100 base pairs are lost from the telomere after every division in normal cells. In 85 to 90% of cancers the expression of the telomerase enzyme is elevated. This allows for the maintenance of telomeres above the threshold length giving cells the ability for continuous replication[5].

The fifth hallmark is the ability to recruit blood vessels. All cells require nutrition to function and survive, and acquire the necessary nutrition cells from within 100 µm of a blood vessel. To become within 100 µm of a blood vessel, the cells acquire the ability to produce necessary proteins that signal for angiogenesis. Angiogenesis is the ability to recruit blood vessels. At first the cells do not have this ability, and this limits their growth. Tumor cells perform angiogenesis by secreting different growth factors which signal for the growth of blood vessels to the area. A few of the signals for angiogenesis are vascular endothelial growth factor (VEGF), transforming growth factor (TGF), tumor necrosis factor (TNF), and platelet-derived endothelial growth factor (PEGF)[9]. Once the cells develop the angiogenic ability, this leads to tumor expansion[3,5,7].

The sixth hallmark is invasion and metastasis. This occurs in the last stage of cancer progression. Cells acquire the ability to undergo the invasion-metastasis cascade. It has been seen that the cell-cell adhesion molecules (CAMs) change[7]. In epithelial cancers, like colorectal cancers, an epithelial-mesenchymal transition occurs. This transition allows the cells to break through the basement membrane and invade the blood or lymph to travel to distant sites in the body, where the cells will colonize[5,7].

These hallmarks are all potential targets for chemotherapy, however, some are more viable than others. Inhibiting hallmarks 1,4 and 5 (the ability to self-proliferate, immortality and angiogenesis) are all current targets for cancer treatment[3,5,7].

Currently, in colorectal cancer, cetuximab and panitumab are chemotherapeutic drugs designed to inhibit endothelial growth factor receptor (EGFR) to suppress proliferation; however, when KRAS is activated in cancer, this treatment has little effect due to KRAS activating EGFRs downstream pathway[7,10]. Bevacizumab and regorafenib target angiogenesis, the fifth hallmark, by inhibiting vascular endothelial growth factor (VEGF) and kinases[11,12]. Currently, drugs targeting the telomerase enzyme are being developed[13–15]. Drugs to target the invasion and metastatic hallmark may eventually be developed, but more understanding of this pathway is needed before designing drugs targeted to invasion and metastasis[3,5,7].

Other than drugs targeting these hallmarks, most chemotherapeutic drugs are designed to damage the DNA[16]. These are more abundant than the drugs targeting the hallmarks. However, there is another hallmark of cancer that until now has not been investigated as a potential target for anti-cancer drugs. This hallmark is the loss of gap junctional intercellular communication (GJIC)[6].

The loss of gap junctions (GJs) and connexins is commonly found in cancers[17]. GJs and some connexins are thought to be tumor suppressors[18]. Gap junctions are involved in the bystander effect. Gap junctions allow for the propagation of small molecules between cells. It has been shown that via the bystander effect contributes to the efficacy of cancer therapy. Using 8-bromo-cyclic-AMP, connexin 43 (gap junction building unit) and GJIC, were up-regulated leading to the increased efficacy of gene therapy[19,20]. These previous studies show that gap junction enhancers have the potential to increase the efficacy of chemotherapy treatments.

Gap Junctions

Gap junctional intercellular communication (GJIC) is the passage of small molecules (>1000 Da) between adjacent connecting cells through gap junction channels, as shown in Figure 1.2[21–24]. The mediation of small molecules through gap junctions is known as the bystander effect. The bystander effect involves the passage of toxic or beneficial compounds to adjacent cells. Small molecules like cAMP, calcium ions, and glucose can pass through gap junctions while large molecules like proteins or complex sugars cannot pass through the gap junctions this suggests maximal pore size of the channel is 1.5 nm in diameter in mammalian cells[25,26]. Through transfer of these different compounds, gap junctions are involved in the regulation of the cell cycle, cell differentiation and cell signaling[27].

Gap junctions are made of the protein known as connexin. There are 21 known isoforms of connexin[28]. The connexin structure consists of 4 hydrophobic transmembrane domains, 2 extracellular, and 3 cytoplasmic loops as shown in Figure 1.2[29,30]. Connexin 43 (Cx43) is the most common connexin studied, as such it is the focus of this project. Six connexins form a hemichannel and 2 hemichannels connect to form a gap junction. Functional gap junctions are found in gap junctional plaques. A plaque consists of hundreds of gap junction channels in

position on the plasma membrane at regions connected to adjacent cells[31]. The loss of gap junctions and connexin 43 is seen commonly in cancer formation[17,32].

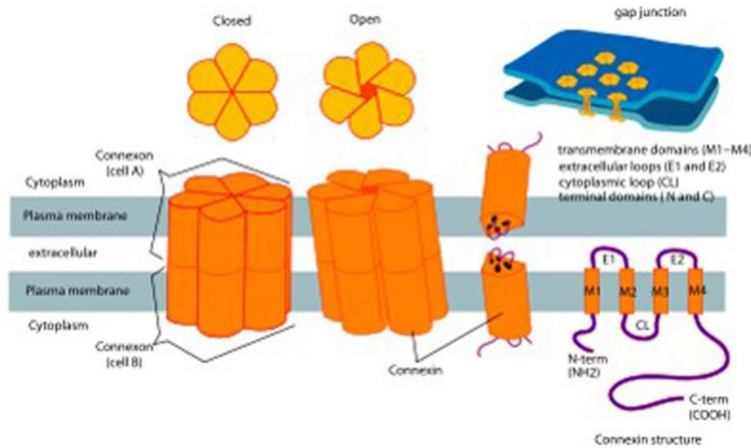

Figure 1.2 Gap junction and connexin structure.
Created by Mariana Ruiz Villarreal[33].

As colorectal cancer forms, there is a decrease in gap junction activity, Cx43 expression, and a shift in localization of Cx43[38]. The half-life of connexin is 1.5 to 3 hours[39]. The regulation of gap junctions occurs through phosphorylation of the connexin carboxyl-terminal domain. Cx43 has been found as 3 different isoforms; P0, P1 and P2. The isoform regulation of Cx43 is via phosphorylation events[31,40–51]. The P0 isoform has been shown to localize on internal membranes like the Golgi apparatus[47,52] The P0 isoform is known to have less phosphorylation than the P2 isoform and is referred to as dephosphorylated[18]. The P1 and P2 isoforms are associated with certain phosphorylation sites. The P1 form has been seen to be phosphorylated at S364/S365 amino acid residues. Phosphorylation at S365 has been shown to be involved in the assembly of gap junctions. The P2 form has been found as 2 different isoforms. One of the P2 isoforms is phosphorylated at S325/S328/S330; this form has been found at gap junctional plaques[48]. When the P2 isoform is phosphorylated at S262 and/or S368, a decrease in gap junction intercellular communication (GJIC) is found[45,52,53]. Kinases (PKC, MAPK, Akt, ect.) are

regulators of gap junctions (GJs) by way of phosphorylation of connexin proteins at multiple phosphorylation sites on the carboxyl-terminal domain[43,45,54–56]. This research will look into the effects of 6-methoxy-8[(3-aminopropyl)amino]-4-methyl-5-(3-trifluoromethyl-phenyloxy)quinolone (PQ1), a gap junction enhancer, on kinases that regulate gap junctions.

PQ1

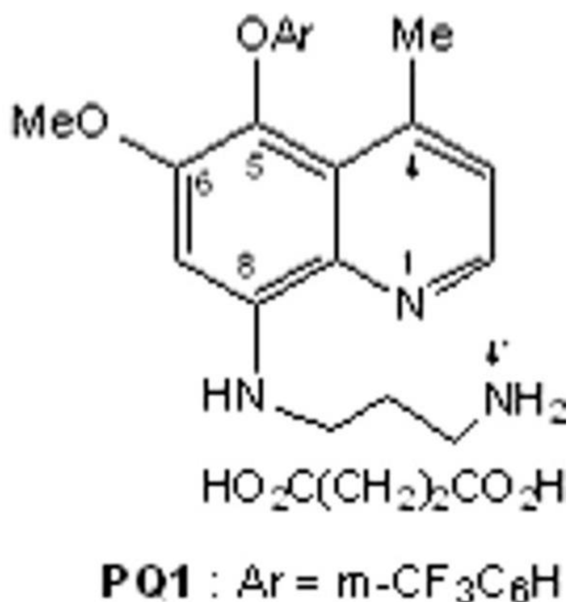

Figure 1.3 Structure of PQ1.
Adapted from Gakhar et al., 2008[57].

PQ1(Figure 1.3) was developed after using the partial crystal structure of gap junctions (GJs) to test substituted quinolones and their ability to interact with the gap junctions. PQ was found to bind to the core of the hemichannel of the gap junction. Interactions (closed contact) at one minimum energy (-0.7 kcal/mol) bound structure were found between the CF_3 group of PQ1 and the H-N of Leu144 (2.5Å) of connexin and OCH_3 group of PQ1 and CH_2 of Phe81 of connexin (2.5Å) were observed. PQ1 has been demonstrated to increase gap junction activity in breast cancer cells. PQ1 caused an 8.5-fold increase in gap junction activity in T47D breast

cancer cells and subsequently a decrease of 70% growth in a xenograft tumor[57]. Oral bioavalability studies indicate that administration of PQ1 via oral gavage has a low toxicity to normal tissue, with no observable adverse effects, while significantly attenuating tumor growth[57].

This study addresses whether overexpression of Cx43 or increase gap junction activity can be achieved in human colorectal cancer cells, SW480. Using transfection and small molecule approach (PQ1), the gap junction activity of SW480 cells was restored. Overall, this study provides evidence, for the first time, that regain of GJIC can be achieved by a small molecule of gap junction enhancer, PQ1, on SW480 colorectal cancer cells.

Chapter 2 - Hypothesis and Objectives

Hypothesis

1. Gap junction intercellular communication can be restored by overexpression of Cx43 and/ or by small molecule PQ1.

2. PQ1 can induce apoptosis at concentrations higher than that needed for GJIC restoration.

Objectives

1. To determine the effect of overexpression of Cx43 on GJIC.

2. To determine the effect of PQ1 on GJIC.

3. To determine PQ1's effect on apoptosis.

Chapter 3 - Overexpression of Cx43 leads to increase in GJIC

Introduction

It has been seen that in colorectal cancer GJIC and Cx43 is decreased[38]. GJIC allows for the direct propagation of small molecules between cells (bystander effect)[19]. Using 8-bromo-cyclic-AMP, Cx43 and GJIC were increased. The increase in GJIC potentiated the effect of suicide gene therapy by way of the bystander effect in breast cancer cells[19]. An increase in GJIC has also been seen after overexpression of Cx43 in breast cancer cells[18,58]. In this chapter, the effect of increasing GJIC on colorectal cancer cell line SW480 is studied.

Methods

Cell Line

The SW480 human colorectal cancer cell line was purchased from American Type Cell Culture (ATCC, Manassas, VA). Cells were grown with 0% CO_2 in Leibovitz's L-15 Medium with 10% Gibco Fetal Bovine Serum (FBS) purchase from Life Technologies (Grand Island, NY, USA).

Western Blot

Cells were seeded to 50% density in a T-25 cm^2 flask for 24 hours and allowed the density to reach 90% prior to treatment. Cells were harvested with lysis buffer (20 mM Tris-HCL pH 7.6, 0.5 mM EDTA, 0.5 mM EGTA, and 0.5% Triton-X 100) (Cell Signaling Technology Inc., Danver, Massachusetts, USA). The lysate mixture was centrifuged at 13,000 rpm (Using an Eppendorf centrifuge 5415R with rotor F-45-24-11, Eppendorf North America, Hauppauge, New York, USA) for 30 minutes at 4˚C, and the supernatant was collected. Total protein concentration was determined using a Bio-Rad protein assay kit (Bio-Rad Life Science Research,

Hercules, California, USA). Twenty-five µg of whole cell extract was separated by 5-10% sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and transferred onto a nitrocellulose membrane. The nitrocellulose membrane was immunoblotted against the protein of interest. The primary antibodies were mouse anti-Cx43 (F-7) antibody, specific for epitope mapping between amino acids 357-381 at the carboxy-terminus domain, and the mouse anti-GAPDH (0411) antibody purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA). Secondary antibodies were anti-mouse and anti-rabbit IgG linked HRP, they were purchased from Cell Signaling Technology (Danver, Massachusetts, USA).

Transfection

Eight x10^5 SW480 cells were seeded into 6-well plates for 24 hours. Cells were transfected with 3.5 µg of Gja1, NM 012567.2, subcloned into pEGFP-N3 vector 21 and Optifect reagent (Life Technologies, Grand Island, NY, USA) in 0% Fetal Bovine Serum (FBS) tissue culture media.

Gap Junction Activity

Scrape Load/Dye Transfer (SL/DT) assay was used to measure gap junction activity. Eight x 10^5 SW480 cells were grown on a cover slip in a 6-well plate. Cells were grown for 24 hours; cells were transfected with a vector Cx43 for 24 hours. Cells were washed with Phosphate Buffered Saline (PBS) 3 times. A mixture of 1% Lucifer yellow and 0.75% Rhodamine dextran was added in the center of the cover slip. Two cuts crossing one another in the center of the coverslips were made. After 3 minutes, cells were washed with PBS 3 times and incubated at 37˚C in tissue culture media for 20 minutes. The cells were then washed with PBS and fixed with 2.5% paraformaldehyde for 30 minutes. Cells were mounted on a slide and then

sealed and visualized under a fluorescent microscope (Nikon Eclipse 80i, Nikon Instruments, Melville, NY, USA)(X-Cite 120 PC fluorescence illumination system, EXFO Photonic Solutions Inc., Mississauga, Ontario, Canada) at 10x objective (Nikon Instruments, Melville, NY, USA). Images were captured using Nikon Digital Sight Fi1 (Nikon Instrument, Melville, NY, USA). The distance between the designated cut and the dye transfer was measured. The distance of dye uptake indicates that cells are active and have allowed the dye to pass from one cell to the next cell.

Proliferation and Viability

Eight x 10^5 SW480 cells were seeded into 6-well plates for 24 hours. Cells were transfected with a vector expressing Cx43. Twenty-four hours later, tissue culture media of respective treatments were saved in 15 mL conical tubes. A volume of 0.5 mL of trypsin was added to the cells for 5 minutes. Cell scrapper and 3 mL of PBS were used to harvest cells. Cells were spun down for 5 minutes at 13,000 rpm; afterwards the supernatant was removed. Volumes of 900 µL PBS and 100 µL of trypan blue were added to the pellet and left to stand for 5 minutes. Cellometer Auto 2000 from Nexcelom Bioscience was used to measure the number of cells for proliferation and viability. All cells were counted toward proliferation.

Results

Transfection of Cx43 leads to increased GJIC in SW480 colorectal cancer cells

Intercellular communication in many organs is maintained via GJIC. As cancer forms, a decrease in GJIC and Cx43 protein are observed [38]. An effective clinical drug targeting GJIC has not been studied for colorectal cancer at this time; thus, ways to increase GJIC in colorectal cancer cells were examined. Cells were transfected with Cx43 expression plasmid for 24 hours.

Western blot analysis showed that 25 ug of Cx43 expression vector was sufficient to increase Cx43 in SW480 cells compared to control or empty vector (Figure 3.1A and 3.1B). These cells were analyzed for gap junction activity after 24 hours of transfection. The results showed a 6-fold increase of gap junction activity in Cx43-transfected cells compared to control cells (Figure 3.1C). Thus, the results suggest that regain of GJIC in SW480 cells can be achieved via transfection of Cx43. Furthermore, a differential pattern of Cx43 isoform was observed. The protein blot analysis showed that there are three distinct isoforms of Cx43: P0, P1, and P2. Isoform expression of Cx43 had shifted from P0 form to P1 form in the Cx43 transfected cells (Figure 3.1D). Overall, these results show an increase in GJIC by overexpression of Cx43.The effects of overexpression of Cx43 on cell viability and proliferation were analyzed to see if an increase in GJIC would affect cell growth and death. A proliferation study of SW480 cells, overexpressed with or without the Cx43 expression vector, showed no significant change compared to empty vector (Figure 3.2A). The viability of SW480 cells overexpressing Cx43 was not found to change (Figure 3.2B). These data demonstrate that the overexpression of Cx43 did not alter the proliferation and viability of SW480 cells, and the change in gap junction activity is due to the overexpression of Cx43.

Discussion

In colorectal cancer, a decrease in GJIC has been found. Thus, chemotherapeutic compounds cannot utilize gap junctions to propagate their effects throughout the tumor. In this study, the effects of overexpression of Cx43, a gap junction protein, were analyzed. The overexpression of Cx43 in SW480 cells resulted in an increase in GJIC, as well as a shift in isoform expression of Cx43 from P0 to P1 (Figure 3.1). These findings are in line with similar studies performed by TenBroek[59]. Experiments were performed to analyze the effects of GJIC on

the proliferation and viability of SW480 cells. As shown in Figure 3.2, increasing GJIC does not affect viability or proliferation of SW480 cells. Since transfection is not a viable therapeutic target, a small molecule approach is needed to increase GJIC *in-vivo*.

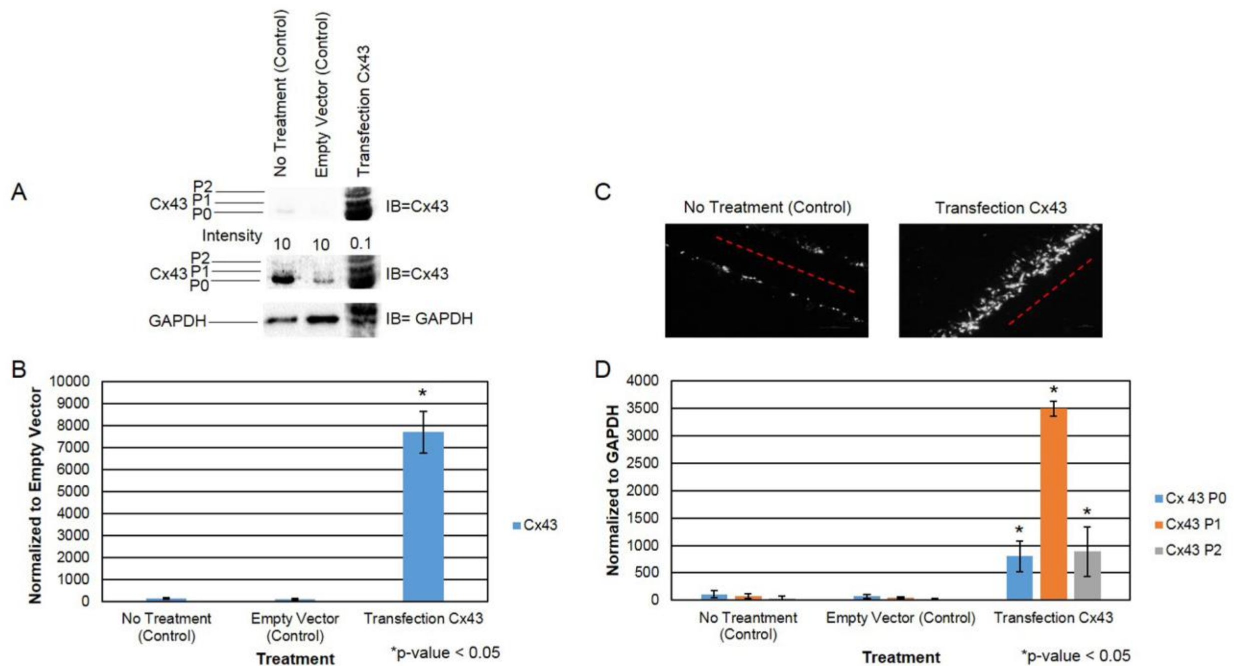

Figure 3.1 Overexpression of Cx43 and its effects on Gap Junction Activity.
Cells were treated with: no treatment (control), transfection of Empty Vector, transfection of Cx43 for 24 hours. A) Levels of Cx43 were examined by western blot analysis using anti-connexin43 (F-7) antibody. GAPDH was used as loading control. B) Graphical presentation of 3 independent experiments showing pixel intensities of total Cx43 normalized to control. *P value is <0.05 compared to control. C) Scrape/ Load Dye transfer after no treatment or overexpression of Cx43. Lucifer yellow dye in cells indicated in white. Red line indicates the point of entry for Lucifer yellow. Dye transfer is measured from point of entry along scape line to outer most cells. D) Graphical presentation shows the ratio of Cx43 isoforms P0, P1 and P2. Data were obtained in 3 independent experiments and are represented as the mean ± SD. *P value is <0.05 compared to control. IB= Immunoblot against Cx43.

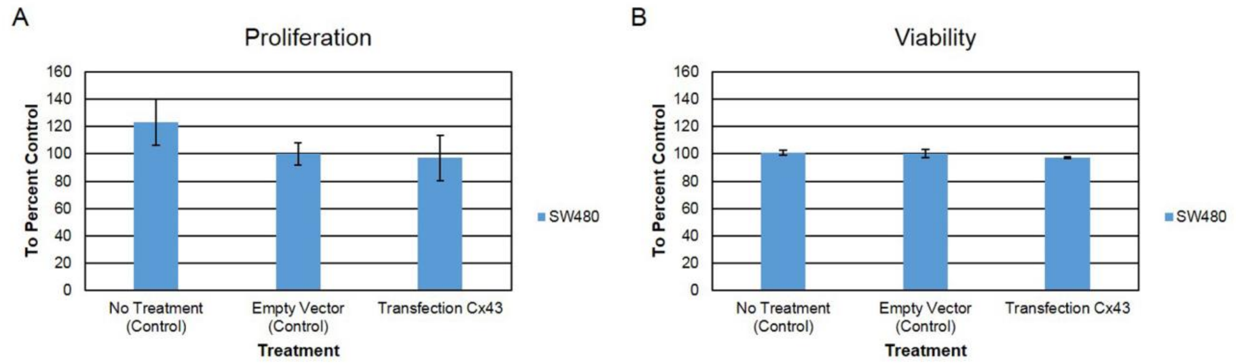

Figure 3.2 Proliferation and Viability of SW480 cells after transfection.

Eight x 10^5 cells were seeded into 6-well plates. Cells were then treated with respective treatments for 24 hours. Treatments were: Control, transfection of Empty Vector, and transfection of Cx43 plasmid. After 24 hours of transfection, viability and proliferation was performed. A) Proliferation of SW480 cells. B) Viability of SW480 cells. Data were obtained in 3 independent experiments and are represented as the mean ± SD.

Chapter 4 - PQ1's effect on GJIC

Introduction

6-methoxy-8[(3-aminopropyl)amino]-4-methyl-5-(3-trifluoromethyl-phenyloxy)quinolone (PQ1) has been shown in breast cancer cells to increase GJIC[57]. This study focuses on the effects of PQ1 on GJIC in colorectal cancer cells and its mechanism. PQ1 has 2 possible mechanisms: the first is by direct binding to Cx43; the second is by regulating kinase activity as gap junctions are regulated by kinases[43,45,54–56]. In this study PQ1's effects on kinase activity is analyzed.

Protein kinase C (PKC) α, p44/42 mitogen-activated protein kinase (p44/42 MAPK) and active protein kinase B (pAkt) were investigated for their role in regulating gap junctions in the presence of small molecule, gap junction enhancer, PQ1. 12-O-Tetradecanoylphorbol-13-acetate (TPA) a GJIC inhibitor decreases GJIC by mimicking diacylglycerol (DAG) and activating PKC. PKC is known to phosphorylate Cx43 at S368, a site that is known for its association with decreased GJIC. However, the effects of PKC on GJIC are dependent on the isoform of PKC (PKCα,β,δ,γ, and ε) PKC α has been shown to both increase and decrease GJIC[50,60].

Studies on the phosphorylation of Cx43 by way of MAPK have mixed results. Early studies found MAPK (using an antibody that detects all MAPK) to be a GJIC down-regulator by phosphorylation at S255, S279 and S282[61]. A recent study found MAPK phosphorylation to lead to the up-regulation of GJIC[62]. p44/42 MAPK was shown to decrease GJIC[63].

The kinase Akt has been shown to stabilize gap junctions via phosphorylation at S373 of Cx43[64]. Dunn et al., found that upon inhibition of Akt by Akt VIII inhibitor or with a dominant negative version of Akt, gap junctions were smaller, and less phosphorylated Cx43 was present[65].

Methods

Cell Line

The SW480 human colorectal cancer cell line was purchased from American Type Cell Culture (ATCC, Manassas, VA). Cells were grown with 0% CO_2 in Leibovitz's L-15 Medium with 10% Gibco Fetal Bovine Serum (FBS) purchase from Life Technologies (Grand Island, NY, USA).

Western Blot

Cells were seeded to 50% density in a T-25 cm^2 flask for 24 hours and allowed the density to reach 90% prior to treatment. Western blot assay was performed as described in Chapter 3. Primary antibodies mouse anti-Cx43 antibody, mouse anti-PKCα (H-7), specific for epitope mapping between amino acids 645-672 at the c-terminus of PKC α, and mouse anti-GAPDH antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA). Primary antibodies purchased from Cell Signaling Technology (Danvers, Massachusetts, USA) were; rabbit anti-phospho-Akt (Ser473) (D9E) specific for endogenous levels of Akt when phosphorylated at Ser473, rabbit anti-caveolin 1 (D46G3), specific for endogenous levels of total caveolin-1, and rabbit anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), specific for endogenous levels of p44 and p42 MAPK when phosphorylated at Thr202 and/or Tyr 204 of extracellular-signal-regulated kinase1 (Erk1), a classical MAPK. Secondary antibodies were anti-mouse and anti-rabbit IgG-linked HRP, they were purchased from Cell Signaling Technology (Danver, Massachusetts, USA).

Translocation Assay

The cells were harvested with $MgCl_2$. The mixture was centrifuged at 35,000 rpm (Using a Beckman TL-100 Ultra Centrifuge with rotor) for 1 hour and the supernatant was collected

(cytoplasmic portion). The pellet was re-suspended with lysis buffer (Cell Signaling Technology Inc., Danver, Massachusetts, USA). The mixture was centrifuged at 13,000 rpm (Using an Eppendorf centrifuge 5415R with rotor F-45-24-11, Eppendorf North America, Hauppauge, New York, USA) for 30 minutes at 4˚C, and the supernatant (membrane fraction) was collected. The lysate was used for western blot analysis as described previously.

Gap Junction Assay

The SL/DT assay was used to measure gap junction activity. Eight x 10^5 SW480 cells were grown on a cover slip in a 6-well plate. The cells were grown for 24 hours; then pre-treated with kinase inhibitors: Calphostin C or Staurosporin (EMD Millipore (Bellerica, Massachusetts, USA)) for 1 hour, followed by treatment with/without 200 nM 12-O-Tetradecanoylphorbol-13-Acetate (TPA) and / or with 50 nM, 200 nM, or 500 nM PQ1 for 1 hour. The SL/DT assay was performed as previously described in Chapter 3.

Immunofluorescence

Cells were seeded at 8 x 10^5 cells on coverslips in 6-well plates. Cells were pre-treated with Calphostin C or Staurosporin for 1 hour, followed by treatment with 200 nM PQ1 for 1 hour. Controls were: no treatment, DMSO, and 200 nM PQ1 for 1 hour. The cells were then fixed with 4% paraformaldehyde for 30 minutes and were washed 3 times with Tris Buffered Saline (TBS) with Tween 20 (TBST). The cells were permeabilized with 0.1% Triton-X 100 for 20 minutes, and were washed 3 times with TBST. The cells were then blocked in 3% Bovine Serum Albumin (BSA) for 1 hour, followed by primary antibody overnight in 3% BSA. The samples were then washed in 3% BSA 3 times, secondary antibody was added for 2 hours at room temperature and the samples were washed with 3% BSA 3 times. Coverslips were placed

on slides using Prolong Gold antifade reagent (Life Technologies, Grand Island, NY, USA). Primary antibody mouse anti-Cx43 was purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA). Secondary antibody Alexa Fluor 488 Rabbit Anti-Mouse IgG (H+L) was purchased from Life Technologies (Grand Island, NY, USA). Software and instrument are the same as used in Gap Junction Activity Assay.

Results

PQ1, gap junction enhancer, increases GJIC in SW480 colorectal cancer cells

The approach of increasing GJIC directly has potential to enhance the efficacy of cancer treatment. Since transfecting all cancer cells with Cx43 is not valid as a therapeutic option, an alternate approach is needed. Recently, gap junction enhancers, a class of substituted quinolines (PQs), have shown to increase GJIC in other cancer cells. Thus, in this study PQ1 was used to increase GJIC in SW480 colorectal cancer cells. SW480 cells were treated with PQ1 at concentrations of 50 nM, 200 nM, or 500 nM for 1 hour. The gap junction activity was measured by scrape load/ dye transfer assay. The results showed that cells treated with 200 nM PQ1 had a 4-fold increase of dye transfer compared to control cells without treatment, or solvent alone (Figure 4.1A and 4.1B). Interestingly, cells treated with PQ1 and GJ inhibitor, TPA, had no increase of gap junction activity compared to DMSO, suggesting that TPA blocks PQ1-mediated GJIC in SW480 cells (Figure 4.1C). Carbenoxolone (CBX) is a known gap junction inhibitor; thus it was used as a control to see if PQ1 effects directly affect the gap junction. CBX is thought to effect the gap junctions by altering local lipid environment[66]. The effects of CBX on the bilipid layer membrane conductance were not studied in this paper. Results show that treatment with 100 µM CBX + 200 nM PQ1 did not show an increase in GJIC when compared to 100 µM

CBX alone or controls (no treatment and DMSO) (Figure 4.1A and 4.1D). This concludes that PQ1 cannot open GJs in the presence of a GJ inhibitor.

Previously, PQ1 was constructed using the structure of the carboxyl-terminus of Cx43[57]. Western blot analysis was used to analyze the effects of PQ1 on gap junction protein Cx43. Figure 4.2 showed the protein expression of Cx43 after treating with 200 nM PQ1 and / or 200 nM TPA for 1 hour. Results showed no significant change in total Cx43 expression after treatment with PQ1 and/or TPA for 1 hour compared to DMSO (Figure 4.2B). Figure 4.2C shows the Cx43 isoform expression in treated cells compared to control cells. After treatment for 1 hour with 200 nM PQ1, a 2-fold decrease in isoform expression of P0 was seen in PQ1 treated SW480 cells compared to DMSO (Figure 4.2C). The expression of the P2 form increased 2-fold after treatment with 200 nM PQ1 for 1 hour compared to DMSO (Figure 4.2C). When treating cells for 1 hour with 200 nM TPA, the isoform profile of Cx43 showed no significant change in the expression of the P2 or P1 isoforms of Cx43 compared to DMSO. The P0 isoform was seen to decrease 2-fold with TPA treatment alone when compared to the DMSO treated P0 isoform. However, there was no significant change in isoform expression after treatment with both TPA and PQ1 for 1 hour, suggesting a potential antagonistic relationship between PQ1 and TPA (Figure 4.2C). Overall, these findings suggest that PQ1's ability to increase GJIC is by acting on the existing Cx43 and not by increasing Cx43 expression.

PQ1's effects on Kinase Activity

Since specific kinases phosphorylate Cx43 and have been found to cause a change in gap junction activity, this study focuses on kinases involved in the PQ1-mediated GJIC. To view the effects of kinase activity on GJIC with treatment of PQ1, SL/DT was performed with kinase

inhibitors Calphostin C (PKC inhibitor) and Staurosporine (inhibitor of PKC and phospholipid/calcium-dependent kinase). Cells were pre-treated for 1 hour with either Calphostin C or Staurosporine then treated for 1 hour with 200 nM PQ1 (Figure 4.3A and 4.3B). Compared to no treatment or DMSO solvent, GJIC had an increase of 50% after 1 hour treatment with 200 nM PQ1. When compared with 1 hour treatment of 200 nM PQ1 to cells pre-treated with Calphostin C or Staurosporin followed by treatment with 200 nM PQ1, GJIC showed a 50% decrease in inhibitor treated cells compared to 200 nM PQ1 treatment alone (Figure 4.3B).

In Figure 4.2A and 4.2C, isoform P2 expression Cx43 was shown to increase, suggesting the formation of GJs on the plasma membrane after PQ1 treatment. In Figure 4.4, GJ plaques are seen in 200 nM PQ1 treated cells compared to no GJ plaques in no treatment or DMSO treated cells. In cells pre-treated with Calphostin C, plaques were also found but at a decreased abundance compared to PQ1 treatment alone. Pre-treated cells with Staurosporin were lacking in GJ plaques compared to cells with PQ1 treatment alone (Figure 4.4). These findings suggest PQ1 activates kinases in order to form GJ plaques and to increase GJIC.

In Figure 4.5, membranous PKC α is not seen to change significantly after treatment with PQ1 or TPA compared to DMSO. While triplicates westerns show no significant change in PKCα expression in the cytoplasmic portion visualization of western blot analysis shows a decrease in PKCα expression in the cytoplasmic samples of TPA with or without PQ1. Further studies will be needed to confirm this but it seems that this change correlates to TPA's ability to activate and the deplete PKC isoforms in cells[67]. PKC α has been shown to decrease GJIC, but it has also been shown to increase GJIC[50,60]. In Figure 4.5, PKC α expression is not seen to change after PQ1 treatment compared to DMSO. This suggests that PQ1 does not affect PKC α but it does not rule out PQ1's potential effects on other PKC's in the SW480 cells, like PKC γ.

Furthermore, MAPK was examined due to the fact that MAPK can phosphorylate Cx43 and subsequently increase or decrease gap junction activity. The results show that an increase of 250% in activated form of MAPK (p44/42) was detected in the presence of 200 nM PQ1 at 1 hour treatment compared to DMSO (Figure 4.6). This suggests that, in the presence of small molecule PQ1, p44/42 MAPK may cause an increase in GJIC in SW480 cells.

The activated form of Akt was analyzed. Akt has been shown to stabilize GJs[65]. After 1 hour of treatment with PQ1, activated Akt increased by 150% compared to DMSO (Figure 4.7A and 4.7B). This suggests that increasing gap junction activity by PQ1 may involve the activation of Akt. However, after performing a translocation assay, active Akt levels were not shown to change in membrane or cytosolic portions after treatment with 200 nM PQ1 for 1 hour (Figure 4.7C and 4.7D). This suggests that either active Akt is indirectly involved in the increase in GJIC, that Akt did not bind strongly enough to the Cx43 to remain in the membrane fraction of the translocation, or that at the 1 hour time point Akt was no longer interacting with Cx43. The literature has shown that phosphorylation can be completed within 15 minutes and that a change in GJIC may not occur for another 1 to 3 hours[68]. This suggests that studies into kinase activity and localization need to be conducted using immunofluorescence and western blot analysis at earlier time points.

Discussion

Gap junctions allow for intercellular communication between adjacent connecting cells. GJs play a major role in the life cycle of cells; they are involved in tissue homeostasis and proliferation, as well other aspects of the cell cycle[69–71]. In cancer cells, there is a significant change of Cx43 localization at the plasma membrane and the cytoplasmic membranes, such as the Golgi apparatus[38,48]. Colorectal cancer cells show a similar profile[72]. A small molecule, PQ1,

was tested as a potential GJ enhancer in SW480 colorectal cancer cells. The results show that PQ1 can increase gap junction activities. PQ1, does not cause an increase in Cx43 expression, it causes an increase isoform P2 which forms functional GJs while decreasing isoform P0 expression causing an increase of Cx43 on the plasma membrane (Figure 4.2 and 4.4) and to form functional gap junctions (Figure 4.1A, 4.1B, 4.2A and 4.2C).

The regulation of gap junctions is controlled by phosphorylation of specific sites (mostly serine and tyrosine sites) on the carboxyl-terminal tail region of the connexins[54–56]. In the presence of TPA, PKCα has been shown to decrease GJIC[50]. However, in some instances PKC α has been shown to increase GJIC[73]. It is known that PKC phosphorylates Cx43 at S368 and S362 *in-vitro* and, subsequently, leads to decreases in GJIC[45,52,53]. PQ1's effects on GJIC are negated in the presence of PKC inhibitor (Figure 4.3 and 4.4); however no significant change of PKC α in the membrane or cytoplasm is seen (Figure 4.5). This suggests that PQ1 may affect PKC's other than PKC α [50].

Previously, MAPK and Akt have demonstrated to modulate Cx43 phosphorylation and, subsequently, increase gap junction activity. Active MAPK has been shown to increase and decrease GJIC; active Akt has been found to stabilize gap junctions[53,74].This study also determined whether the increase of gap junction activity by PQ1 was due to the activation of MAPK and Akt. Interestingly, PQ1 was shown to cause an increase in p44/42 MAPK and activated Akt (Figures 4.4 and 4.5). Activated Akt was not found to increase in the plasma membrane after treatment with PQ1 (Figure 4.4). This suggests 3 possibilities: 1) is that PQ1's ability to increase GJIC is not through activation and translocation of Akt to the plasma membrane, 2) that PQ1 does cause this translocation but the binding of Akt to Cx43 is not strong

enough for Akt to stay with the membrane or 3) it might be the case that Akt had already disassociated from Cx43 after phosphorylating the protein[68].

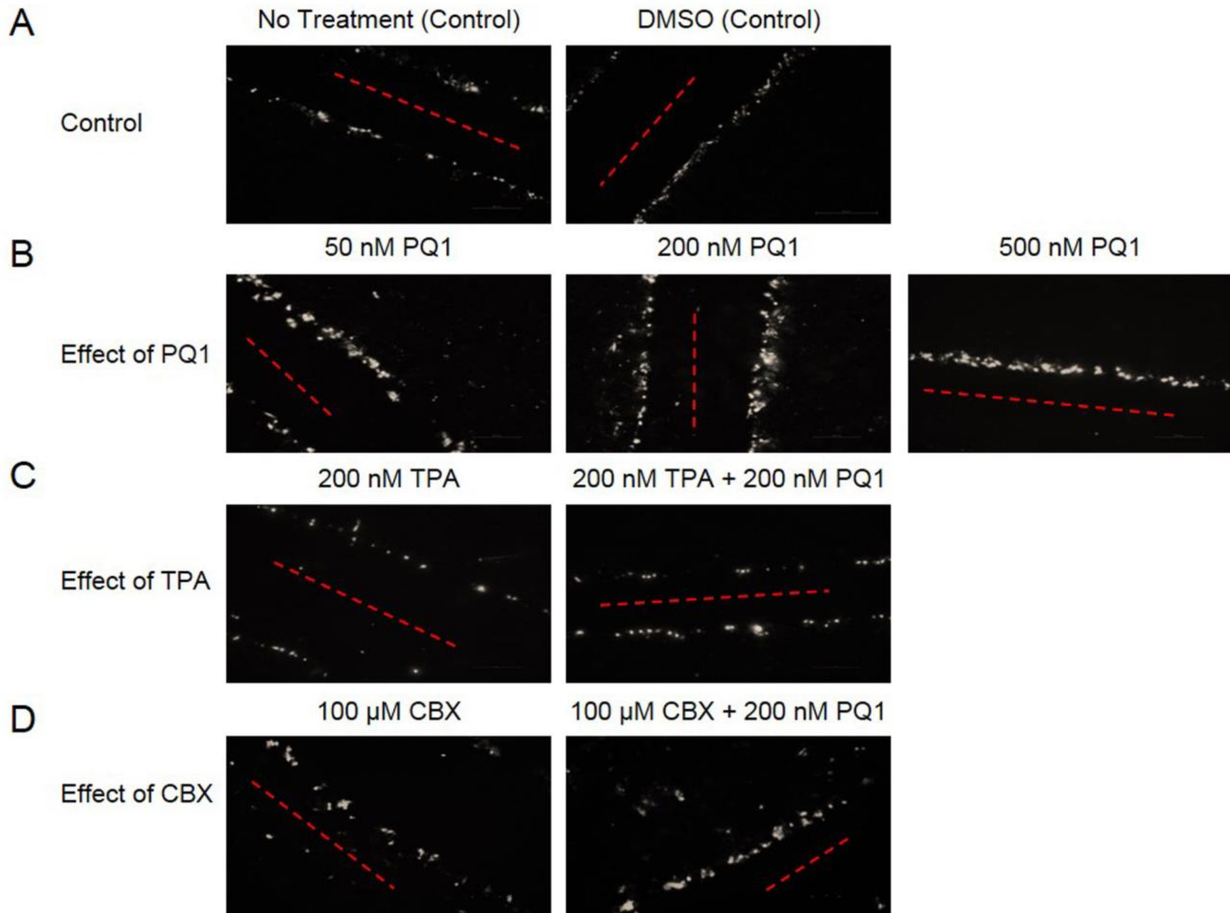

Figure 4.1 Gap junction activity of SW480 cells.

Eight 10^5 SW480 cells were seeded into 6-well plates. A) SW480 cells were treated with no treatment (control) and DMSO (control) for 1 hour. B) SW480 cells were treated with 50 nM PQ1, 200 nM PQ1, or 500 nM PQ1 for 1 hour. C) SW480 cells were treated with 200 nM TPA or 200 nM TPA + 200 nM PQ1 for 1 hour. SL/DT was performed after 1 hour of TPA or PQ1 treatment. Lucifer yellow dyes in cells indicates in white. Red line indicates the point of entry for Lucifer yellow. Dye transfer is measured from point of entry along scape line to outer most cells.

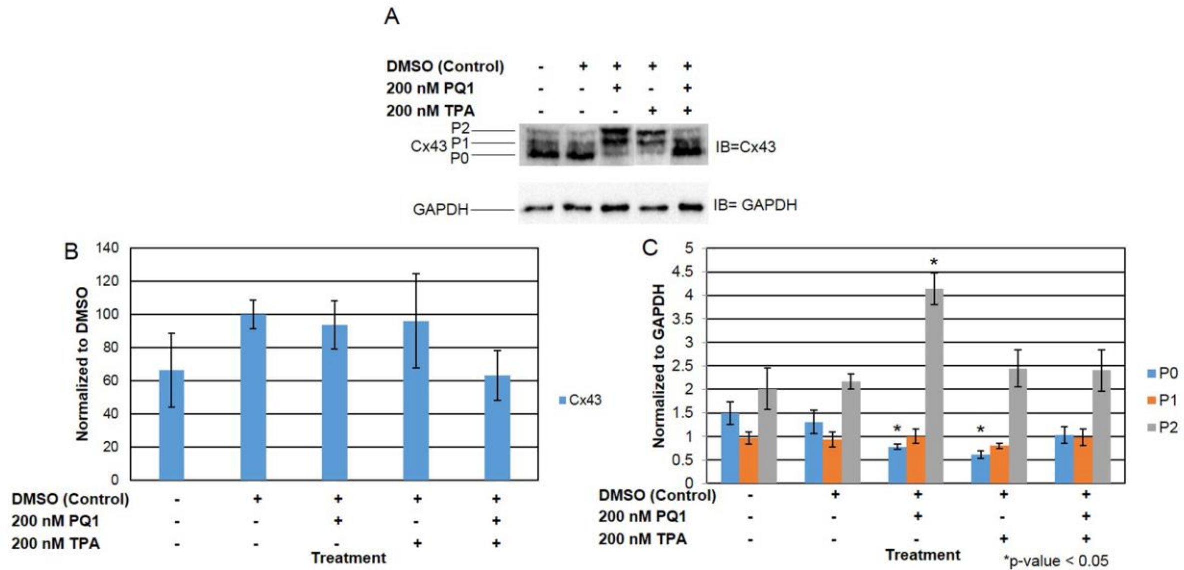

Figure 4.2 PQ1 changes isoform expression of Cx43.

Cells were treated with: no treatment (control), DMSO, 200 nM PQ1, 200 nM TPA, and 200 nM PQ1 + 200 nM TPA. Cells were treated for 1 hour. A) Levels of Cx43 were examined by Western blot analysis using anti-connexin43 (F-7) antibody. GAPDH was used as loading control. B) Graphical presentation of 3 independent experiments showing pixel intensities of total Cx43 normalized to control. *P value is <0.05 compared to control. C) Graphical presentation shows the ratio of Cx43 isoforms P0, P1 and P2. Data were obtained in 3 independent experiments showing pixel intensities of Cx43 isoforms to GAPDH and are represented as the mean ± SD. *P value is <0.05 compared to control. IB= Immunoblot against Cx43.

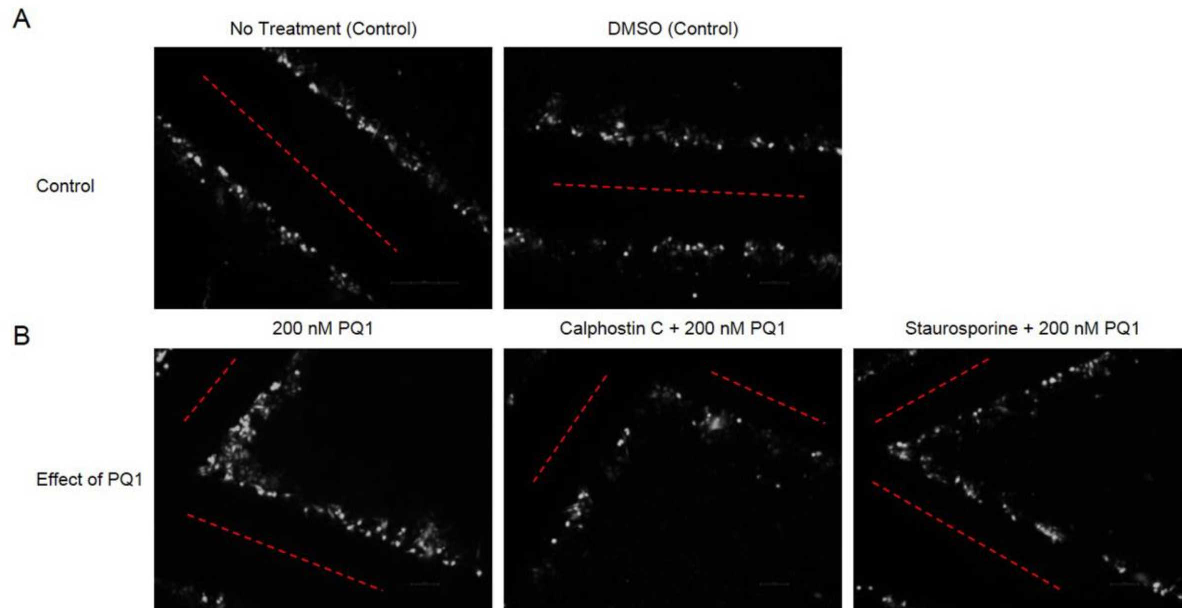

Figure 4.3 Gap junction activity of SW480 cells.

Eight x 10^5 SW480 cells were seeded into 6-well plates. A) SW480 cells were treated with no treatment (control) or DMSO (control) for 1 hour. B) SW480 cells were pre-treated with kinase inhibitors (Calphostin C or Staurosporine) for 1 hour. After pre-treatment, cells were treated with 200 nM PQ1 for 1 hour. SL/DT was performed after 1 hour of PQ1 treatment. Lucifer yellow in cells indicates in white. Red line indicates the point of entry for Lucifer yellow. Dye transfer is measured from point of entry along scape line to outer most cells.

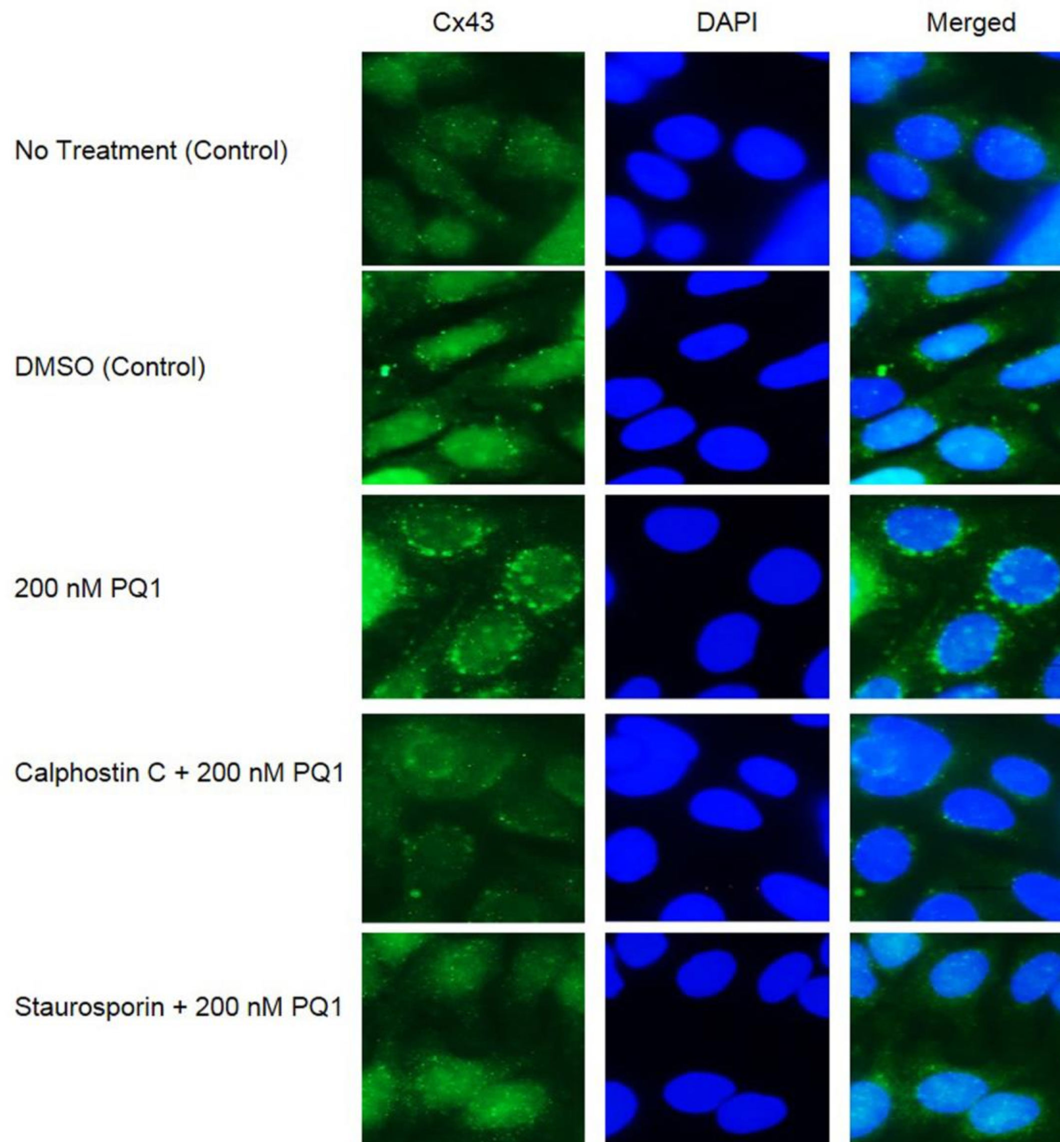

Figure 4.4 PQ1's effect on GJ plaques.

Eight x 105 SW480 cells were seeded onto coverslips in 6-well plates. SW480 cells were treated with no treatment (control) or DMSO (control) for 1 hour. Designated SW480 cells were pre-treated with kinase inhibitors (Calphostin C or Staurosporine) for 1 hour followed by 1 hour treatment with 200 nM PQ1. Controls were: no treatment, DMSO, and 200 nM PQ1. Immunofluorescence was performed using mouse anti-Cx43. Green indicates Cx43 and blue indicates the nuclei.

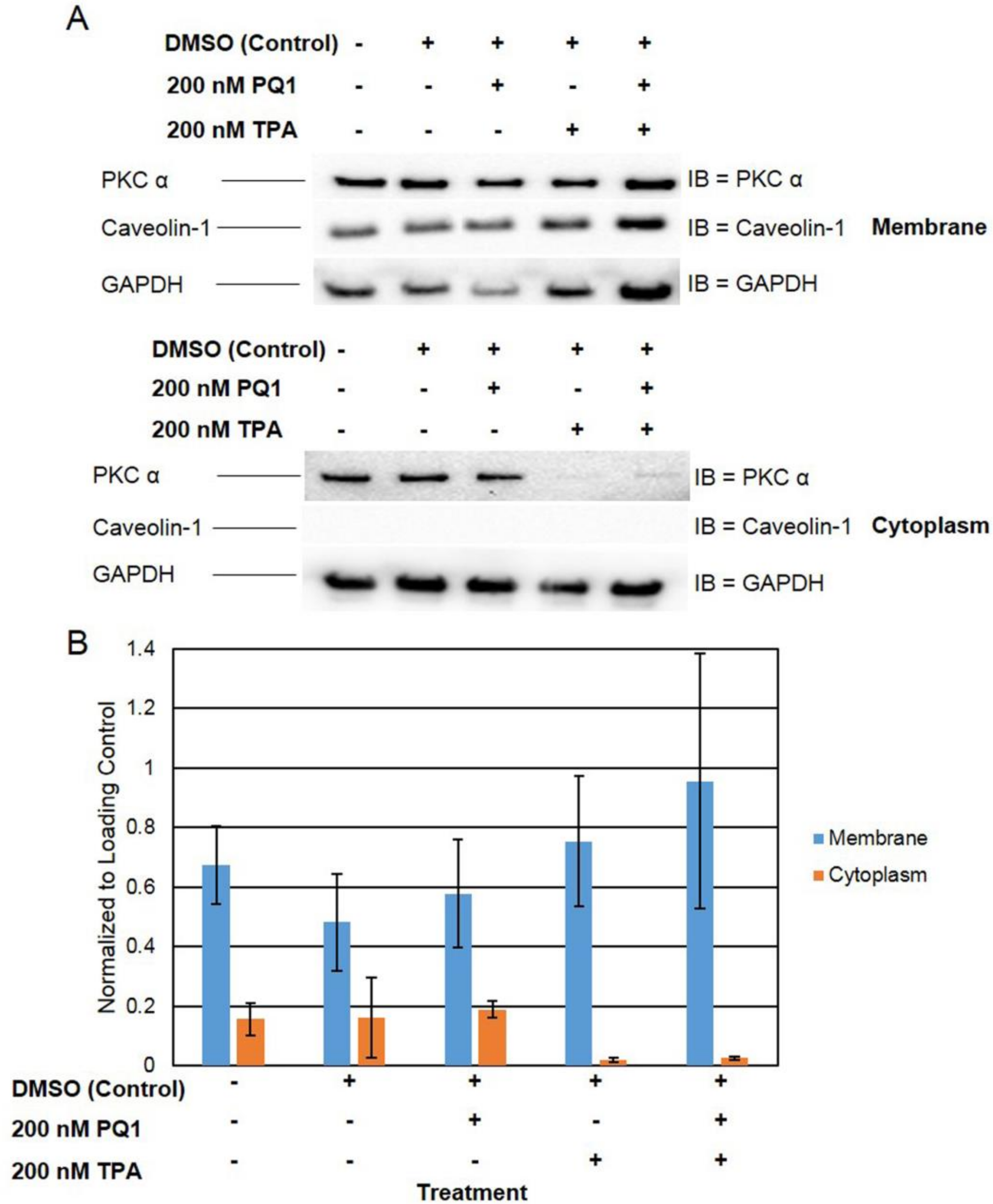

Figure 4.5 PQ1's effects on PKCα.

Cells were treated with: no treatment (control), DMSO, 200 nM PQ1, 200 nM TPA, or 200 nM PQ1 + 200 nM TPA. Cells were treated for 1 hour. A) Level of PKC α was examined by western blot analysis after translocation assay was pre-formed. Membrane and cytosol were separated. GAPDH was used as a loading control. Caveolin was used as negative control. Level of PKC α was examined by western blot analysis using anti-PKC α antibody. B) Graphical presentation of

3 independent experiments showing pixel intensities of PKC α normalized to GAPDH and are represented as the mean ± SD. IB= Immunoblot against PKC α.

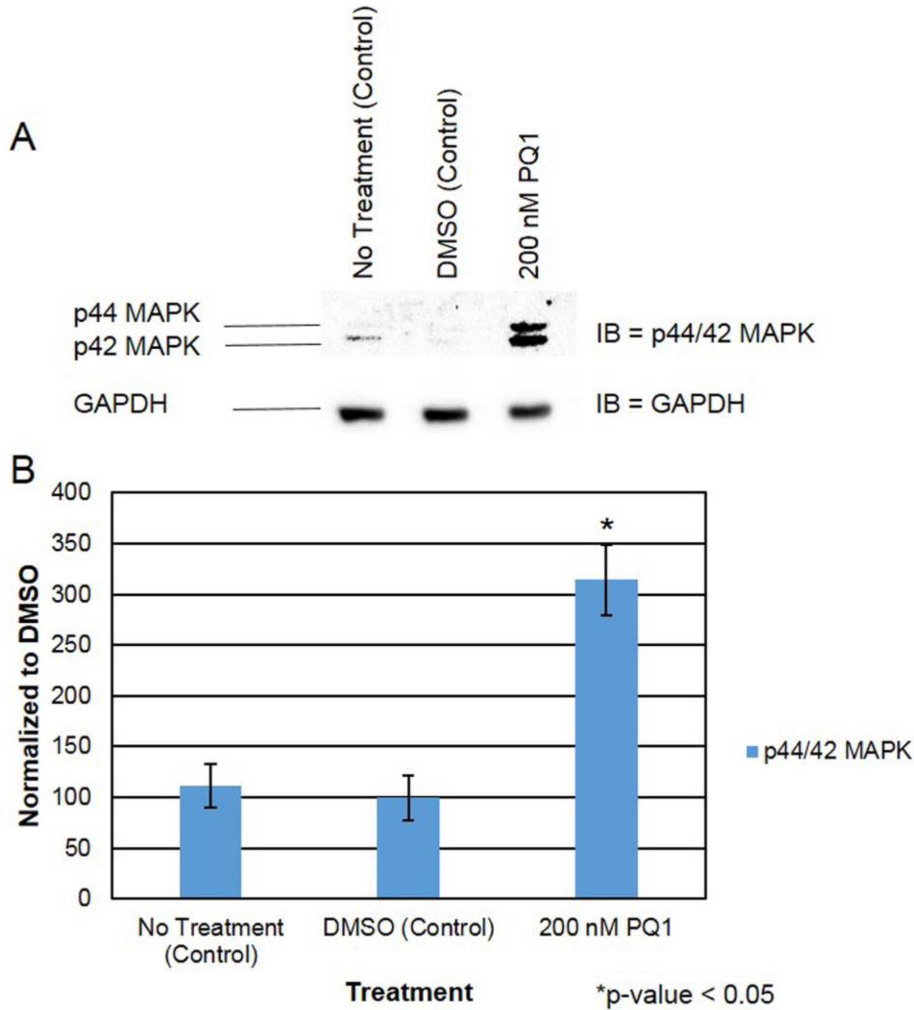

Figure 4.6 PQ1's effect on p44/42 MAPK expression.

Cells were treated with: no treatment (control), DMSO, or 200 nM PQ1. Cells were treated for 1 hour. A) Level of p44/42 MAPK was examined by western blot analysis using anti-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody. GAPDH was used as a loading control. B) Graphical presentation of 3 independent experiments showing pixel intensities of p44/42 MAPK normalized to control. *P value is <0.05 compared to control. IB= Immunoblot against p44/42 MAPK.

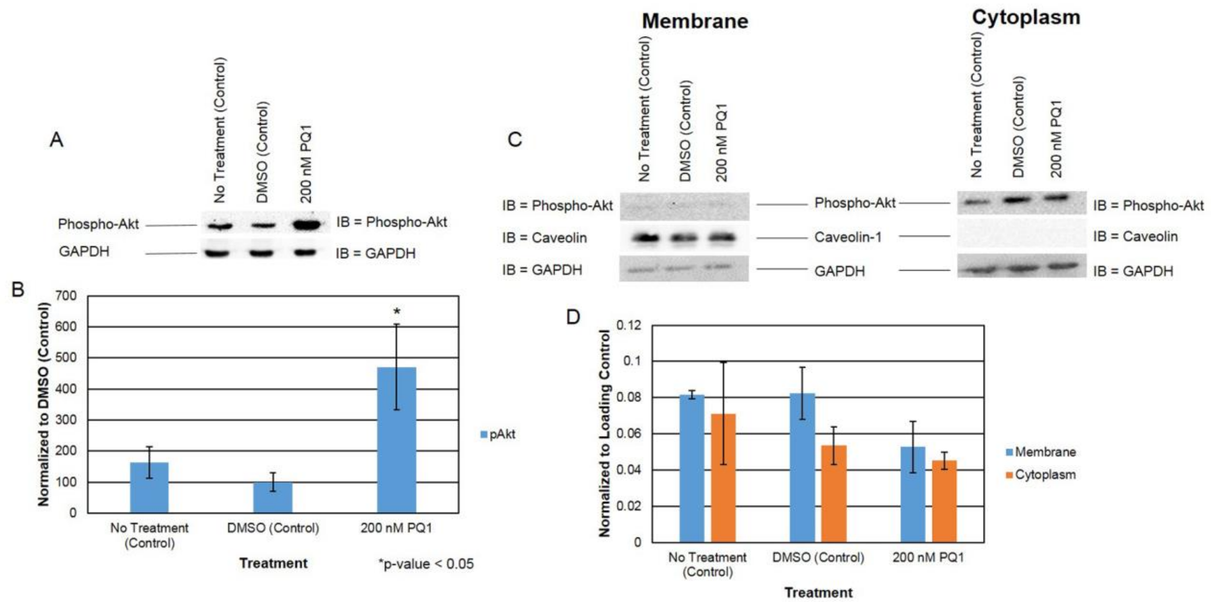

Figure 4.7 PQ1's effect on active Akt expression.

Cells were treated with: no treatment (control), DMSO, or 200 nM PQ1 for 1 hour. A) Level of active Akt was examined by western blot analysis using anti-phospho-Akt (Ser473) (D9E) antibody. GAPDH was used as loading control. B) Graphical presentation of 3 independent experiments showing pixel intensities of active Akt normalized to control. C) Level of active Akt was examined by western blot analysis after translocation assay performed using anti-phospho-Akt (Ser473) (D9E) antibody specific for activated Akt. Membrane and cytosol were separated. GAPDH was used as a loading control. Caveolin was used as negative control. D) Graphical presentation of 3 independent experiments showing pixel intensities of active Akt normalized to GAPDH. IB= Immunoblot against active Akt.

Chapter 5 - PQ1's effect on the Apoptotic pathway

Introduction

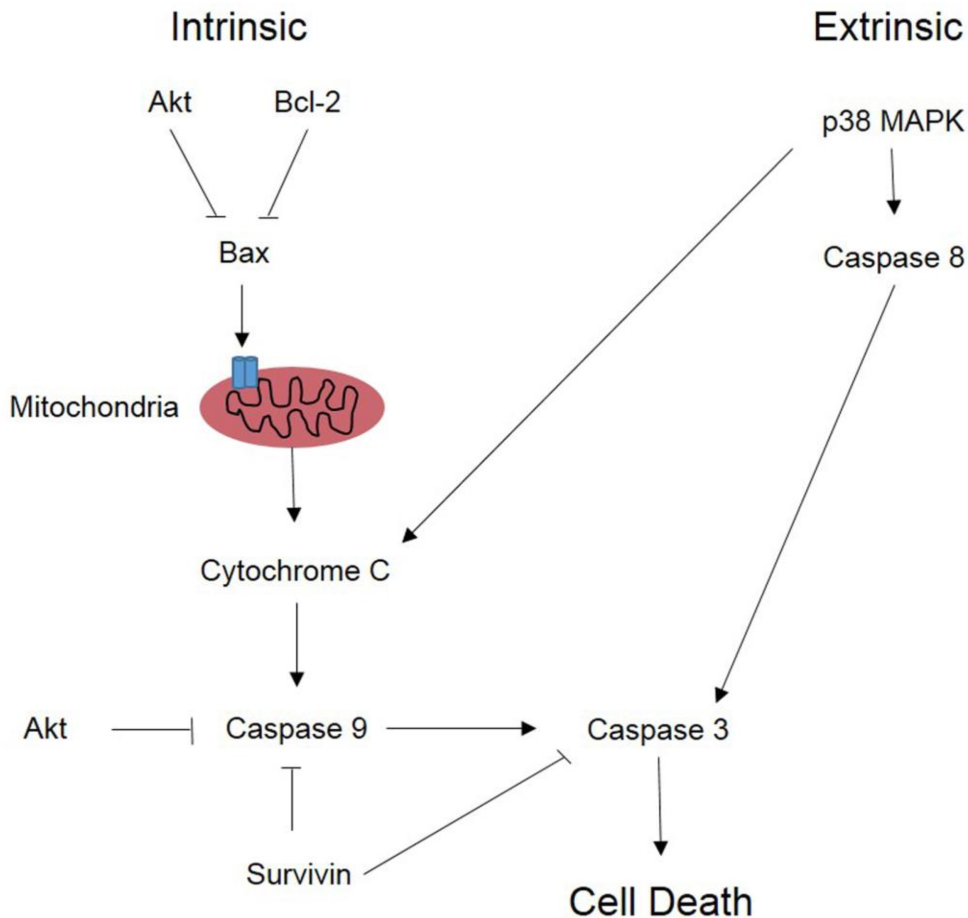

Figure 5.1 Intrinsic and extrinsic apoptotic pathway.

Interestingly, PQ1 has been shown to induce apoptosis via caspase 8 and caspase 9 in T47D breast cancer cells[75]. The regulation of apoptosis is complex. This study focuses on a few specific proteins known to be involved in the apoptosis pathway. Figure 5.1 shows the intrinsic and extrinsic apoptosis pathway and the proteins that are focused on in this study. Previous

studies on PQ1's effects on apoptosis in breast cancer have shown activation of both caspase 8 and caspase 9, which lead to the cleaving and, therefore, activation of caspase 3[75,76]. Caspase 3 is an executioner caspase cleaved and activated by initiators: caspase 8 and caspase 9. The executioner caspases cleave multiple structural and repair proteins[76]. Caspase 3 was shown to be essential for apoptosis-associated chromatin margination (condensed masses of chromatin move toward periphery of the nucleus), DNA fragmentation, and nuclear collapse[76]. Caspase 9 is the initiator caspase required for apoptosis signaling through the mitochondrial pathway. Caspase 9 activation occurs after the release of cytochrome c from the mitochondria[76].

Akt, Bcl-2, and Bax all regulate the intrinsic cell death pathway that leads to the activation of caspase 9. Akt (anti-apoptotic) has multiple targets in the apoptotic pathway. One target of Akt is Bax, a pro-apoptotic protein that signals for the release of cytochrome c from the mitochondria when oligomerized. Akt inhibits Bax and prevents release of the cytochrome c[77,78]. Another anti-apoptotic protein Bcl-2 can dimerize with Bax preventing it from homodimerizing and oligomerizing with a second Bax protein to cause the release of cytochrome c[79]. However, when death signals are present in the cell, both Bcl-2 and Bax are activated, but the ratio of Bax/Bcl-2 dictates the balance of apoptosis/ survival[80]. A third Akt target is caspase 9. Akt directly phosphorylates caspase 9, keeping it from being cleaved and activated[81].

Another kinase p38 MAPK is pro-apoptotic. p38 MAPK (part of the extrinsic apoptosis pathway) is activated by tumor necrosis factor (TNF) after activation it leads to the cleaving of caspase 8[82,83,84]. p38 MAPK also indirectly leads to caspase 9 activation by causing p53 production leading to the release of cytochrome c from the mitochondria[83,85]. Both caspase 8 and caspase 9 activation lead to the cleaving and activating of caspase 3[76]. Another protein, survivin

(anti-apoptotic) is an anti-apoptotic protein with conflicting information on its mechanism. Some studies show it inhibiting caspase 9 while others show it inhibiting caspase 3[86,87].

In this thesis a few pro and anti-apoptotic proteins are studied to understand if apoptosis is activated and if so, what is the pathway that PQ1 utilizes for the induction of apoptosis? There are 3 known methods of cell death: apoptosis, necrosis and necroptosis (programmed necrosis). Apoptosis is programmed cell death due to a signaling cascade. Necrosis is cell death due to external factors and does not use specific proteins, however recently a third method necroptosis was discovered. It was discovered that some parts of necrosis once thought to be passive and not regulated actually have specific mechanisms, this is termed necroptosis[88]. Does PQ1 cause cell death by apoptosis, necrosis, or necroptosis in SW480 colorectal cancer cells?

Methods

Cell Line

The SW480 human colorectal cancer cell line was purchased from American Type Cell Culture (ATCC, Manassas, VA). Cells were grown with 0% CO_2 in Leibovitz's L-15 Medium with 10% Gibco Fetal Bovine Serum (FBS) purchase from Life Technologies (Grand Island, NY, USA).

Proliferation and Viability

The assay was performed as previously described in Chapter 3. Cells were pre-treated for with inhibitors of caspase 3, caspase 8 or caspase 9 for 1 hour, followed by 5000 nM PQ1 treatment for 23 hours. Controls were no treatment, DMSO, or 5000 nM PQ1 for 24 hours.

Flow Cytometry

Two x 10^5 cells were grown in 12-well plates. Cell were dosed with: no treatment (control), DMSO (control), 200 nM PQ1, 500 nM PQ1, 1000 nM PQ1, 5000 nM PQ1, or 10000 nM PQ1 at time points of 1 hour, 4 hours, 8 hours, and 24 hours. After treatment, media was collected. Ethylenediaminetetraacetic acid (EDTA) was added to cells for 5 minutes. The cells were gathered using a 22 gauge needle and added to the saved media. Cells were centrifuged at 1,500 rpm (Using an Eppendorf centrifuge 5415R with rotor F-45-24-11, Eppendorf North America, Hauppauge, New York, USA). Cells were washed with PBS 3 times and centrifuged between washes for 5 minutes. Dead Cell Apoptosis kit with Annexin V Alexa Fluor 488 and Propidium Iodide (PI) from Life Technologies (Grand Island, NY, USA) was used. Samples were then ran on the FACS Calibur by BD Biosciences (San Jose, CA, USA). FCS Express 4 Flow Cytometry by De Novo Software (Los Angeles, CA, USA) was used to analyze the flow cytometry data.

Western Blot

Cells were seeded to 50% density in a T-25 cm^2 flask for 24 hours and allowed the density to reach 90% prior to treatment. Western blot assay was performed as described in Chapter 3. Primary antibodies GAPDH (0411), Bax (B-9) specific for amino acids 1-171 of Bax α, and Bcl-2 (C-2) specific for amino acids 1-205 of Bcl-2, were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA). Primary antibodies survivin (71G4B7) detects endogenous total survivin and p38 MAPK detects endogenous p38α, β, γ were purchased from Cell Signaling Techonolgy (Danvers, Massachusetts, USA). Secondary antibodies were anti-mouse and anti-rabbit IgG linked HRP, they were purchased from Cell Signaling Technology (Danver, Massachusetts, USA).

Immunofluorescence

The assay was performed as described in Chapter 4. Primary antibodies mouse anti-Bax (B-9) and mouse anti-Bcl-2 (C-2) were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA). Rabbit anti-cleaved caspase 3(Asp175) recognizing the 17/19 fragment of activated caspase 3 was purchased from Cell Signaling Techonolgy (Danvers, Massachusetts, USA). Secondary antibodies were Alexa Fluor 488 rabbit anti-mouse IgG (H+L) and Alexa Fluor 568 goat anti-rabbit IgG (H+L) were purchased from Life Technologies (Grand Island, NY, USA).Secondary antibodies were anti-mouse and anti-rabbit IGg HRP linked, they were purchased from Cell Signaling Technology (Danver, Massachusetts, USA).

Results

In the previous chapter 4, PQ1's activation of Akt was shown (Figure 4.7). However, besides being a stabilizer of GJs by way of Cx43; Akt is a known regulator of apoptosis. Akt regulates apoptosis by various proteins along the intrinsic apoptotic pathway. To test for the possibility of PQ1 activating the apoptosis pathway, SW480 cells were initially treated with increasing concentrations of PQ1 to find the IC_{50} of PQ1 in SW480 cells. Figure 5.2A shows the proliferation of SW480 cells after 24 hours of PQ1 treatment. At 5000 nM PQ1, proliferation decreased by 50% (Figure 5.2A). Viability was also tested and the IC_{50} was found to be 5000 nM (Figure 5.2B). Figure 5.2B shows cell death, but does not determine if the death is by apoptosis, necrosis, or necroptosis.

To determine if death was due to apoptosis we utilized flow cytometry using nnexin V 488 and Propidium Iodide (PI) (Figure 5.3). Annexin V binds to both phosphatidylserine (PS) and phosphatidylethanolamine (PE) which are expressed on the cell surface during apoptosis. PI binds to nucleic acid, it is impermeable to cell membranes so will not be present in live cells,

only in cells that are dead. In Figure 5.3, control (no treatment and DMSO) samples showed the largest concentration of cells to be negative for both Annexin V and PI (alive not signaling for apoptosis), a few cells were single positive for Annexin V (alive and signaling for apoptosis), and the rest of the population were double positive, meaning both Annexin V and PI were present (cells were dead with signals for apoptosis present). After 4 hours of treatment with 200 nM PQ1 sample findings were similar to control samples. At 4 hour treatment of 500 nM, 1000 nM, or 5000 nM PQ1 treatment an increase in single positive Annexin V cells and double positive cells are seen (Figure 5.3). The highest percentages of single Annexin V positive and double positive cells are seen after 5000 nM treatment. When treated for 4 hours with 10000 nM PQ1, very few single positive Annexin V cells were found and an increase in double positive cells were seen (Figure 5.3). These results suggest that apoptosis is activate in SW480 cells after treatment with PQ1. Due to these findings, a concentration of 5000 nM PQ1 at the 4 hour time point was used in subsequent assays.

Major components of the apoptotic pathway are caspase 3, caspase 8 and caspase 9. To test PQ1's effects on the apoptotic pathway, SW480 cells were pre-treated with caspase 3, caspase 8, or caspase 9 inhibitors and, subsequently, treated with 5000 nM PQ1 for 24 hours (Figure 5.4). Results show no significant change in proliferation at 5000 nM PQ1 or pre-treated cells compared to DMSO (Figure 5.4A). Viability is seen to decrease to 60% after treatment with 5000 nM PQ1 after 24 hours. When pre-treated with inhibitors of caspase 3, 8, or 9 no change in viability compared to DMSO is seen (Figure 5.3A and 5.3B). These findings suggest that both caspase 8 and 9 are activated in response to 5000 nM PQ1 and lead to the cleaving of caspase 3.

Active Akt (anti-apoptotic) inhibits Bad and Bax. Using western blot analysis, active Akt is not shown to change (Figure 5.5). Bax is a pro-apoptotic protein. In Figure 5.6A and 5.6B,

Bax increases by approximately 20% compared to DMSO at both 5000 nM and 10000 nM PQ1; however, sample size of 3 shows no statistically significance compared to controls. Analyzing Bax via immunofluorescence, it shows Bax decreasing at 5000 nM and 10000 nM PQ1 treatment compared to DMSO (Figure 5.7). However, Bcl-2 at 1000 nM increases by 20%, and 5000 nM PQ1 increases by approximately 50% compared to DMSO, although neither appear significant after 3 replications (Figure 5.8A and 5.8B). At 10000 nM PQ1, Bcl-2 is not seen to change (Figure 5.8A and 5.8B). Visualization of Bcl-2 by immunofluorescence shows no change in Bcl-2 expression at any concentration compared to DMSO (Figure 5.9). Results of Bax/Bcl-2 expression levels analyzed by western blot analysis are summed up in Table 5.1. The ratio of Bax to Bcl-2 determines if that part of the apoptotic pathway is activated or inhibited. Bcl-2 increases at 1000 nM PQ1 on both the western blot, suggesting survival. At 5000 nM PQ1, the western blot analysis showed a larger increase in Bcl-2 than Bax, once again suggesting survival. Western blot analysis at 10000 nM PQ1 showed no change in Bcl-2, and Bax was seen to increase by 20% compared to DMSO, suggesting apoptosis. However, due to the data not being significant after triplicates were performed, more studies are needed. The Bax anti-body used was not specific to the oligomerized form of Bax which is the form when Bax causes the release of cytochrome c; thus, data found does not necessarily correlate to the release of cytochrome c and apoptosis.

Caspase 3 is an executioner protein that is essential for apoptosis[27,76]. Figure 5.10 showed an increase in cleaved caspase 3 with increasing concentrations of PQ1. Cleaved caspase 3 did not increase until 5000 nM and 10000 nM PQ1 treatments (Figure 5.10). This suggests apoptosis is happening. p38 MAPK has been shown to be pro-apoptotic through both caspase 8 and caspase 9[89–92] cleavage. p38 MAPK is not seen to change (Figure 5.11). Survivin is thought to

inhibit Caspase 3 and possibly inhibit caspase 9, an initiator caspase that leads to the cleaving of caspase 3. Survivin is not seen to change (Figure 5.12). These results suggest that apoptosis by way of PQ1 is not regulated by p38 MAPK or survivin.

Discussion

PQ1 was shown to activate Akt, a known GJ stabilizer and a regulator of apoptosis, in Chapter 4 after treatment with 200 nM PQ1 compared to DMSO (Figure 4.7A and 4.7B). This suggested a possible link of PQ1 regulating GJs and influencing the apoptotic pathway[77,78,80,81,93]. Akt inhibits apoptosis by inhibiting pro-apoptotic proteins along the intrinsic apoptotic pathway. Viability studies (Figure 5.2B) were used to find the IC_{50} of PQ1 and allowing the testing of a small range of concentrations to using Annexin V and PI by flow cytometry to learn if apoptosis takes place in SW480 cells after treatment with PQ1. Flow studies suggested that after 4 hours treatment with 5000 nM PQ1 treatment apoptosis was starting to occur (Figure 5.3).

To learn if PQ1 affected the extrinsic or intrinsic apoptotic pathway, viability was performed using caspase 8 and caspase 9 inhibitors (Figure 5.4). It was shown that both the caspase 8 and caspase 9 pathways were activated. Active Akt has been found to inhibit Bax and caspase 9[77,78,80,81,93]. The inhibition of Bax leads to fewer Bax molecules for Bcl-2 to inhibit[80]. Akt increases free Bcl-2, while decreasing Bax molecules leading to a decreased ratio of Bax to Bcl-2 and promoting survival[80]. Akt also inhibits caspase 9 which is activated downstream of Bax and Bcl-2[76,81]. Activation of caspase 9 leads to cleaving and activation of caspase 3, an apoptosis executioner protein[76].

The data on Akt suggested that PQ1 has no effect on active Akt expression, as concentration increases to doses needed to signal for apoptosis (Figure 5.5A and 5.5B). Data on Bax and Bcl-2 suggested that at 1000 nM concentrations, anti-apoptotic factor Bcl-2 was

elevated while Bax saw no change. At 5000 nM PQ1, both Bax and Bcl-2 increased the same amount, suggesting that if apoptosis is occurring, it is mediated by alternate proteins. When 10000 nM PQ1 was used as treatment, Bax was increased and Bcl-2 stayed the same, suggesting apoptosis (Figures 5.6, Figure 5.8 and Table 5.1).

Figure 5.4 suggests activation of apoptosis by way of the caspase pathway. Caspase 3 can be regulated by multiple proteins. In this study, the intrinsic and extrinsic pathways were analyzed using various proteins. The protein survivin has been documented to inhibit apoptosis[83,86]. Interestingly, survivin is unchanged in this case.

The protein p38 MAPK (intrinsic and extrinsic) is a pro-apoptotic protein and has been shown to be activated by TNF[82]. This protein mediates both caspase 8 and caspase 9[83,84,86]. The results show no change in active p38 MAPK suggesting that PQ1 does not use the extrinsic apoptosis signaling cascade to activate p38 MAPK.

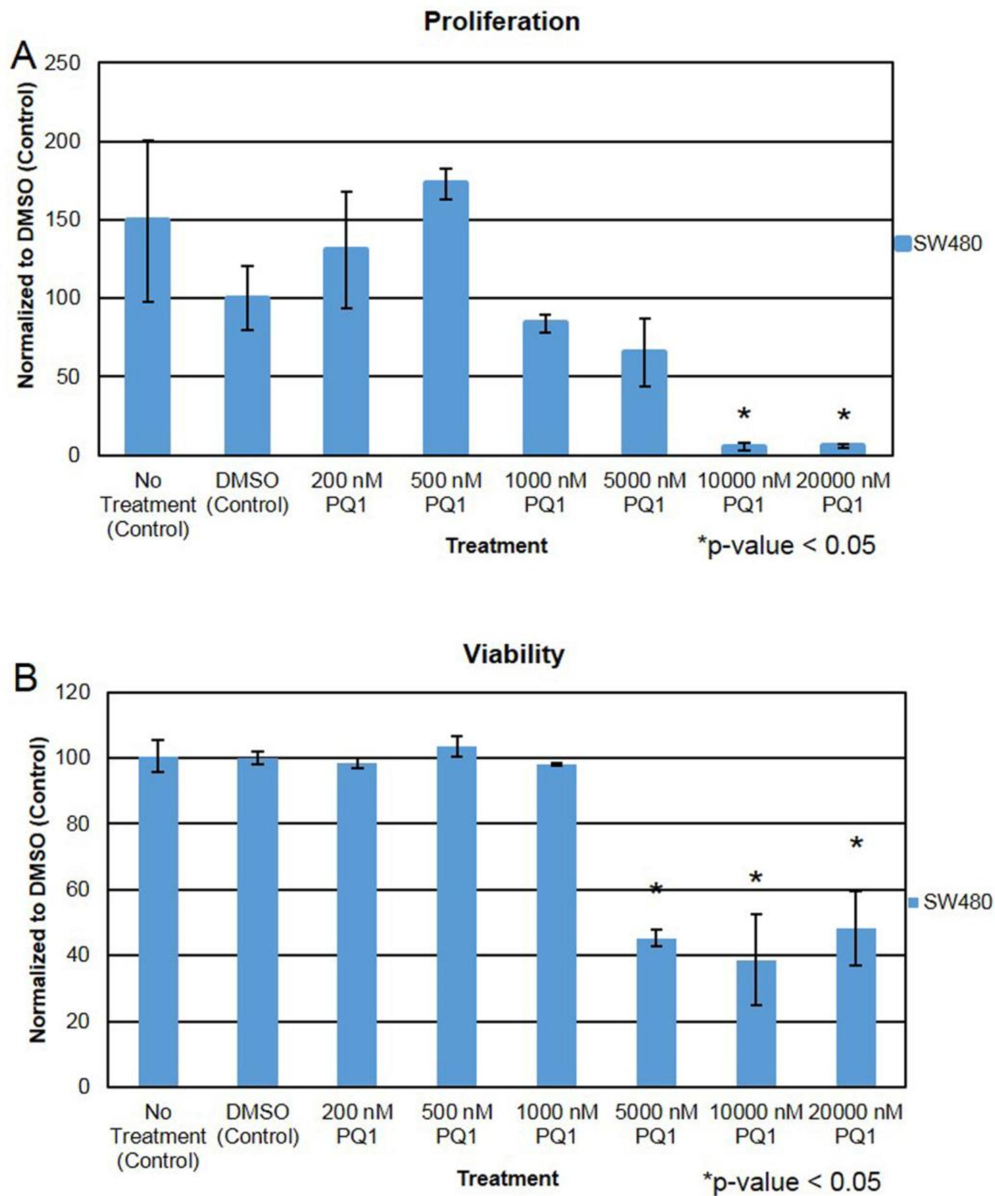

Figure 5.2 Proliferation and Viability of SW480 cells after treatment with PQ1.
Eight x 10^5 SW480 cells were seeded into 6-well plates. The cells were then treated with respective treatments for 24 hours. Treatments were: No treatment (control), DMSO (control), 200 nM PQ1, 500 nM PQ1, 1000 nM PQ1, 5000 nM PQ1, 10000 nM PQ1, or 20000 nM PQ1. After 24 hours viability and proliferation was performed. A) Proliferation of SW480 cells. B) Viability of SW480 cells. Data were obtained in 3 independent experiments and are represented as the mean ± SD. *P value is <0.05 compared to control.

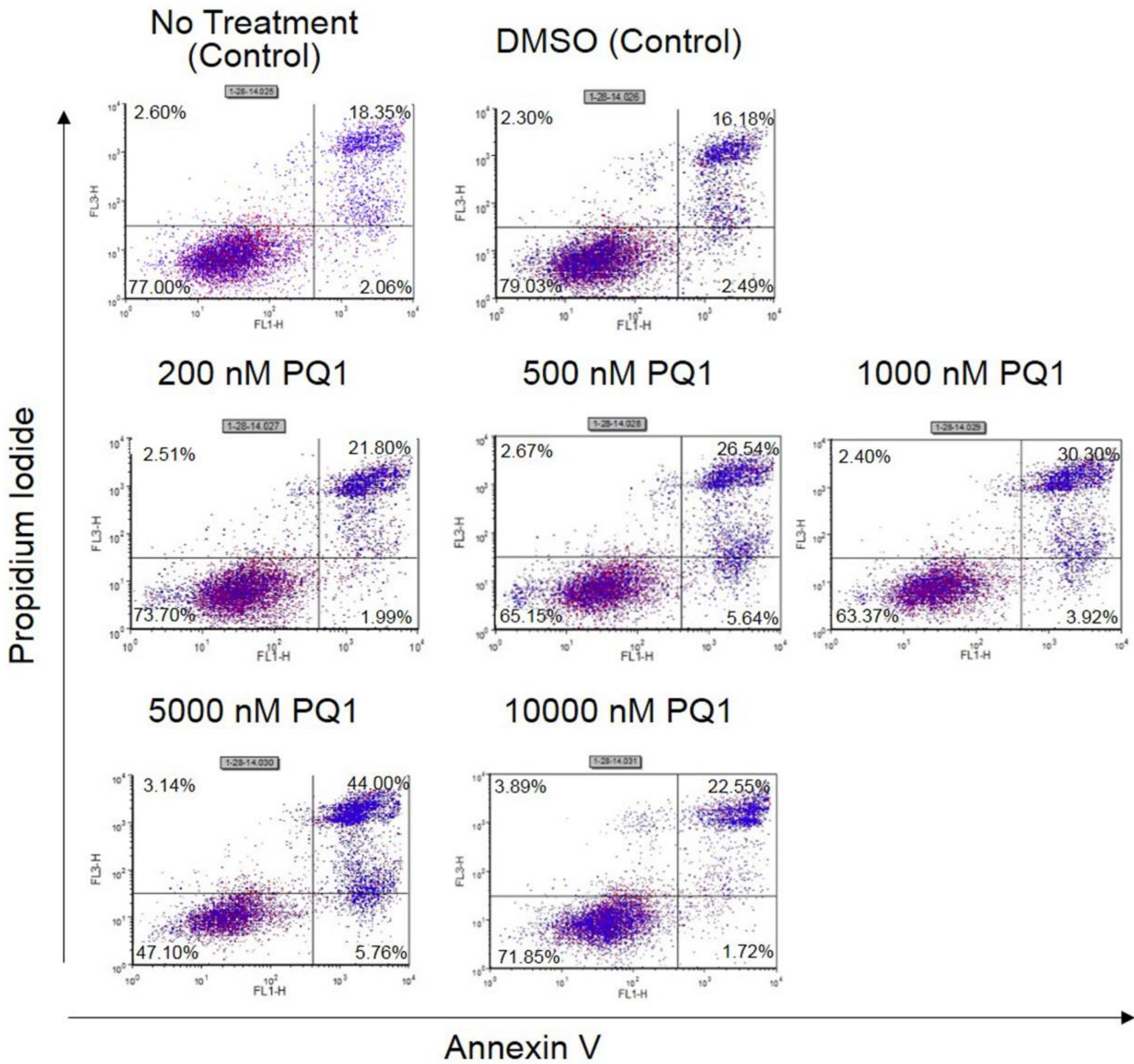

Figure 5.3 Flow Cytometry of Annexin V and PI.

Two x10^5 cells were seeded into 12-well plates. Cells were then treated with respective treatments for 4 hours. Treatments were: No treatment (control), DMSO (control), 200 nM PQ1, 500 nM PQ1, 1000 nM PQ1, 5000 nM PQ1, or 10000 nM PQ1. After 2,4,8 and 24 hours, flow cytometry was performed only 4 hour time point shown as the 4 hour time point was the only time point to show possible apoptosis signaling.

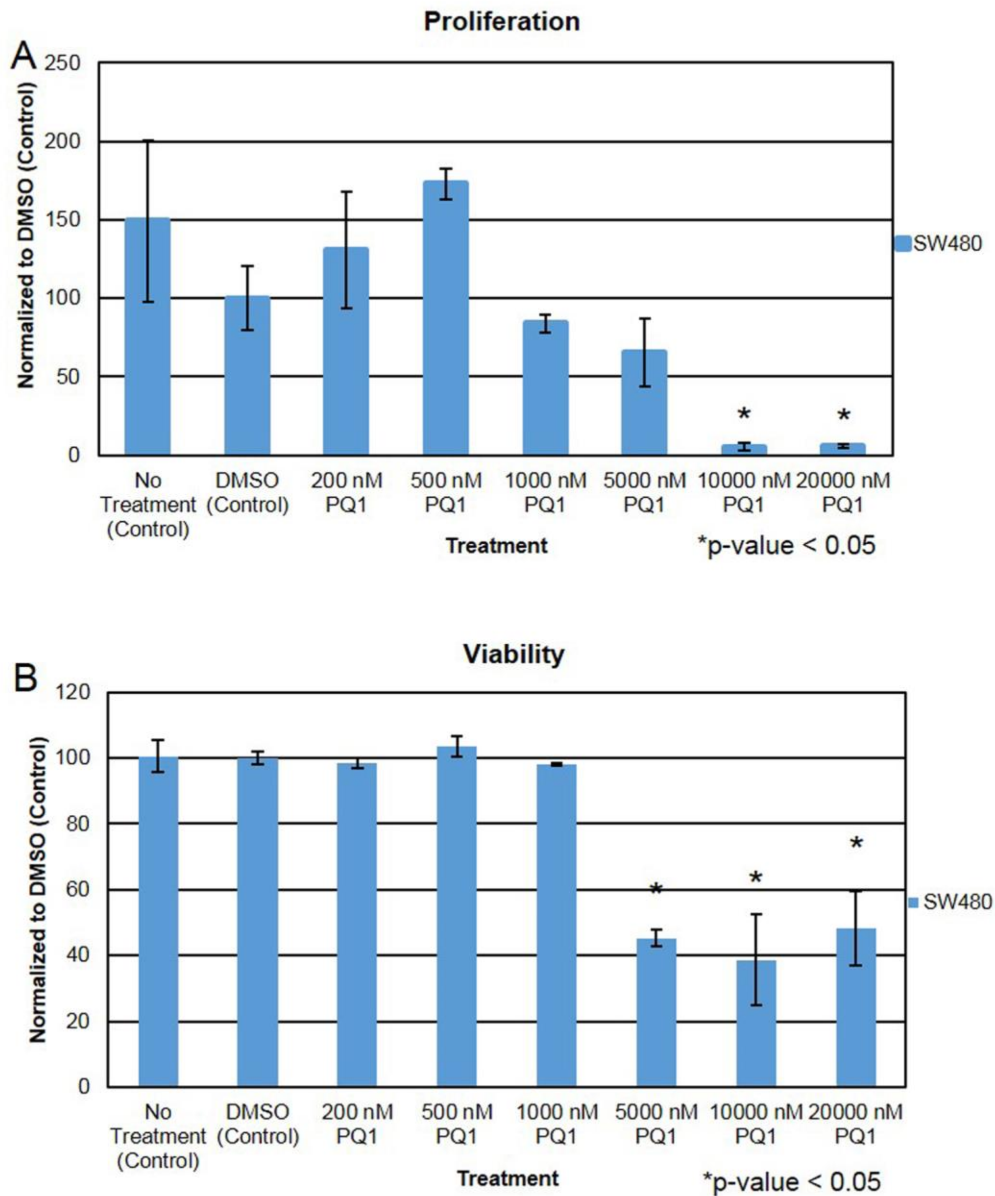

Figure 5.4 Proliferation and Viability of SW480 cells after treatment with caspase inhibitors.

Eight x 10^5 cells were seeded into 6-well plates. Cells were then treated with respective treatments for 24 hours. Treatments were: No treatment (control), DMSO (control), 5000 nM PQ1, 5000 nM PQ1 + Caspase 3 inhibitor, 5000 nM PQ1 + Caspase 8 inhibitor, or 5000 nM PQ1 + Caspase 9 inhibitor. After 24 hours, viability and proliferation was performed. A) Proliferation of SW480 cells. B) Viability of SW480 cells. Data were obtained in 3 independent experiments and are represented as the mean ± SD. *P value is <0.05 compared to DMSO (Control).

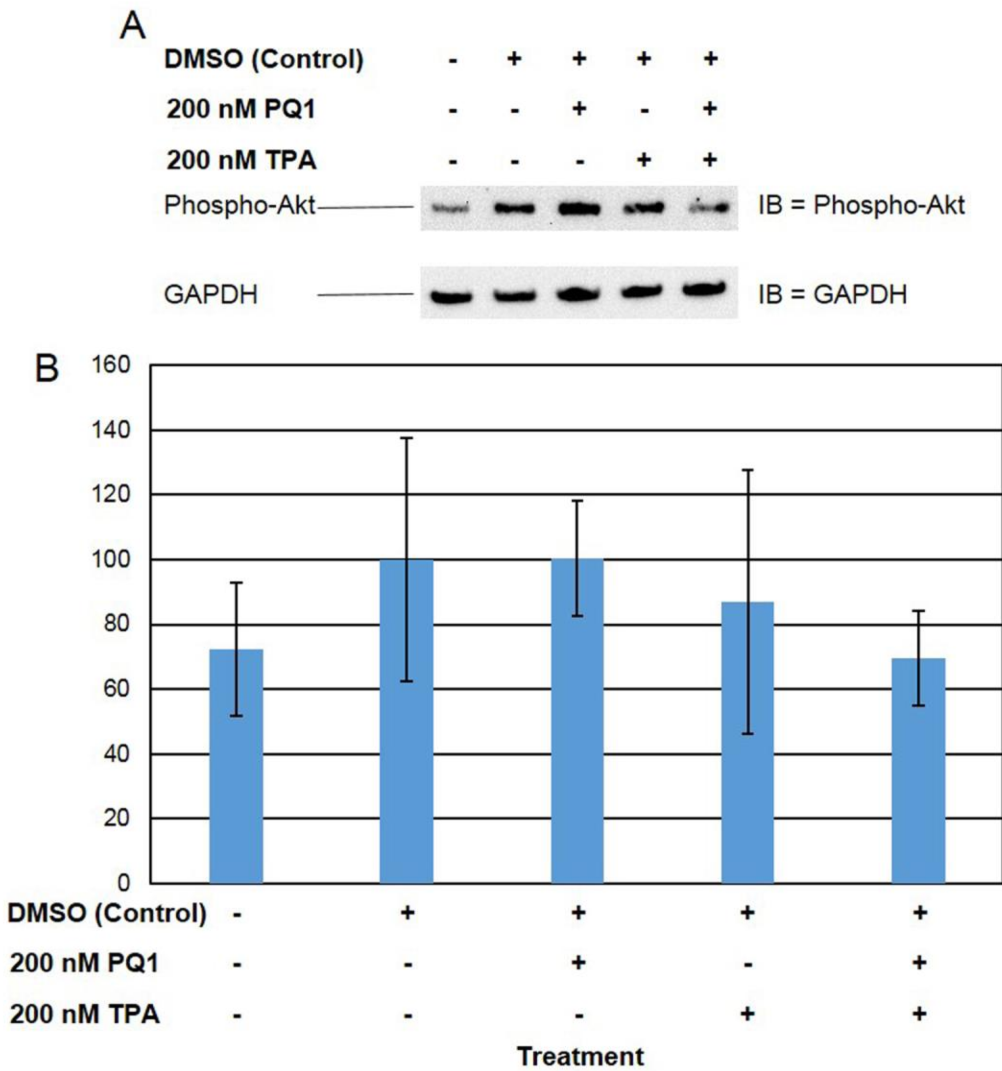

Figure 5.5 PQ1's effect on Akt activation.

Cells were treated with: no treatment (control), DMSO (control), 1000 nM PQ1, 5000 nM PQ1, or 10000 nM PQ1 for 4 hours. A) Level of active Akt was examined by western blot analysis using anti-phospho-Akt (Ser473) (D9E) antibody. GAPDH was used as a loading control. B) Graphical presentation of 3 independent experiments showing pixel intensities of activated Akt normalized to control. IB= Immunoblot against active Akt.

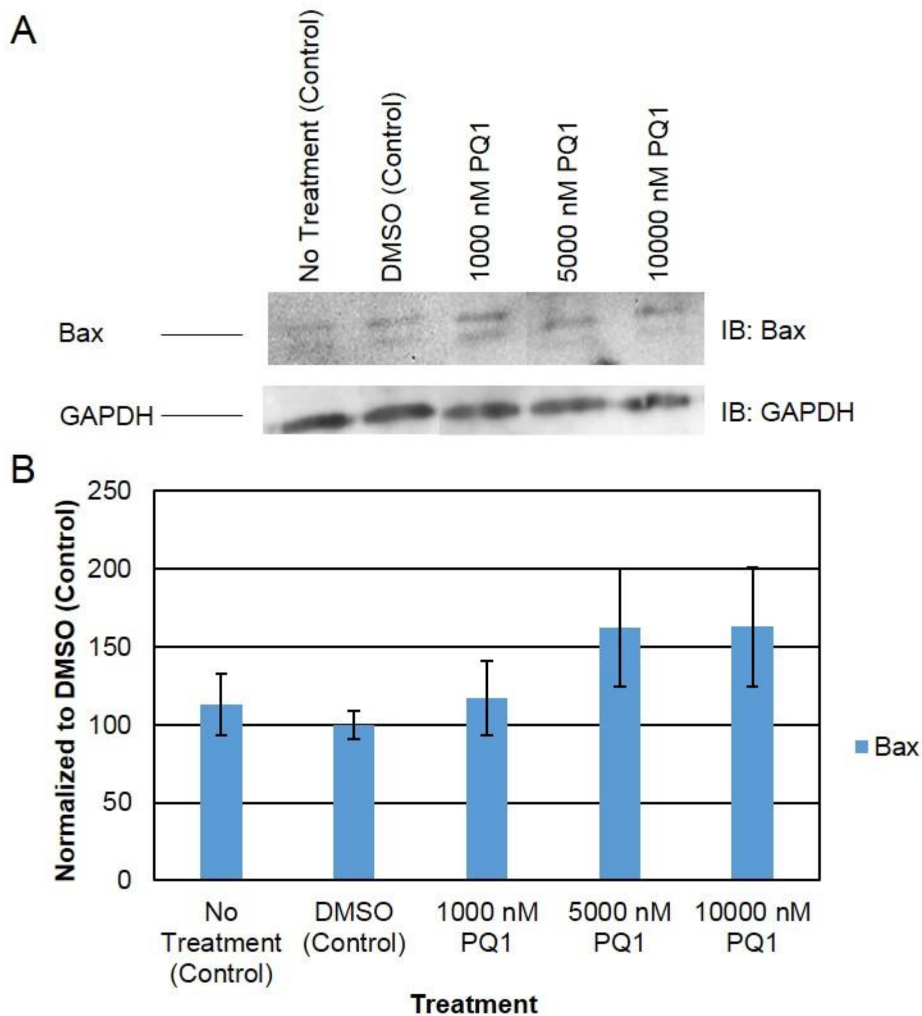

Figure 5.6 PQ1's effect on Bax.

Cells were treated with: no treatment (control), DMSO (control), 1000 nM PQ1, 5000 nM PQ1, or 10000 nM PQ1 for 4 hours. A) Level of Bax was examined by western blot analysis using anti-Baxantibody. GAPDH was used as a loading control. B) Graphical presentation of three independent experiments showing pixel intensities normalized to control. IB= Immunoblot against Bax.

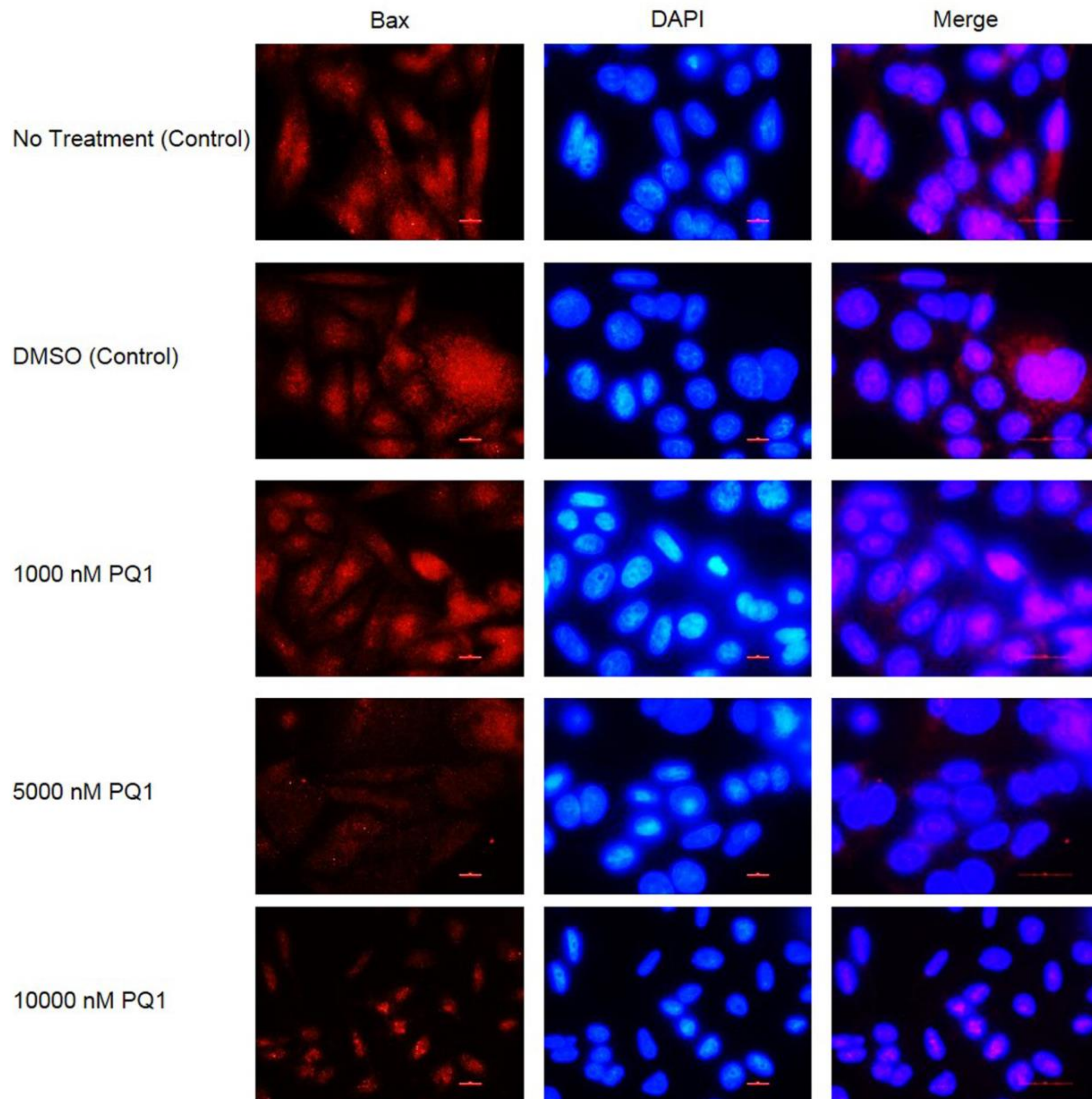

Figure 5.7 Visualization of PQ1's effect on Bax.

Eight x 10^5 SW480 cells were seeded onto coverslips in 6-well plates. Cells were treated with: No Treatment (control), DMSO (control), 1000 nM PQ1, 5000 nM PQ1, or 10000 nM PQ1 for 4 hours. Immunofluorescence was performed using anti-Bax. Red indicates Bax and blue indicates the nuclei. Immunofluorescence was performed in triplicate.

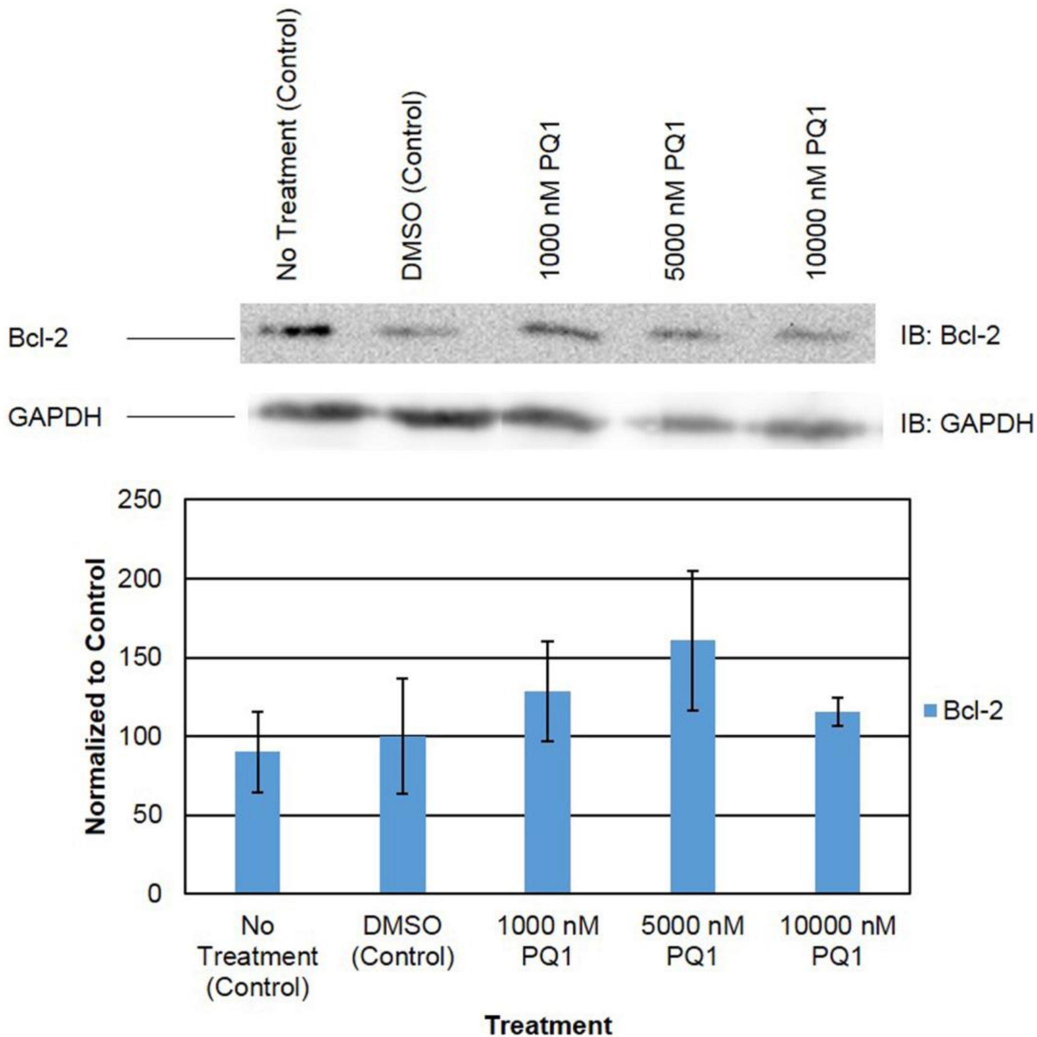

Figure 5.8 PQ1's effects on Bcl-2.

Cells were treated with: no treatment (control), DMSO (control), 1000 nM PQ1, 5000 nM PQ1, or 10000 nM PQ1 for 4 hours. A) The level of Bcl-2 was examined by western blot analysis using Bcl-2 (C-2) antibody. GAPDH was used as a loading control. B) Graphical presentation of three independent experiments showing pixel intensities normalized to control. IB= Immunoblot against Bcl-2.

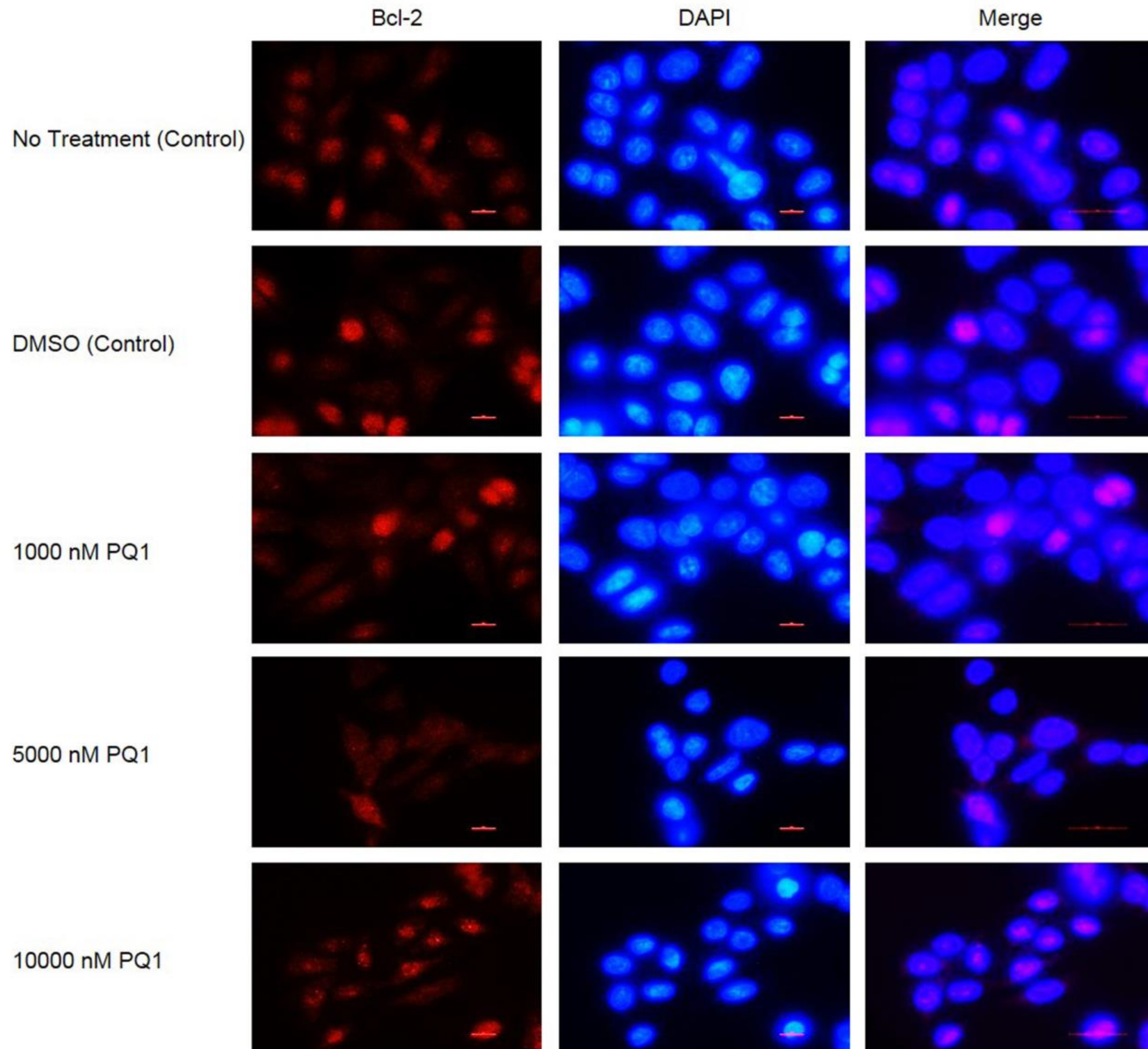

Figure 5.9 PQ1 has no visual effect on pro-apoptotic protein Bcl-2.

Eight x 10^5 SW480 cells were seeded onto coverslips in 6-well plates. Cells were treated with: No Treatment (control), DMSO (control), 1000 nM PQ1, 5000 nM PQ1, or 10000 nM PQ1, for 4 hours. Immunofluorescence was performed using anti-Bcl-2 antibody. Red indicates Bcl-2 and blue indicates the nuclei. Immunofluorescence was performed in triplicate.

	PQ1 Concentration		
Protein	1000 nM	5000 nM	10000 nM
Bcl-2	20%	50%	0%
Bax	0%	50%	20%

Table 5.1 PQ1's effect on Bcl-2 and Bax expression.

Compilation of data from Figure 5.5B and 5.7B.

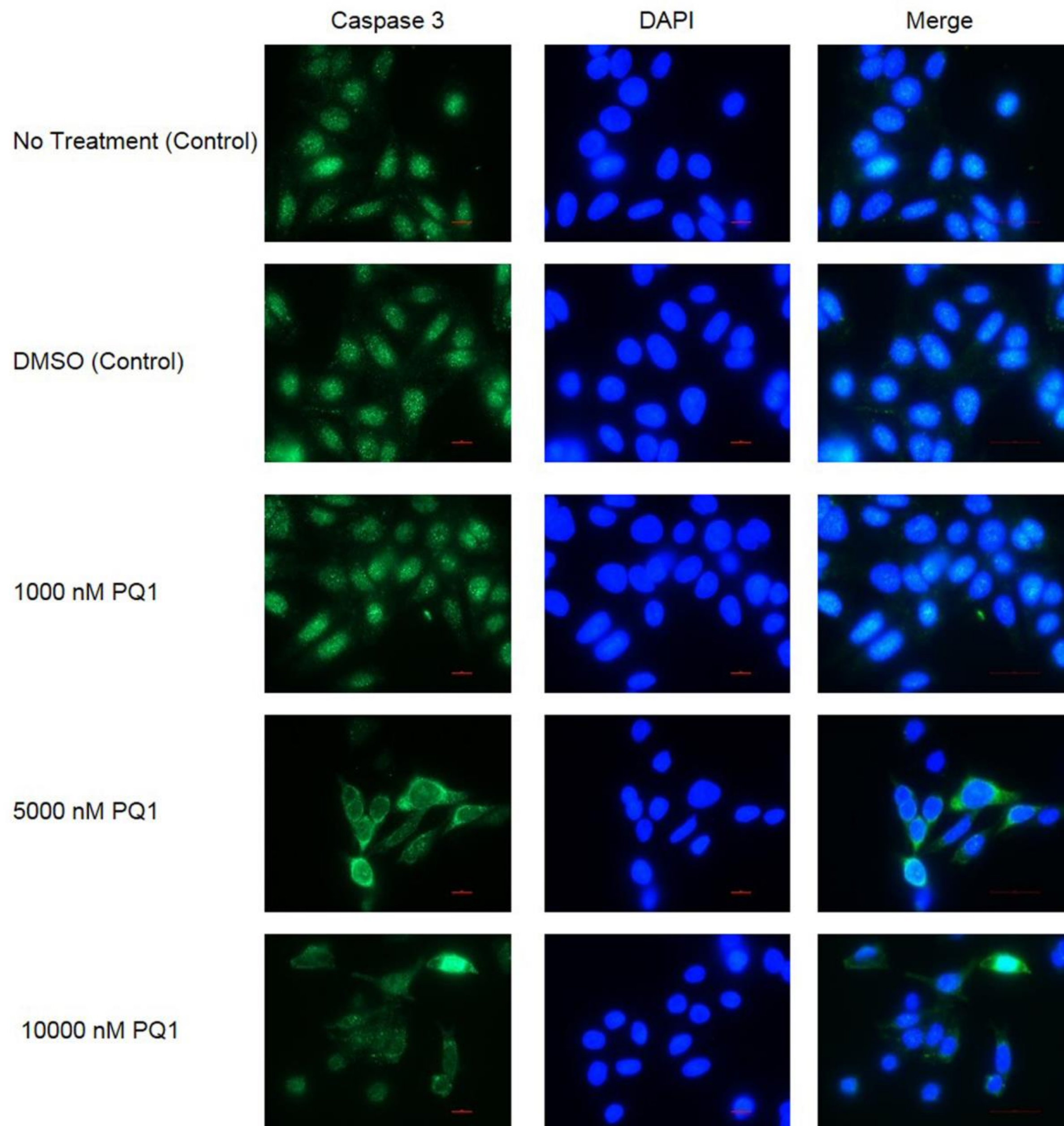

Figure 5.10 PQ1's effect on caspase 3.

Eight x 10^5 SW480 cells were seeded onto coverslips in 6-well plates. Cells were treated with: No Treatment (control), DMSO (control), 1000 nM PQ1, 5000 nM PQ1, or 10000 nM PQ1 for 4 hours. Immunofluorescence was performed using anti-cleaved caspase 3. Green indicates anti-cleaved caspase 3 and blue indicates the nuclei. Immunofluorescence was performed in triplicate.

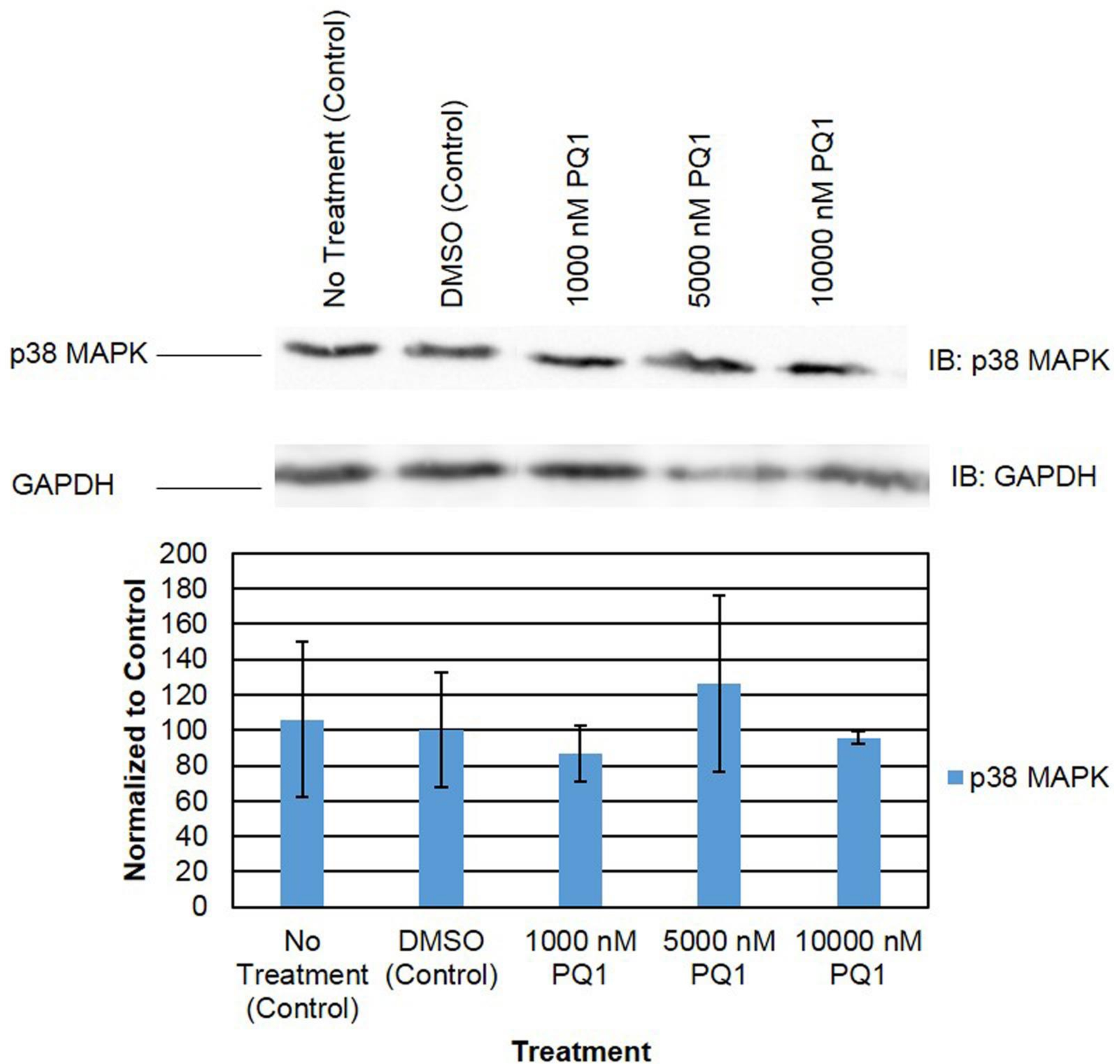

Figure 5.11 PQ1's effects on p38 MAPK.

Cells were treated with: no treatment (control), DMSO (control), 1000 nM PQ1, 5000 nM PQ1, or 10000 nM PQ1 for 4 hours. A) Level of active p38 MAPK was examined by western blot analysis. GAPDH was used as a loading control. B) Graphical presentation of 3 independent experiments showing pixel intensities normalized to control. IB= Immunoblot against active p38 MAPK.

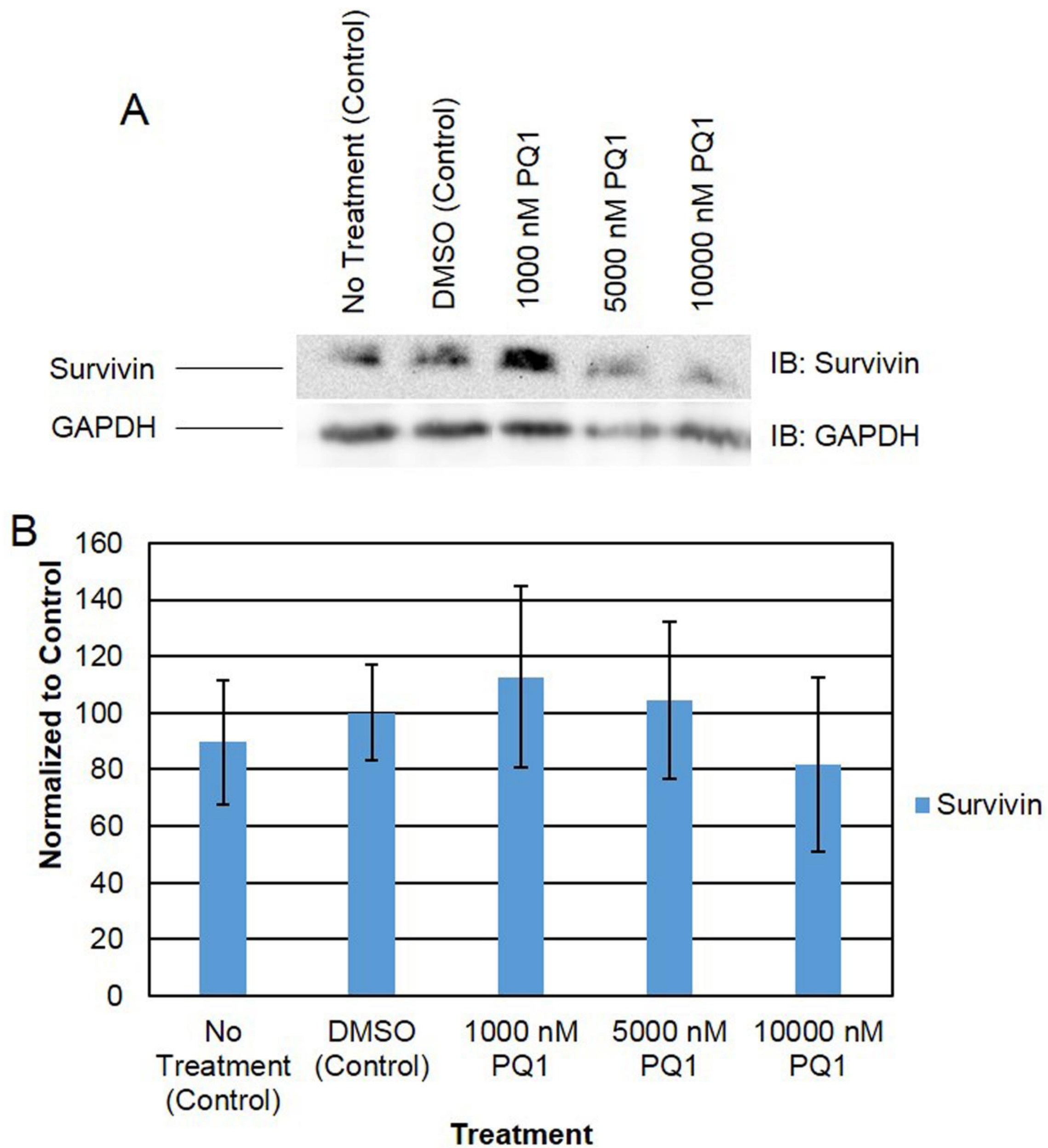

Figure 5.12 PQ1's effect on Survivin.

Cells were treated with: no treatment (control), DMSO (control), 1000 nM PQ1, 5000 nM PQ1, or 10000 nM PQ1 for 4 hours. A) Level of survivin was examined by western blot analysis using anti-survivin antibody. GAPDH was used as a loading control. B) Graphical presentation of 3 independent experiments showing pixel intensities of survivin normalized to control. IB= Immunoblot against Survivin.

Chapter 6 - Conclusion/ Future Studies

PQ1's Mechanism for increasing GJIC

During cancer formation, a decrease in GJIC occurs. Cx43, a GJ protein, is found to decrease and become localized on cytoplasmic membranes like the Golgi apparatus. Currently, no anti-cancer drugs target gap junctions. In this study, transfection was used to test SW480 colorectal cells to see if an increase in GJIC could occur. When cells were overexpressed with Cx43, GJIC was increased with no effect in cell viability and proliferation, compared to control without transfection. This suggests that increasing GJIC could increase the efficacy of other chemotherapeutic drugs by allowing for their propagation through open gap junctions.

Since overexpression of Cx43 via transfection is not a viable therapeutic option, a small molecule needed to be developed that caused the increase in GJIC. Using the known structure of the carboxyl-terminus domain of Cx43, the small molecule, PQ1, was developed. PQ1 increases GJIC by increasing the P2 isoform expression of Cx43. PQ1 led to an increase in the P2 Cx43 isoform which correlates to Cx43 in gap junction plaques.

The mechanism of how PQ1 causes the change in Cx43 isoforms and the increase in GJIC has yet to be elucidated. As PQ1 was created using the carboxyl-terminus domain structure of Cx43 one possibility was its direct binding to the domain, causing a shift in the isoform of Cx43. The second is by indirect means. PQ1 may cause the change in isoform expression by activating the kinases that regulate gap junctions by phosphorylating the carboxyl-terminus domain of Cx43. Of the 2 possible mechanisms, the kinase mechanism was the focus of this study.

Kinase inhibitors specific for PKC and phospholipid/calcium dependent kinase caused a reversal of PQ1's effects on GJIC and on GJ plaque formation. Western blot analysis was then

used to test for the expression of active kinases. PKC α, shown to increase and decrease GJIC, was analyzed for translocation. No change in PKC α localization was found. p44/42 MAPK, shown to be a GJ inhibitor, was analyzed and seen to increase in expression. This is the first study in SW480 cells to suggest that p44/42 MAPK may lead to an increase in GJIC. Active Akt, a GJ stabilizer, had an increase in expression however, when testing to see if Akt was translocated to the plasma membrane to phosphorylate GJs, no change was seen.

These results have shown that PQ1 regulates GJIC through kinase activity. Due to the inhibitors specificity towards PKC and the fact that PKC α did not translocated to the membrane, it suggests, that while PKC plays a role in the increase in GJIC caused by PQ1, another PKC other than PKC α is most likely used by PQ1 to increase GJIC. Active Akt was seen to increase but it was not seen to increase in the membrane portion of the lysate. This could be due to it not phosphorylating the Cx43, the binding may not have been strong enough for it to have stayed with the membrane portion during the translocation, or it may have no longer been bound to Cx43 on the membrane. The literature has shown that phosphorylation can take as little as 15 minutes while GJ formation and opening may not happen for hours[68].

Future studies will test the kinases (PKC's, pAkt and MAPKs) at different time points (15 minutes, 30 minutes and 45 minutes). Phosphorylation sites known to increase GJIC like S325/S328/S330 will be mutated and GJIC will be tested after treatment with PQ1 to find the sites which PQ1's kinases phosphorylate in order to cause the increase in GJIC.

PQ1's effect on Apoptosis

The second part of this study focused on the possibility of PQ1 activating the apoptosis pathway at higher concentrations. Proliferation and viability at increasing PQ1 concentrations decreased compared to DMSO. The flow cytometry data suggested apoptosis at 4 hours with

5000 nM of PQ1. Proliferation and viability using inhibitors for caspase 3, caspase 8 and caspase 9 concluded that PQ1 was activating the apoptotic pathway.

The study analyzed several proteins known to cleave caspase 8 and caspase 9 which leads to the activation of caspase 3 and then to cell death. The link between GJ regulation and apoptosis by pAkt was analyzed. However pAkt expression was unchanged.

To further analyze apoptosis, the Bcl-2/ Bax ratio was examined. The findings showed that Bcl-2 expression increases at 1000 nM PQ1, Bcl-2 and Bax expression increase at 5000 nM, and Bax expression increases at 10000 nM PQ1. However, in order for Bax to release cytochrome c it has to oligomerize with other Bax proteins. Since the Bax antibody used was not specific for oligomerized Bax, we cannot say that the increased Bax levels caused the release of cytochrome c. PQ1 activates may activate proteins upstream of Bcl-2 and Bax that also lead to the activation of other apoptosis proteins. Other proteins could be Bak, Bad and p53, all pro-apoptotic proteins[83,84,93]. Bak is an integral membrane protein on the outer membrane of the mitochondria. When activated, it leads to the release of cytochrome c[94].

Besides Bax and Bcl-2, p38 MAPK and survivin were also analyzed. p38 MAPK activates both the caspase 9 and the caspase 8 pathways. Survivin is thought to inhibit caspase 9 in some literature and, in other literature, it inhibits caspase 3[86,87]. Neither protein was found to change in the presence of PQ1.

Future studies into the mechanism of PQ1 and the apoptosis pathway will be performed. Analysis of other apoptosis proteins like p53, p21, Bak, Bad and cytochrome c are future targets for elucidation of the apoptosis pathway. This study has shown PQ1 activating apoptosis, future studies will elucidate how this occurs.

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