The Effect of Naphthoquinones on Gap Junctional Intercellular Communication

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

in
Pharmaceutical Sciences

Faculty of Pharmacy and Pharmaceutical Sciences
University of Alberta

ABSTRACT

Gap junctions are groups of channels that connect two neighboring cells, allowing for the passage of small molecules, such as nutrients and signalling factors, between cytosols. Gap junctional channels consist of building blocks called connexins. Cancer cells exhibit a low basal level of gap junctional intercellular communication (GJIC), and experimental animals that lack certain connexins were shown to develop cancer at faster rates than their healthy counterparts. Here, we investigate the effect of synthetic and natural naphthoquiones on connexin43 and on GJIC in order to identify potential modes of interference of quinoid compounds with cellular pathways that control GJIC. WB-F344 rat liver epithelial cells were exposed to synthetic and natural naphthoquinones. Phosphorylation of connexin-43, the epidermal growth factor receptor (EGFR) and extracellular signal-regulated kinases (ERK-1, -2), were analysed by Western blotting. Naphthoquinone toxicity profiles were established using neutral red uptake for assessment of cell viability. Assessment of GJIC was performed by microinjection of a channel-permeant fluorescent dye, Lucifer yellow, into single cells and microscopic analysis of its spreading to cells adjacent to injected cells. Of the naphthoquinones tested, menadione (2-methyl-1,4-naphthoquinone, MQ), 2-methoxy-1,4-naphthoquinone (MNQ) and 2,3-dimethoxy-1,4-naphthoquinone (DMNQ) caused a significant phosphorylation of connexin-43 at different concentrations. In line with this, GJIC was significantly downregulated after 20 min of exposure to MQ, MNQ or DMNQ. In conclusion, Redox-cycling naphthoquinones (with exclusive redox-cyclers, such as DMNQ, and alkylating/redox-cycling naphthoquinones, such as MQ and MNQ) stimulate connexin phosphorylation and a loss of GJIC.

Preface

All the work presented in this thesis was conducted by Omar Alomair except for the experiment titled "Gap junctional communication analysis using dye transfer assay" on page 49 and 50 which was performed by Xiaoqing Hou. Omar Alomair prepared chemicals for that experiment and conducted the statistical analysis.

Dedication

This work is dedicated to my most beloved

person in the whole world

My mother

Aljawhara abdulaziz Almohana

Thank you for your unwavering support and

love throughout my journey

AKNOWLEDGMENTS

I would like to extend my appreciation to my supervisor Dr. Lars Oliver Klotz for his support and guidance, and for giving me the opportunity to work under his supervision.

I would like also to express my gratitude toward my supervisory committee members Dr. Sharon Marsh and Dr.Sujata Persad for their valuable suggestions and advices.

I want also to thank my previous lab colleagues for their help and support.

I would like to acknowledge the Faculty of Pharmacy and Pharmaceutical Sciences at the University of Alberta for providing excellent learning environment.

Many thanks are also extended to Saudi Arabian Cultural Bureau in Canada and the Government of Saudi Arabia for providing me with the financial support.

Finally, my sincere indebtedness is due to Dr. Arno Siraki for his guidance and assistance.

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LIST OF ABBREVIATIONS

AKT	Protein kinase B
APS	Ammonium peroxodisulfate
ATP	Adenosine triphosphate
BE	Bystander effect
CHF	Congestive heart failure
CK1	Casein kinase 1
Cx43	Connexin 43
DMNQ	2,3-Dimethoxy-1,4-naphthoquinone
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinases
GJIC	Gap junctional intercellular communication
MAPK	Mitogen-activated protein kinase
MeONQ	2-methoxy-1,4-naphthoquinone
MQ	Menadione
PBS	Phosphate-buffered saline
PKA	Protein kinase A
PKC	Protein kinase C
RNA	Ribonucleic acid
ROS	Reactive oxygen species

SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBS	Tris-buffered saline
TBST	Tris-buffered saline plus tween
TEMED	N,N,N',N'-Tetramethylethylenediamine
TNFa	Tumor necrosis factor alpha
ZO-1	Zonula occludens-1

CHAPTER 1

INTRODUCTION

1.1 Background: Gap junctions and cell-cell communication

Gap junctions are collections of channels that can be found between cells in the vicinity of each other, allowing for the passage of nutrients and signaling molecules from one cell to another, as long as these substances weigh less than 1 kDa (Goodenough & Paul, 2009; Laird, 2006). These channels are assembled through the locking of two semi-channels, called connexons (Goodenough & Paul, 2009). Each connexon consist of six connexin proteins that are located in the cell membrane in transmembranal formation and are present virtually in all cells and tissues (Goodenough & Paul, 2009) (Figure 1.1). The connexin family of proteins is represented in the human genome with 21 variants, which explains the enormous diversity between connected channels and the attributed biological functions, which becomes more apparent with the different diseases associated with their abnormalities (Laird, 2006).

The 21 different connexins that the human genome can encode are differentiated with a nomenclature that distinguishes each one with a number that represents the molecular mass (Laird, 2006). For example, connexin 43 (Cx43) is one of the most commonly expressed members of this family and a one that can be found all over the human body, but more so in the liver and the heart, while Cx26 on the other hand, is expressed mainly in skin tissue (Mese, Richard, & White, 2007). Each gap junction can be established with building units that are made of a single connexin type (forming a homomeric structure) or a collection of different varieties (forming a heteromeric composition), this heterogeneity in the structure of gap junctions contributes to a wide variety of physiological functions, manifested in the ability of certain channels to pass secondary messengers or ions, while other gap junctions may not permit these molecules to traverse between connected cells (Mese et al., 2007).

Countless physiological processes have a strong association with normally functioning connexins and the homeostasis of gap junctional intercellular communication (GJIC) (Giepmans, 2004). GJIC has a pivotal role in allowing action potential to proliferate in heart tissue, facilitating child delivery, propagating electrical signals in brain neurons, and last but not least regulating the influx of electrolytes and the consequent contraction in lenses (Beyer & Berthoud, 2014; Doring et al., 2006; Klotz, 2012; Sohl et al., 2005; van der Velden & Jongsma, 2002). Moreover, mice lacking Cx43 have abnormal heart development exhibited through the obstruction of normal blood movement within ventricles, contrastingly, excessive Cx43 production through enhancing mice gene expression resulted in embryonic neuronal and heart defects (Willecke et al., 2002). Additionally, Cx32 knockout mice show significant difficulty in extracting glucose from glycogen to respond to sympathetic nervous system activation (Willecke et al., 2002). Likewise, mutations in Cx45 and Cx50 have a strong association with an early onset of cataract formation (Beyer & Berthoud, 2014).

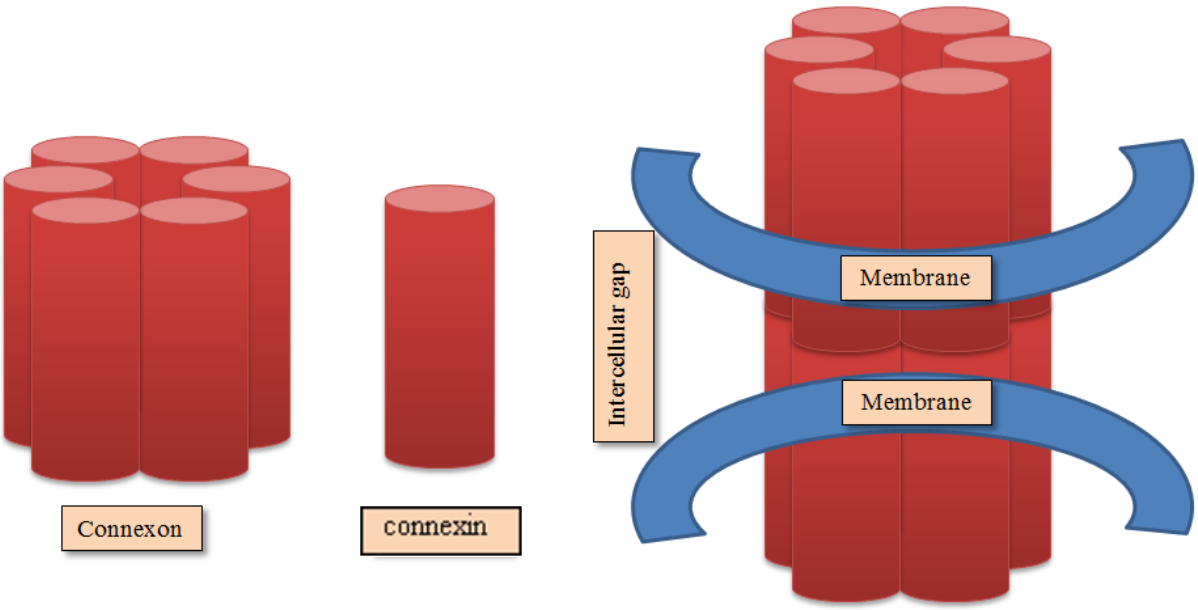

Figure 1.1 Structure of a gap junctional channel. An illustration of the gap junction which consists of two hexameric hemi-channels connecting to each other to create a single intercellular conduit. Each hemi-channel is called connexon, and a connexon is formed through linking of a family of transmembrane proteins termed connexins. The channel can be homomeric in nature when it consist of similar connexins, or heteromeric if the involved connexins differ (Goodenough & Paul, 2009).

Several studies have demonstrated the involvement of connexin in cancer cell development, which is demonstrated through a substantial decrease in connexin expression in different Tumors (Kar et al., 2012). Interestingly, inserting the gene encoding for the suppressed connexin 43 into malignant cells led a halt of cancer growth (Charles et al., 1992). Maintaining high number of functioning gap junctions guarantee the movement of apoptotic triggering molecules between clusters of malignant cells, which in turn assists the body in the fight against cancer. In similar

fashion, drugs targeting cancer exploit the gap junction task of passing substances between a group of connected cells in what is known as 'bystander effect' (BE) (Kandouz & Batist, 2010). By keeping the channel open and improving the cell to cell communication, anticancer drugs can spread within tumor tissue unchallenged. While the high basal level of operating connexins has been shown to be beneficial in the fight against cancer at an early onset of tumor development, later stages of carcinogenesis that is accompanied with aggressive metastasis show abnormally elevated level of connexins (Kamibayashi et al., 1995).

1.2 Connexin 43

1.2.1 Structure and lifecycle

Connexin 43 (Cx43) is a 381 amino acid protein considered to be the most abundantly expressed connexin in the mammalian body, and accordingly the most investigated and researched member of the connexin family (Izzo et al., 2006; Klotz, 2012). Cx43 consists of 4 domains crossing the cellular membrane; 2 protein loops extend to the extracellular space, while only one loop located inside the cell, all of which are stabilized with disulfide bonds (Sohl & Willecke, 2004). At the same time, N and C termini protrude into the cytoplasmic space (Kumar & Gilula, 1996). The general structure of Cx43 was found to be similar to the protein formation and arrangement of both Cx32 and Cx26 with no countering findings regarding the structures of the reset of the connexin protein family (Goodenough et al., 1988; Hertzberg et al., 1988; Laird & Revel, 1990) (Figure 1.2).

Cx43, and the rest of the subtypes of the group, exhibit a unique characteristic in the form of a distinctively short half-life, reported to be within 1 to 2 hours (Laird et al., 1991). After Cx43

RNA translation in the ribosome, it gets inserted into the endoplasmic reticulum (ER) where proper folding takes place, from there Cx43 is transported through the cell tubular network to the trans-Golgi compartment to initiate Cx43 oligomerization (Thomas et al., 2005). Microtubules begin the process of appropriately positioning Cx43 into the cell membrane with the help of diverse connexin-binding proteins (Laird, 2006; Thomas et al., 2005).

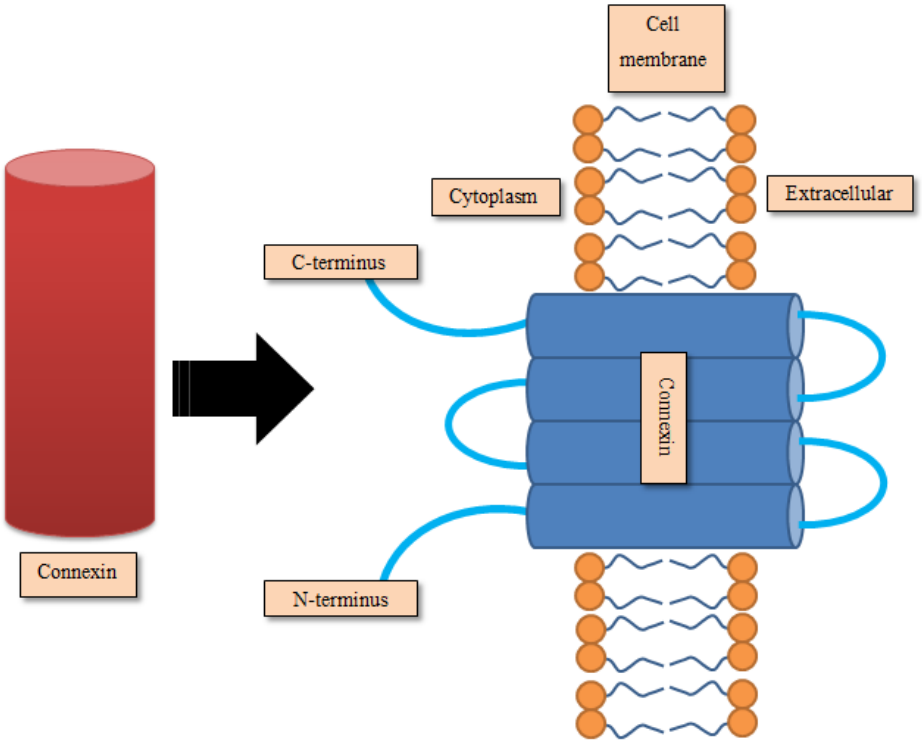

Figure 1.2 Molecular structure of connexin 43. Diagram of Cx43 protein in which each cylinder equates to a protein domain that is located in the cell membrane. Three loops are present (two in the extracellular space and one in the cytoplasmic counterpart), with C and N termini located inside the cell. Adapted from (Sohl & Willecke, 2004).

1.2.2 Cx43 distribution and functions

The principle connexin in cardiomyocytes is Cx43, and it has the burden of maintaining sequenced electrical conduction through the adaptive response to Ca2+ levels, where low level of Ca2+ has been established to induce the opening of gap junctions and vice versa (De Vuyst et al., 2011; Ponsaerts et al., 2012). Furthermore, any decrease in Cx43 level that is coexisting with abnormal distribution has been observed in patients diagnosed with cardiac diseases, particularly congestive heart failure (CHF), ischemia, and arrhythmia (Severs et al., 2008).

In the liver, Cx43 possesses a considerable significance as the sole connecting connexin in hepatic progenitor cells or "oval cells" in the early stage of liver maturation (Paku et al., 2004). These early types of hepatocytes require extensive energy expenditure which explains the expression of Cx43 in them, since some findings have attributed a comparatively higher ability of Cx43 to pass more ATP (adenosine triphosphate) than other connexin subtypes (Naves et al., 2001).

Superficially, Cx43 is down regulated in the area adjacent to an injured skin tissue and that, in effect, hinders the passage of immune response and inflammatory elements such as neutrophils, macrophages, and tumor necrosis factor alpha (TNFa) throughout the following 2 days of contusions (Grek et al., 2014). Contrariwise, the blood vessels near the site of lesions exhibit a high level Cx43 formation permitting an a easier building of connective tissue over the wound (Coutinho et al., 2003).

The involvement of Cx43 of the healing process gives a hint of its significance in cellular homeostasis and the abnormalities that could spin from any disruption of that balance, and cancer is one of these abnormalities. This involvement of Cx43 in tumor development is associated either directly with the role of Cx43 in GJIC (GJIC-dependent) or the possibility of Cx43 interaction with tumor modulating molecules (GJIC-dependent) (Mroue et al., 2011). The GJIC-dependent class of action is related to gap junction ability to pass apoptotic substances to affected tissue or the previously mentioned "bystander effect", where a treatment targets the gap junction to alter the passage of therapeutic agents against connected malignant cells. An example of this is the ability of ganciclovir (guanosine analogue) to freely move between rat glioma

tumor cells when they are transfected with Cx43 gene, leading to significant decrease in tumor size (Sanson et al., 2002). On the other hand, GJIC-independent mechanism of fighting cancer can be attained through the capacity of Cx43 protein to suppress the volume of n-cadherin, which is a transmembrane protein involved in the metastasis of breast cancer (Li et al., 2008). Having explored some precedents where the level of cellular interactions play integral part in maintaining a healthy cellular network, it is no wonder that understanding factors and mechanism involved in modulating gap junction is crucial to further enhance our understanding of the relationship between GJIC and different medical conditions.

1.3 Cx43 posttranslational modifications

1.3.1 Glycosylation

Glycosylation is a protein change that takes place after protein synthesis where a glycan polysaccharide is enzymatically added to the protein (Paulson & Colley, 1989). Glycosyl transferases facilitate this binding and it can be either an N-linked glycosylation when they are connected to a nitrogen terminal of asparagine, or an O-linked glycosylation if the linkage is with a serine or threonine hydroxyl moiety (Freeze & Sharma, 2010). The main purposes of glycosylation, which takes place usually in Golgi apparatus or endoplasmic reticulum (ER), are enhancing protein folding stability and controlling trafficking inside the cell (Roth et al., 2010). As opposed to other forms of modification, particularly phosphorylation and ubiquitination, proteins in the connexin family do not undergo glycosylation (Martin & Evans, 2004). However, it is worth noting that some studies have found that inhibiting glycosylation led to a collateral increase in Cx43 opening via elevating cAMP levels (Wang & Mehta, 1995).

1.3.2 Ubiquitination

Cytoplasmic ubiquitination system controls the degredation of proteins by the adding ubiquitin modules (regulatory proteins) to an N-terminal of protein to initiate the proteasome complex to breakdown the tagged protein (Lichtenstein et al.,2011). Cx43 has been shown in numerous finding to be affected by the ubiquitination regulatory system. For instance, Cx43 presence in the plasma membrane, and its distribution, are increased after treatment with N-acetyl-l-leucyl-l-leucinyl-norleucinal, a chemical that interferes with the proteasome system (Laing & Beyer, 1995). Moreover, employing the carcinogen 12-O-tetradecanoylphorbol 13-acetate (TPA) mediated a surge in the amount of ubiquitinated Cx34 and the consequent internalization and degradation, both of which ceased after the addition of proteasomal inhibitors (Leithe & Rivedal, 2004).

In spite of the previously explained results pointing to the ubiquitination as a decomposition path of Cx43, other findings hint to an opposing conclusion. In a recent study, it was established that CIP75-bound Cx43 (CIP75 is a ubiquitin-like protein that attaches to a substrate undergoing ubiquitination) does not get ubiquitinated following a co-immunoprecipitation (Su et al., 2010).

1.3.3 Phosphorylation

Phosphorylation is the most important form of posttranslational modifications, governing the normal physiological function of gap junctions and the hemostasis of intercellular communication. Phosphorylation of Cx43 occurs by protein kinases that are capable of adding phosphate groups to the amino acids tyrosine (Y), serine (S), and threonine (T) of the Cx43 protein moiety (Severs, 2007). Phosphorylation can lead to enhancement or retardation of expected cellular functions resulting from phosphorylation induced alteration of protein charge, water insolubility and modifying protein structure which is clearly displayed in the case of Cx43 by the opening or the closure of the gap junction (Davis, 2011).

Western blotting analysis of the characteristic of Cx43 phosphorylation by sodium dodecyl sulfate/ Polyacrylamide gel electrophoresis (SDS/PAGE) shows that Cx43 is phosphorylated simultaneously at different sites (Laird et al., 1991). Electrophoretic separation of Cx43 is visualized by a number of isoforms with different migrating speeds across the separating gel; these include a rapidly moving non-phosphorylated form denoted as P0, trailing bands named P1 and P2 (the numbers after P refer to the number of phosphorylated sites) and finally the hyper phosphorylated form Pn (n= more than two phosphorylated sites) which is the slowest migrating isoform (Figure 1.3) (Berthoud, Ledbetter, Hertzberg, & Saez, 1992; Crow, Beyer, Paul, Kobe, & Lau, 1990).

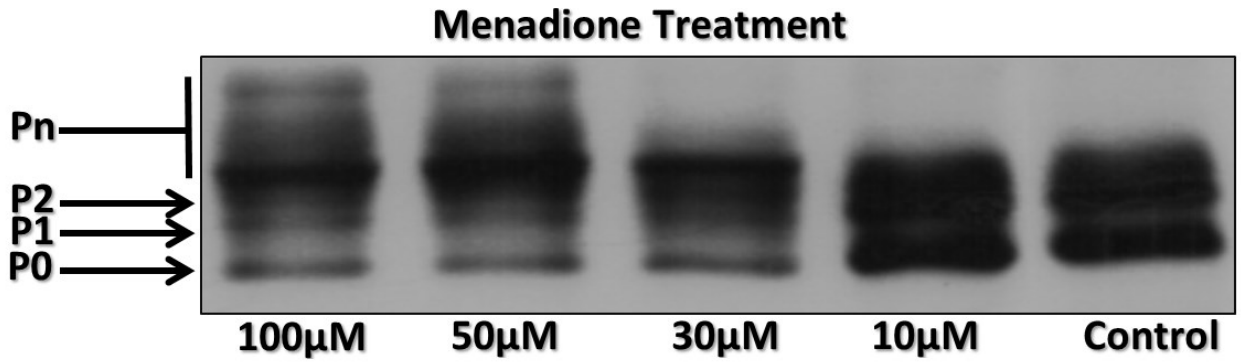

Figure 1.3 Cx43 migrations before and after the treatment with phosphorylation triggering chemical. Above is a blot of total Cx43 proteins following menadione treatment which is known to cause Cx43 phosphorylation and a reduction in GJIC. P0 is the unphosphorylated isoform, P1&2 are the singly and doubly phosphorylated isoforms, respectively. Pn is the multi-phosphorylated isoforms.

Cx43 phosphorylation has taken the front seat in the interest of many researchers due to substantial findings pointing to how essential it is for the normal life cycle of Cx43. Changing Cx43 function through phosphorylation affects Cx43 movement in the cytosol, positioning in the plasma membrane, internalization for degradation, and the opening and closing of gap junction (Laird, 2005; Martin & Evans, 2004). This posttranslational modification has been found to be performed by different kinases including: protein kinase C (PKC), protein kinase A (PKA), casein kinase 1 (CK1), calmodulin protein kinase, proto-oncogene tyrosine-protein kinase Src , protein kinase B (AKT) and mitogen-activated protein kinase (MAPK) the main interest of this thesis (Solan & Lampe, 2009).

The main target of kinases in Cx43 is the carboxyl end situated in the cytoplasm, but the N-terminus of Cx43 was shown to be phosphorylatable (Chen et al.,). Cx43 loops positioned inside the plasma membrane lack serine residues and are therefore not a target of serine phosphorylation (Solan & Lampe, 2005). In contrast to other types of connexins, Cx43 has the highest number of sites (serine, threonine, and tyrosine) possibly subjected to kinase action, almost half of which reside in the intracellular C-terminus (Martin & Evans, 2004). Furthermore, techniques utilized in quantifying and visualizing Cx43 phosphorylation changes, such as western blotting and immunohistochemistry, have established that Cx43 is phosphorylated at more than one site, which complicates the understanding of connexin phosphorylation, but confirms the significance of multisite phosphorylation in regulating a Cx43 task inside the cell (Cooper & Lampe, 2002).

The aggregation of the Cx43 hemichannel ahead of the insertion into the gap junction is controlled through the stimulation and the inhibition of the carboxylic tail, for that reason mutant Cx43 lacking phosphorylation sites showes diminished Cx43 gathering in the cell membrane, and accordingly a reduction in gap junction formation (Johnson et al., 2012). Enzymatically, PKA boosts the phosphorylation of Cx43 at the S364 site promoting the building and interlocking of gap junctions, while CK1 phosphorylation of different serine sites located in the COOH-terminus enhanced the channel stability (Cooper & Lampe, 2002; TenBroek et al.,2001). Conversely, c-SRC hinders gap junction assembly as a consequence of Cx43 phosphorylation which is the binding site of zonula occludens-1 (ZO-1), a chaperone protein involved in the trafficking of Cx43 to cell membrane (Toyofuku et al., 2001).

The carboxyl terminal of Cx43 inside the cell manages the state of the permeability of the gap junction, seeing that its enzymatic phosphorylation or dephosphorylating can lead to the closure or the opening of the gap hemichannel (Johnstone et al., 2012). This can explain how the truncation of position 239 located in the C-terminus did not prevent the formation of normal channel but the inability to open it (De Vuyst et al., 2007). Similarly, the closure of gap junctions formed by Cx43 was mediated through the phosphorylation of S368 by Protein kinase C and S279/S282 by MAPK (Chandrasekhar & Bera, 2012). Overall, GJIC is promoted with phosphorylation if the kinases involved are CK1 and PKA, at variance with v-SRC, PKC and MAPK which phosphorylate Cx43 causing marked decrease in GJIC (Pahujaa et al., 2007; Solanet al., 2005) (Figure 1.4).

Cx43 GJIC reduction is noticeable with a surge in PKC level preceding the phosphorylation of S368 in Cx43, evident by the resulting drop in intercellular electrical conductance (Ek-Vitorin et al., 2006). In a similar fashion, PKC phosphorylation of S262 results in a marked increase in cancer proliferation and tumor size, implying that abnormal GJIC levels are associated with carcinogenesis (Doble et al., 2004). Growth factors binding to epidermal growth factor receptors (EGFR) set off a chain of complicated events that include the activation of MAPK/ ERK (extracellular signal-regulated kinases) stream, which are known to trigger the phosphorylation of Cx43 at S255/S279/S282 and thereupon a significant blockage of gap junction (Rivedal & Opsahl, 2001).

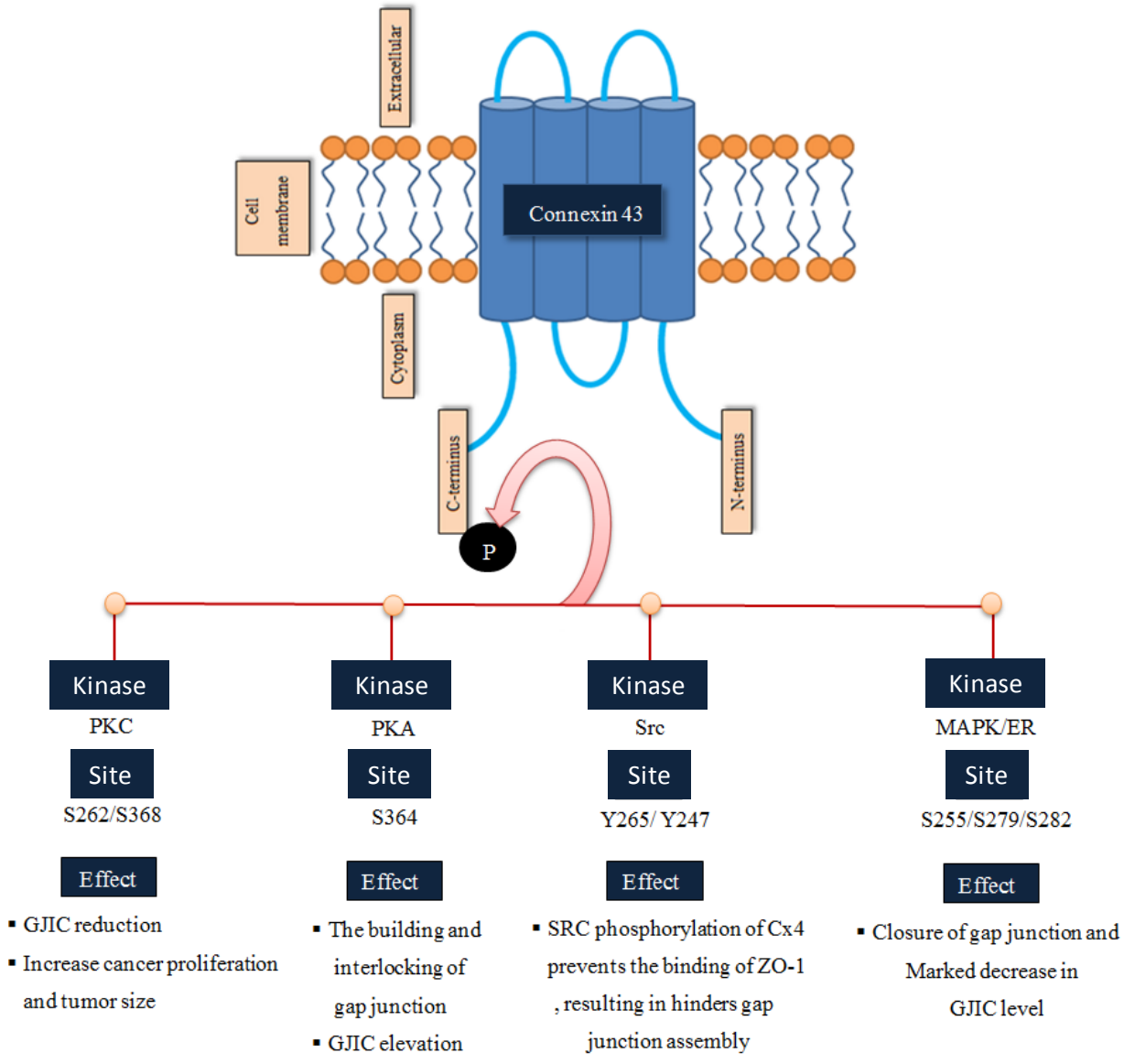

Figure 1.4 Main kinases targeting Cx43. Protein kinase C (PKC) phosphorylates Cx43 at S368 causing an increase in cancer proliferation and tumor size (Doble et al., 2004; Ek-Vitorin et al., 2006). Protein kinase A (PKA) target S364 of the C-terminus of Cx43, promoting the building and interlocking of gap junctions into the cell membrane (TenBroek et al., 2001). Sarcoma Proto-oncogene tyrosine-protein kinase (Src) and Specifically c-Src, hampers the normal

positioning of Cx43 inside the membrane as consequence of the phosphorylation of Cx43 at Y265, which is the binding site of zonula occludens-1 (ZO-1), a chaperone protein involved in the trafficking of Cx43 to cell membrane (Toyofuku et al., 2001). Mitogen-activated protein kinases is triggered primary through the activation of growth factor receptors, and result in the phosphorylation of Cx43 at S255/S279/S282 bringing about a discernible decrease in gap junction intercellular communication (GJIC) (Rivedal & Opsahl, 2001). In general, PKA leads to an elevation of GJIC in opposition to the other 3 kinases which reduce the basal level of cell-to-cell interaction (Pahujaa et al., 2007; Solan & Lampe, 2005).

The MAPK/ERK pathway is of special interest since it is very susceptible to any redox imbalance inside the cell. By its nature, oxidative stress - which arises from disparity between the cell antioxidant defense system and the overproduction of reactive oxygen species – modulates GJIC levels. Oxidative stress interferes with phosphatases that negatively regulate the normal behavior of EGFR and its downstream MAPK/ERK family of kinases that interact with Cx43 (Berg et al., 2004; Kamakura et al., 1999). The aspects of Cx43 phosphorylation and its effects on normal CJIC function can be studies through the usage of specific chemicals that can simulate similar outcome, such as naphthoquinones, the main focus of this project.

1.4 Natural and synthetic naphthoquinones: overview

The main interest of this study is to correlate the physiochemical effect of several synthetic and natural naphthoquinones with awide range of toxicity profiles and medicinal applications with their ability to attenuate the typical level of GJIC. In this part of the first chapter we are briefly going to go over the 9 naphthoquinones employed in this project: menadione, 1,2-naphthoquinone, 1,4-naphthoquinone, 2-methoxy-1,4-naphthoquinone (MeONQ), 2,3-Dimethoxy-1,4-naphthoquinone (DMNQ), plumbagin, juglone, lawsone and lapachol. Structurally, these quinones can function as coenzymes, pigments, antibiotic agents and vitamin K (Castro et al., 2008) (Figure 1.5).

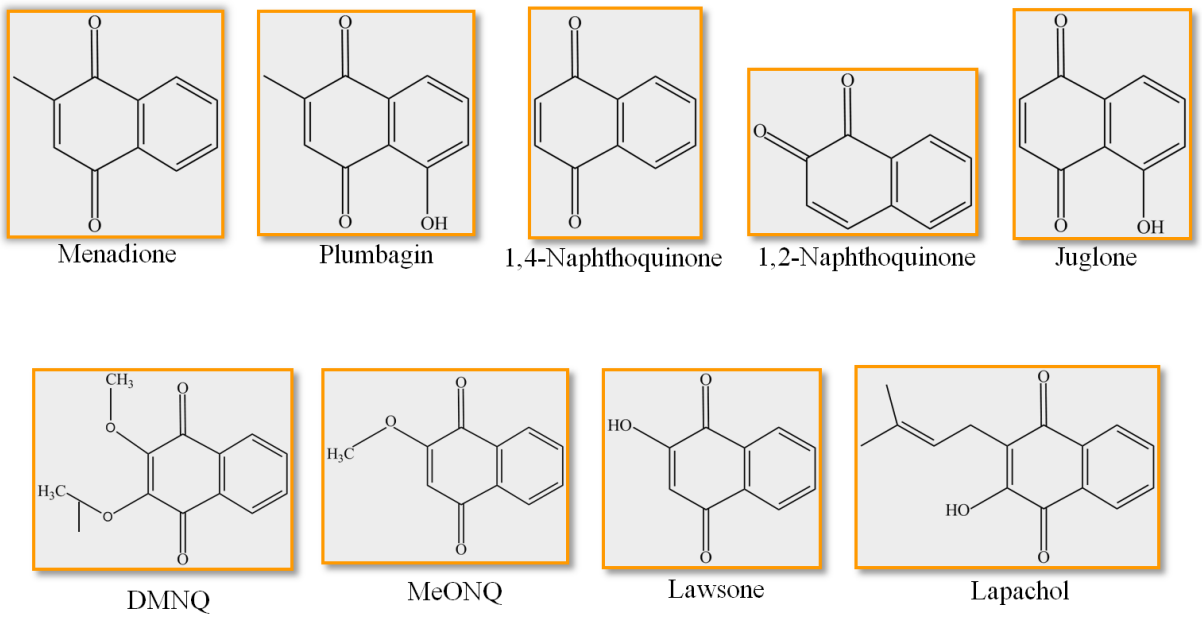

Figure 1.5 Structures of the utilized synthetic and natural naphthoquinones

Menadione (2-methyl-1,4-naphthoquinone) or vitamin K_3, a synthetic naphthoquinone, has been utilized in the clinical setting for its ability to exert similar physiological effects as vitamin K. After ingestion, menadione is converted enzymatically with the help of colon bacteria to vitamin K_2, which is the form of vitamin K stored in fat tissue, and therefore it was and in some cases still is used as a vitamin K replacement (Thijssen et al., 2006). The reason for the current limited use of menadione as a cheap alternative to Vitamin K is the number of scientific reports giving evidence to the toxicity of menadione. This toxicity usually surface as a result of menadione redox recycling potential that is accomplished via NAD(P)H:quinone oxidoreductase which offsets the redox state balance with the release of a high number of superoxide and hydrogen peroxide molecules (Baillie & Rettie, 2011; Criddle et al., 2006). Additionally, menadione has the distinct properties that allow it to directly target nucleophilic structures within the cell, mainly protein, leading to alkylation and thus, rendering them physiologically dysfunctional (Sun et al., 1990). The previously stated toxicity characteristic of menadione made it a prime tool for many researchers interested in understating how chemicals produce cellular toxicity through numerous mechanisms resembling menadione induced oxidative stress and interaction with biological matters.

1,2-naphthoquinone is an atmospheric chemical but also can be created in the body with the help of CYP1A2 isoform of the hepatic cytochrome P450 system that biotransforms it from naphthalene (Stohs et al., 2002). The strong electrophilic nature of 1,2-naphthoquinone facilitates the targeting of biological components of the cell with ease, for example, glutathione S-transferase (a major detoxifying enzyme that enhances the conjugation of reduced glutathione to toxicant) was shown to be disrupted with 1,2-naphthoquinone in the lens leading to cataract

formation (Kroner et al., 1991). In the same way, 1,2-naphthoquinone covalently binds to protein-tyrosine phosphatases (PTPs), specifically PTP1B, in guinea pig trachea inducing uncontrolled activation of EGFR (which is negatively controlled by PTP) and subsequently a constriction of its airway (Iwamoto et al., 2007).

1,4- naphthoquinone is a synthetic naphthoquinone and has mainly an industrial importance in the form of a precursor in the synthesis of anthraquinone, a major starting material for large scale dying manufacturing (Bovey & Kolthoff, 1948). Additionally, 1,4-naphthoquinone has been utilized as a polymerization controller, firming agent for polyesters, corrosion protector, and as a menadione derivative (Bovey & Kolthoff, 1948; Lin & Sartorelli, 1976). Biologically, It has been described as a strong antibacterial agent with potent activity against *K. pneumoniae* and *E. coli* that surpass kanamycin (aminoglycoside bacteriocidal antibiotic) effects on these Gram-negative strains (Tandon et al., 2005). Additionally, a recent study on amyloidosis (the buildup of amyloid proteins inside organs, which is associated with metabolic diseases like common dementias and type II diabetes) demonstrated the potent activity of 1,4-naphthoquinone as an amyloid inhibitor (Gong et al., 2014).

The naturally occurring 2-methoxy-1,4-naphthoquinone (MeONQ) can be extracted from the plants *Impatiens balsaminaand* and *Impatiens glandulifera*, known colloquially as garden balsam and Policeman's Helmet, respectively (Lobstein et al., 2001). It has a wide range of medical uses that include: antipruritic activity which gives it a chance of being a promising pruritus treatment, anti-inflammatory, and antiallergic action (Lien et al., 1996; Oku, Kato, & Ishiguro, 2002). Moreover, hepatocellular carcinoma was found to shrink following a treatment with MeONQ

obtained from *Impatiens balsamina* leaves (Ding, Jiang, Chen, Lv, & Zhu, 2008). Likewise, a bactericidal activity with strong affinity to Gram-positive bacteria has been investigated for MeONQ with favorable outcomes (Yang et al., 2001). In human, oral ointment containing MeONQ reduce mouth cavity lesions and cuts appearing in human immunodeficiency virus (HIV) positive individuals (Blignaut et al., 2006).

In contrast to MeONQ, 2,3-dimethoxy-1,4-naphthoquinone (DMNQ) is not found in nature by synthesized synthetically and has used extensively in research when redox recycling potential is of big consequence. The redox recycling ability of DMNQ is more useful due to the fact that DMNQ is a pure redox recycler and does not reach out to nucleophiles such as thiol moiety of many enzymes, in contrast to menadione which is both a redox cycler and alkylator (Seung, Lee, Lee, Park, & Chung, 1998). Inherently, the oxidative stress generated by DMNQ introduction to an *in vitro* system caused a strong appearance of chromosomal abnormalities, micronuclei, apoptosis, DNA single strand breaks, and tissue damage (Bresgen et al., 2003; Morgan, Hartley, & Cohen, 1992). Lastly, The release of reactive oxygen species by DMNQ incubation has the potential to induce a signaling cascade event that involve the activation of MAPK/ERK kinases (MEK) 1/2 as an aftermath of EGFR stimulation (Abdelmohsen, Gerber, von Montfort, Sies, & Klotz, 2003).

Plumbagin derives its name from the plant plumbago in which plumbagin was isolated for the first time but can also be traced in *juglans nigra* (eastern black walnut) (van der Vijver, 1972). Historically it has been used in South America as a herbal medicine against numerous infectious diseases, mainly against bacteria and yeast contiguous cases (de Paiva, Figueiredo, Aragao, &

Kaplan, 2003). The minimum inhibitory concentration of plumbagin against carcinogenic bacteria, notably *Streptococcus mutans* (Gram-positive bacteria found normally in oral cavities), was the lowest out of the other tested naphthoquinones (Didry, Dubreuil, & Pinkas, 1994). Other biological and physiological actions reported for plumbagin include antimalarial, anti-inflammatory, and increase in heart contractility effects (Checker et al., 2010; Itoigawa, Takeya, & Furukawa, 1991; Likhitwitayawuid, Kaewamatawong, Ruangrungsi, & Krungkrai, 1998). Similar to other toxic naphthoquinones, recent findings illustrate how oxidative stress level following plumbagin treatment are significantly elevated and the thiol level is contrastingly reduced leading to marked cytotoxicity and growth halt of cancerous cells (Gaascht et al., 2014).

Black walnut also contain another naphthoquinone of interest in this project, juglone (5-hydroxy-1,4-naphthalenedione). Juglone can significantly stunt normal plants growth if they are planted or grown near black walnut tree (Michael & Rosie). This phenomenon, where one plant produces a chemical that does not affect it but is toxic to other plants, is called allelopathy (Zeng, 2014). Juglone is used industrially as an herbicide and a dying agent in food and cosmetics due to its strong dark yellowish color and its insolubility in water. Medicinally, antiviral and bactericidal effects has been reported in numerous studies such as the inhibitory action on enzymes secreted from the ulcer causing-bacteria *Helicobacter pylori* (Kong et al., 2008; Vardhini, 2014). Similar to plumbagin, juglone demonstrates a strong affinity to cause overload of ROS and thiolstatic effects and as such it has been studied for its potential cancer treatment (Seshadri, Rajaram, & Rajaram, 2011).

Henna drawing and hair dying is a tradition in Arabic and Indian cultures used in celebratory events like weddings. The henna leaves (Lawsonia inermis) contain a strong coloring agent in the form of the naphthoquinone lawsone (Bailey & Bailey, 1976). The coloring effect of lawsone on skin and hair is due to the interaction with keratin protein in a process known as the Michael reaction (Kraeling, Bronaugh, & Jung, 2007). Some findings have reported a respectable antibacterial and antifungal activity of lawsone and its derivatives (Rahmoun et al., 2012). Whereas *in vitro* lawsone is believed to be a non-redox-recycling napthoquinone due to not being a substrate for NADPH-cytochrome c reductase, other research points to the enhancement of the redox recycling activity of lawsone in the abundant presence of xanthine oxidase enzyme (Osman & van Noort, 2003).

The last naphthoquinone we are going to review is lapachol (2-Hydroxy-3-(3-methylbut-2-enyl)naphthalene-1,4-dione), an extract from different types of *Bignoniaceae* (Lira et al., 2008). Therapeutic applications of lapachol such as its viability as an antifungal, and bacterial agents were established in a number of findings (Duarte et al., 2000). Furthermore, lapachol shows a high anti-metastatic activity and it is promising potential for adjuvant therapy of certain cancers. (Balassiano, De Paulo, Henriques Silva, Cabral, & da Gloria da Costa Carvalho, 2005). To conclude, the interaction of the aforementioned naphthoquinones with kinases that are involved in modulating GJIC and Cx43 phosphorylation can lead to better understating of gap junction regulation and the applicability of their action in finding new therapeutic pathways, leading to effects on cancer development and other diseases that have strong associations with abnormal level of cellular communication.

1.5 Naphthoquinone-induced phosphorylation of Cx43

Taking menadione as a model in this project, it is used extensively in oxidative stress and cancer research since it can produce physiological damage through its ability to function as a redox-recycler and thus form reactive oxygen molecules, or via its capability to bind to cellular matters such as proteins in alkylating fashion, This disturbance in the homeostasis inside the cell by menadione leads to cellular death (Bolton, Trush, Penning, Dryhurst, & Monks, 2000). Menadione chemical action inside the cell was found to activate kinases that modulate a verity of cellular function, one of those kinases is extracellular signal-regulated kinase (ERK 1/2) which, we previously noted, is one of the kinases that targets and phosphorylates Cx43 (Osada, Saji, & Osada, 2001).

The mechanism of menadione activation of ERK1/2 has been reported to be related to the activation of epidermal growth factor receptor through the inactivation of phosphotyrosine phosphatases (PTPs) that negatively regulate EGFR induction, which is the starting point of signaling cascade that involves ERK1/2 (Klotz et al., 2002). In the case of menadione it was demonstrated that it is an alkylating property of menadione rather than the ROS production that was the cause of EGFR activation, and this finding was supported through the use of dicoumarol, an inhibitor of NAD(P)H:quinone oxidoreductase, preventing the formation of hydroquinone and semiquinone and the resultant superoxides (O_2^-) and hydrogen peroxide (H_2O_2) molecules leaving the alkylating pathway to take effect (Abdelmohsen, Stuhlmann, Daubrawa, & Klotz, 2005). See figure 1.6 for more details.

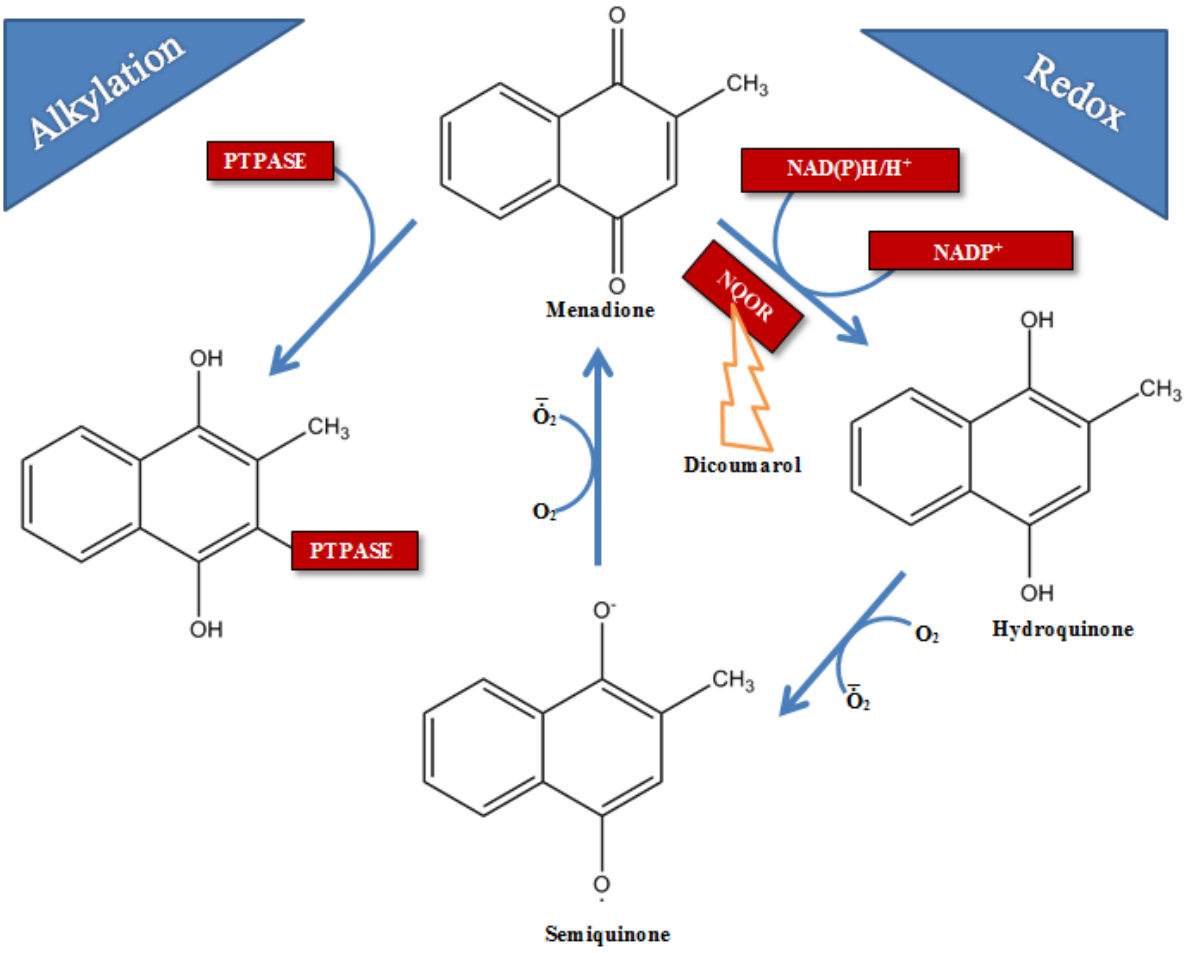

Figure 1.6 Menadione metabolism. Diagram illustrating the 2 main mechanisms of menadione action inside the cell and the role of dicoumarol in the inhibition of NQOR and PTPase alkylation by menadione. Adapted from (Klotz et al., 2002).

As the EGFR/ERK system is implicated in the phosphorylation of Cx43, using a variety of synthetic and natural naphthoquinones related to menadione, an already established trigger of the cascade, with various chemical properties and toxicity profiles, is important in enhancing our understanding of how gap junctions are modulated by signaling molecules, and the involvement of connexin proteins, especially Cx43, in the impairment of normal function of GJIC.

1.6 Rationale, Hypothesis and Objectives

1.6.1 Rationale

The connexin family of proteins, including the most abundant isoform connexin 43, has attracted substantial research interest in recent years. As a building block of gap junctions, connexins have the responsibility to maintain a normal level of gap junctional intercellular communication (GJIC). Normal GJIC levels are essential in multiple physiological processes such as the proliferation of action potential in heart tissue, propagation of electrical signals in brain neurons, healthy skin, and normal healing processes (Goodenough & Paul, 2009). Additionally, cancer development and connexin relationships have inspired many investigators to elucidate the connection between abnormalities in GJIC level and tumor progression. Connexin proteins are involved in carcinogenesis directly, with connexins' role in GJIC (GJIC-dependent) or indirectly, in the form of connexin binding with tumor modulating molecules (GJIC-dependent) (Mroue et al., 2011). The GJIC-dependent connexin interaction with cancer has been termed "the bystander effect" wherein an antitumor drug is allowed to move between connected cells after altering gap junction passage of such chemicals (Sanson et al., 2002).

As explained in the previous part of this chapter, Cx43 (the most common connexin and the main focus of this study) can be a target of phosphorylation by a number of kinases, principally the EGFR/ERK cascade. This cascade is initiated by way of reactive oxygen species or alkylators targeting PTPases that regulate the EGFR activation level. Menadione which is a member of the naphthoquinones family has already been extensively investigated in this regard and shown to be a strong alkylator of PTPases (Klotz et al., 2002). According to these results, we expect other synthetic and natural napthoquinones with wide range of differences in toxicity and chemical

properties, to have capability to alter GJIC level through interference of EGFR activation and its downstream kinases ERK1/2.

1.6.2 Hypothesis

Through the indirect activation of epidermal growth factor receptor (EGFR), synthetic and natural naphthoquinones initiate a signaling cascade downstream of EGFR involving extracellular signal-regulated kinases 1/2 (ERK 1/2), and end with the phosphorylation of connexin 43.

1.6.3 Objectives

- To establish toxicity profiles of nine naphthoquinones.
- To visualize and quantify the level of gap junction intercellular communication after the exposure of cells to different naphthoquinones.
- To examine Cx43 phosphorylation in cells treated with different naphthoquinones.
- To examine EGFR and ERK1/2 induction in cells following incubation at various concentrations and different time points.
- To elucidate the mechanism of Cx43 phosphorylation using EGFR and ERK1/2 inhibitors.

CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals

Dulbecco's modified Eagle's low glucose medium (DMEM, 1g/L glucose), phosphate-buffered saline (PBS), non-essential amino acids, Polysorbate 20 (tween 20), ammonium persulfate, tetramethylethylenediamine (TEMED), sodium dodecyl sulfate (SDS), and Lucifer yellow fluorescent dye were bought from Sigma Aldrich (St. Louis, MO, USA). Sigma Aldrich also delivered the naphthoquinones: menadione, plumbagin, juglone, lawsone, lapachol, 1,4-napthoquinone, 1,2-napthoquinone, and MeONQ. DMNQ was purchased from Enzo Life Sciences (Burlington, ON, Canada). Acrylamide and sodium dodecyl sulfate polyacrylamide gel electrophoresis material were obtained from Bio-Rad Laboratories (Hercules, CA, USA). Caledon Labs (Halton Hills, ON, Canada) was the source of glycerol and hydrochloric acid. Invitrogen (Carlsbed, CA, USA) provided penicillin/streptomycin mixture, tris hydroxymethyl aminomethane (TRIS) and trypsin. Purchases were made to Fisher Scientific (Toronto, ON, Canada) to acquire Glycine and sodium hydrochloride. Antibodies against Cx43 and β-actin were procured from Sigma Aldrich (St. Louis, MO, USA). The rest of the primary antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). The source of horseradish peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibodies was Dianova (Hamburg, Germany), while the anti-mouse one was obtained from GE Healthcare Life Sciences (Baie d'Urfe, QC, Canada). Supersignal west pico and femto chemiluminescent substrates used in western blotting detection were purchased from Thermo Scientific (Rockford, Il, USA). GE Healthcare Bio-Sciences Corp (Piscataway Township, NJ, USA) supplied fetal calf serum used in preparing growth media. The EGFR inhibitor Tyrphostin AG 1478 was procured from Enzo Life Sciences. Selleck Chemicals (Boston, Mass, USA) was the source of ERK 1/2 inhibitor U0126.

2.2 Methods

2.2.1 Cell culturing

WB-F344 rat liver epithelial cells were utilized in this project. WB-F344 cell line carries a unique stem-cell like characteristic such as its ability to perform similarly to hepatocyte progenitor cells (Coleman et al., 1997). The other important trait of WB-F344 cells is the overexpression of connexin 43, the most abundant connexin in mammalian bodies and the main focus of this study. WB-F344 cells were cultured to 60-70% confluency before exposure to different naphthoquinones. The 60-70% confluency proved to be the showed the most consistent result in term of the toxicity and signaling activation following various treatments. The cells were grown in low glucose Dulbecco's modified Eagle's medium (DMEM, 1g/L glucose) mix with 10% (v/v) fetal calf serum, 1% (v/v) non-essential amino acids and 1% (v/v) penicillin/streptomycin. The low glucose medium was chosen to prevent cellular differentiation. The cells were incubated at 37 °C with 5% (v/v) CO2. Samples were collected after exposure using trypsin 0.5% (5g/l) to dislodge the cells from the growth flask.

2.2.2 Neutral red uptake assay

To assess the toxicity of synthetic and natural naphthoquinones, neutral red uptake assay was used. Neutral red uptake assay measure cellular toxicity via the ability of cells to absorb the Neutral red dye into lysosomes (Repetto, del Peso, & Zurita, 2008). Where normal cells uptake the dye readily, unhealthy cells show otherwise. To perform the assay, 24-well plates were grown to 90-100% confluency and then treated with different naphthoquinones in serum free media at different concentrations in multiples of four. The treatment duration was one hour. After the treatment duration expires, media containing naphthoquinones were replaced with fresh

serum-free media carrying neutral red dye at 1:25 concentration and the put in an incubator at 37 °C for 2 hours. After the 2 hours pass, the plates are washed with PBS prior to adding fixing solution made of ethanol, acetic acid, and water in 50:1:49 ratios, respectively. The resultant colored solution is pipetted into 96-well plates and the plates are then are inserted into Absorption reading machine to measure absorption at 55nm wavelength with background reading at 405nm. Absorption is then plotted against cell numbers.

2.2.3 Microinjection assay

WB-F344 cells were gown to at least 80% confluence on 9.5 cm^2/wells then exposed to naphthoquinones in serum free media for 1 hour. PBS is used afterward to wash the cells and to cover the cells for the microinjection. To visualize and quantify the level of gap junction intercellular communication (GJIC), fluorescent dye Lucifer Yellow at 10% (w/v) in 0.33 M lithium chloride was injected into cells using an Eppendorf FemtoJet microinjection system (Hamburg, Germany); the cells to be injected were selected with the help of micromanipulator. The number of cells that fluoresce around the injected cells are counted 1 minute after injection since it was the time point where we saw the optimal fluorescence. For each dish, 10 cells were given the dye and the mean of the number of fluorescing cells were established.

Determination of GJIC was performed by Xiaoqing Hou in the laboratory of Dr. Klotz.

2.2.4 Western blot

6-well plates were used to grow WB-F344 cells and they were treated with different concentrations of naphthoquinones at varying time courses. The wells were washed before and after treatment with PBS prior to collecting the sample using 2x Laemmli buffer (125 mM

Tris/HCl pH 6.8, 4% (w/v) SDS, 20% glycerol, 100mM dithiothreitol) with a pinch of bromophenol blue to color the sample. The lysing process was conducted on ice to minimize protein degradation. The collected samples were then stored in -20 °C fridge. Before carrying out gel electrophoresis, samples were broken down using a sonicator for 4 seconds, followed by centrifuging at maximum speed at 4 °C for 10 minutes. The samples were then heated for 5 minutes at 95 °C to facilitate loading the samples. The samples were then loaded into SDS-polyacrylamide gels with 12.5% w/v gel concentration for connexin 43 samples and 10% w/v gel concentration for detecting EGFR and ERK 1/2 phosphorylation. After running the gels, the samples were transferred to nitrocellulose membranes. 5% (w/v) non-fat milk was used to block the membrane after finishing the transfer process for 45 minutes to 1 hour. Next, primary antibodies were incubated in 5% (w/v) non-fat milk with the membranes for immunofluorescence detection. Antibodies used: anti-total-connexin 43 at 1:5000 dilution, anti-EGFR at 1:1000 dilution, anti-ERK 1/2 (T202/Y204) at 1:1000 dilution, anti-total-EGFR at 1:1000 concentration, anti-total-ERK at 1:1000 concentration, and finally anti-β-actin antibodies which were used at 1:5000 dilution. All the primary antibodies were polyclonal in nature. The incubation time for all the aforementioned antibodies was 24 hours in 4 °C room on a rotator except for anti-β-actin which was incubated for 1 hour at room temperature because β-Actin signal is very strong. Afterwards, membranes were washed off the primary antibodies with a solution made of a mixture of Tris-Buffered Saline and 0.1% (v/v) Tween 20 (TBST). The membranes were washed 3 times in 30 minutes with 10 minutes between each wash. Next, the membranes were covered with 5 ml of 5% (w/v) non-fat milk containing horseradish peroxidase (HRP)-conjugated anti-rabbit and mouse secondary antibodies. The incubation with the secondary antibodies was conducted while shaking at room temperature for 1 hour. Similar to the

first incubation with the primary antibodies, the membranes are washed again with TBST 3 times with 10 minutes between each wash. Supersignal femto chemiluminescent substrates were added to the membranes after the washing for 5 minutes followed by inserting the membranes into a General Electric chemifluorescence imager to obtain pictures of protein signals from the membranes.

2.2.5 Statistical analysis

GJIC data from the microinjection experiment are expressed as means ± standard deviation while the data from the inhibitors assay are presented as means ± standard error. The toxicity profiles of the naphthoquinones are presented as means ± standard deviation. One-way ANOVA followed by Tukey posthoc analysis was used to study controls and treatments results. A p-value less than 0.05 was the cut off for statistical significance. IBM SPSS Statistics 20 software was utilized to perform statistical tests and ImageQuant LAS 500 program was used to extract densitometry data from western blot pictures.

CHAPTER 3

RESULTS

3.1 Effects of synthetic and natural naphthoquinones on WB-F344 cell viability

WB-F344 cells were treated with the nine naphthoquinones previously described. The concentrations used were 1, 3, 10, 30 and 100 μM, while the treatment interval was 1 hour. The concentrations and treatment durations choice was based on previously published results, mainly on the effects of menadione. Additionally, early results obtained from experimenting with different concentrations allowed us to picture the toxicity potential of the different naphthoquinones.

3.1.1 Toxicity profiles following 1 hour treatment

The toxicity of naphthoquinones was assessed immediately after the treatment of WB-F344 for 1 hour. All naphthoquinone incubations were carried out in serum-free media. Lapachol and lawsone showed toxicity profiles similar to control (DMSO) treatments at all concentrations. Cells exposed to MeONQ or DMNQ were 10% and 20% less viable than control cells at 30 μM and 100 μM, respectively. Menadione showed a marked toxic effect on the cells at 30 μM with 40% viability compared to the control. Additionally, at 100 μM, menadione caused 100% cell death. 1,2-naphthoquinone results were similar to menadione with about 10% better viability across all the concentrations used. 1,4-napthoquinone was more toxic than 1,2-napthoquinone at 10 μM, with 60% less viability than control, and 90% cell death at 30 μM. Furthermore, viability of cells exposed to 1,4-napthoquinone was less than 10% at 30 μM and no surviving cells were observed at 100 μM. Last, plumbagin and juglone showed significant toxicity even at 10 μM, where juglone caused complete cellular death and plumbagin decreased viability to 10% of the control. Results are shown in Figure 3.1.

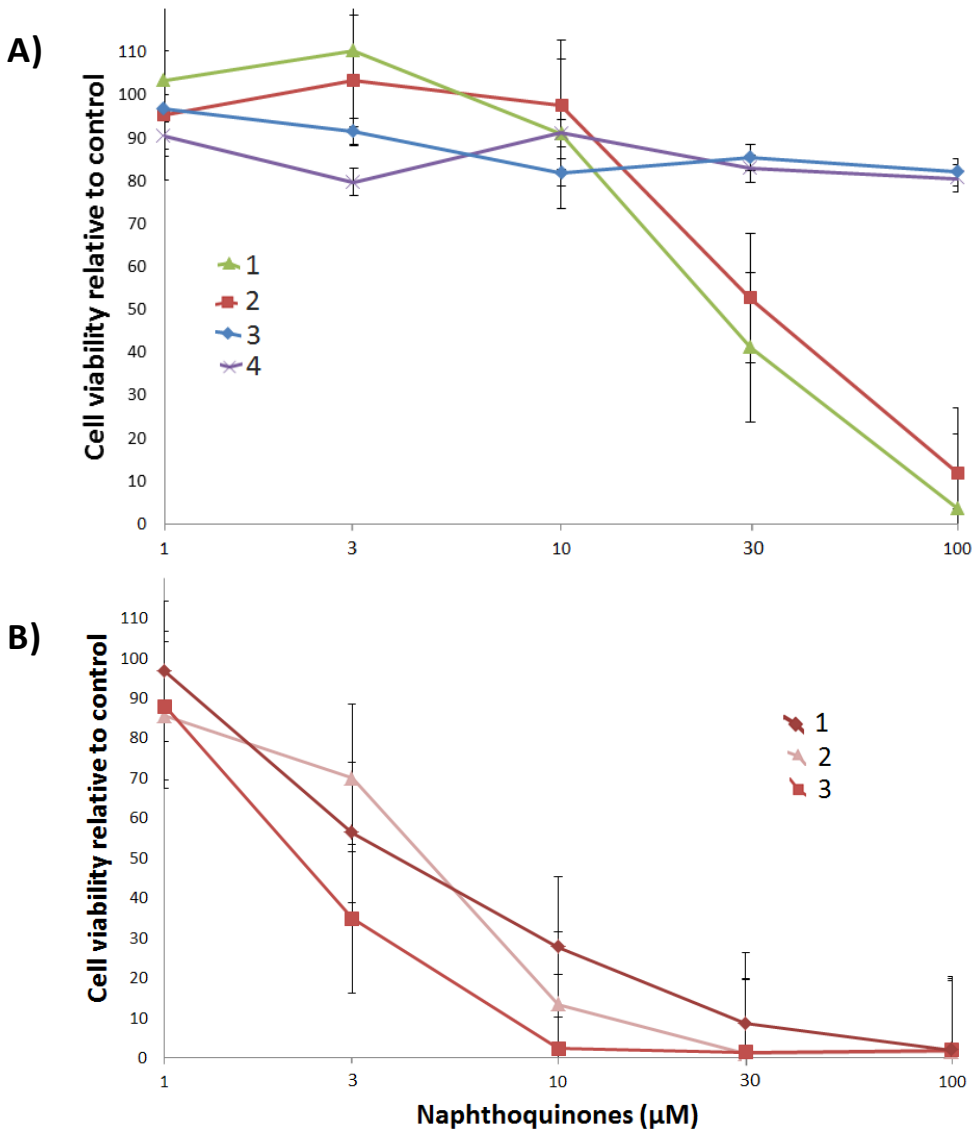

Figure 3.1 Effect of naphthoquinones on cell viability after 1 hour incubation. A) Cytotoxicity of naphthoquinones: 1- menadione; 2- 1,2-NQ; 3- MeONQ; 4- DMNQ at different concentrations relative to the control**. B)** Cell viability following treatments with: 1- 1,4-NQ; 2- plumbagin; 3- juglone. All data are presented as means ± SD and results of 3 independent experiments. Lawsone and lapachol were not toxic (data not shown).

3.1.2 Toxicity profiles 24 hours post 1 hour treatment

To investigate the post treatment toxicity of naphthoquinones, cells were treated with naphthoquinones at 1, 3, 10, 30 and 100 μM, and plumbagin and juglone at 0.5, 1, 1.5, 2, 3 μM. Cells were incubated in the presence of naphthoquinones diluted in serum-free media for one hour. Naphthoquinones were washed away using PBS and the cells were covered again with fresh serum-free media. The cells were incubated at 37 °C for 24 hours, followed by analysis of viability employing the neutral red uptake assay. Lawsone showed toxicity similar to treatment with vehicle (DMSO), whereas lapachol was slightly more toxic, with 10% less viability across the concentrations used. MeONQ and DMNQ demonstrated similar results with 60-70% cell death being observed at 100 μM. 1,2-naphthquinone saw significant decrease in cell viability between10 μM and 30 μM. Menadione led to almost complete cell death at 30 μM while 1,4-naphthoquinone was very toxic even at 10 μM with 10% cell viability. In the case of plumbagin and juglone, 1.5 μM caused complete cellular death. Results are shown in Figure 3.2.

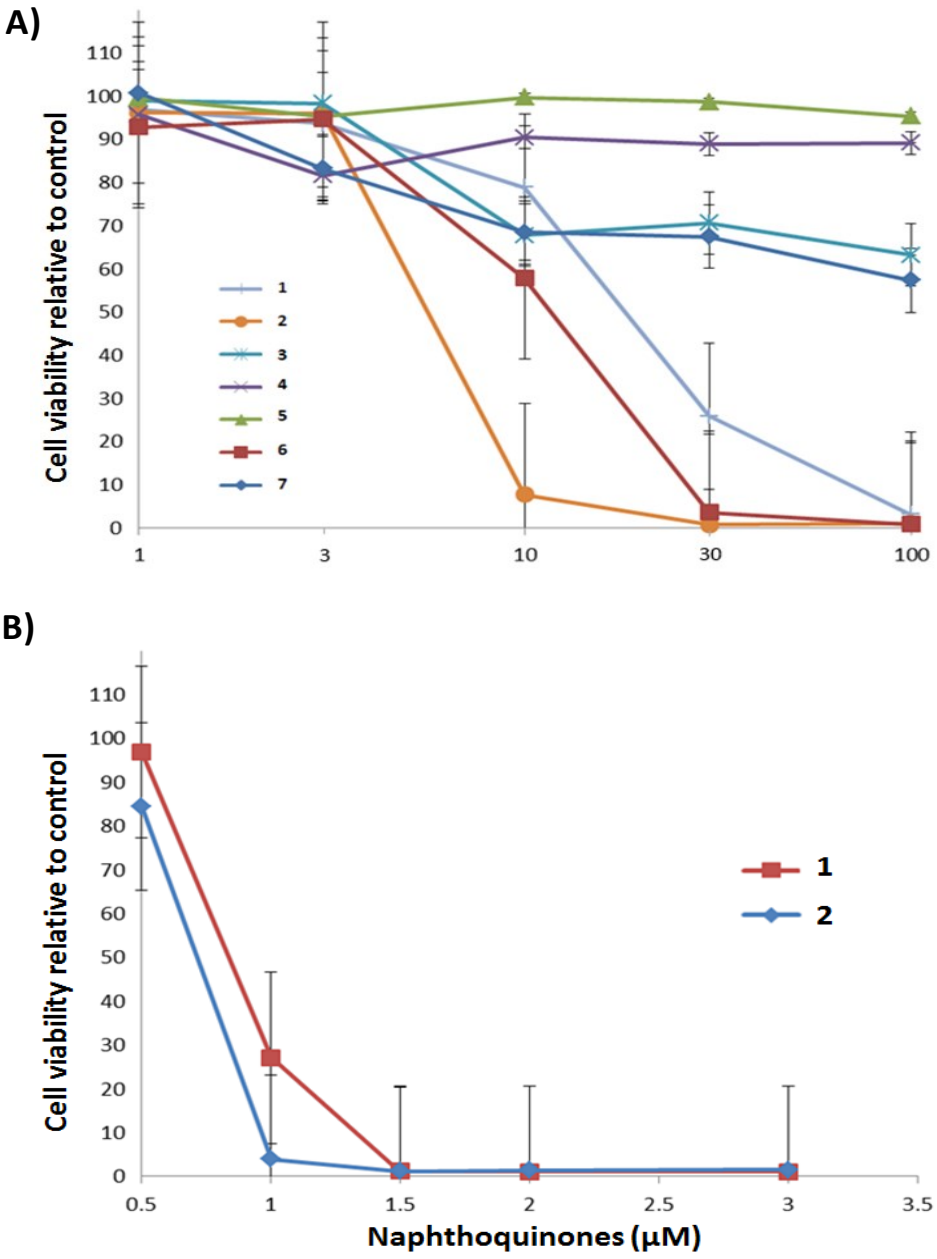

Figure 3.2 Effect of naphthoquinones on cell viability 24 hours post 1 hour treatment. A) Cytotoxicity of naphthoquinones: 1- 1,2- NQ; 2- 1,4- NQ; 3- DMNQ; 4- Lapachol; 5- lawsone; 6- menadione; 7- MeONQ at different concentrations relative to control. **B)** Cell viability following treatments with: 1- plumbagin; 2- juglone. All data are presented as means ± SD and results of 3 independent experiments.

3.2 Analysis of gap junctional intercellular communication using a dye transfer assay

WB-F344 rat liver epithelial cells were exposed to menadione (MQ), MeONQ, DMNQ, lapachol, and lawsone at 10 μM for 1 hour, followed by analysis of gap junctional intercellular communication using the microinjection/dye transfer assay. The numbers of fluorescent cells were counted 1 minute following the dye injection. Lucifer Yellow dye transfer was significantly hindered in the case of menadione, MeONQ and DMNQ with 17.9%, 19.4% and 28.6% less communicating cell than the control, respectively. Lapachol and lawsone had no effect on the number of communicating cells and were similar to control. The rapid loss of cellular communication tested with the rate of the dye diffusion suggests the participation of mechanisms other than transcriptional ones. Cx43 phosphorylation by menadione has been found to cause a decrease in cellular communication in previous published work (Klotz et al., 2002). The comparable results of MeONQ and DMNQ suggest the possible involvement of Cx43 phosphorylation in attenuating GJIC. We were not able to attain the microinjection assay with the other naphthoquinones due to the toxicity of these compounds. Results are shown in Figure 3.3. N.B. the experiment was performed by Xiaoqing Hou, Omar Alomair helped in preparing chemicals and conducting the statistical analysis.

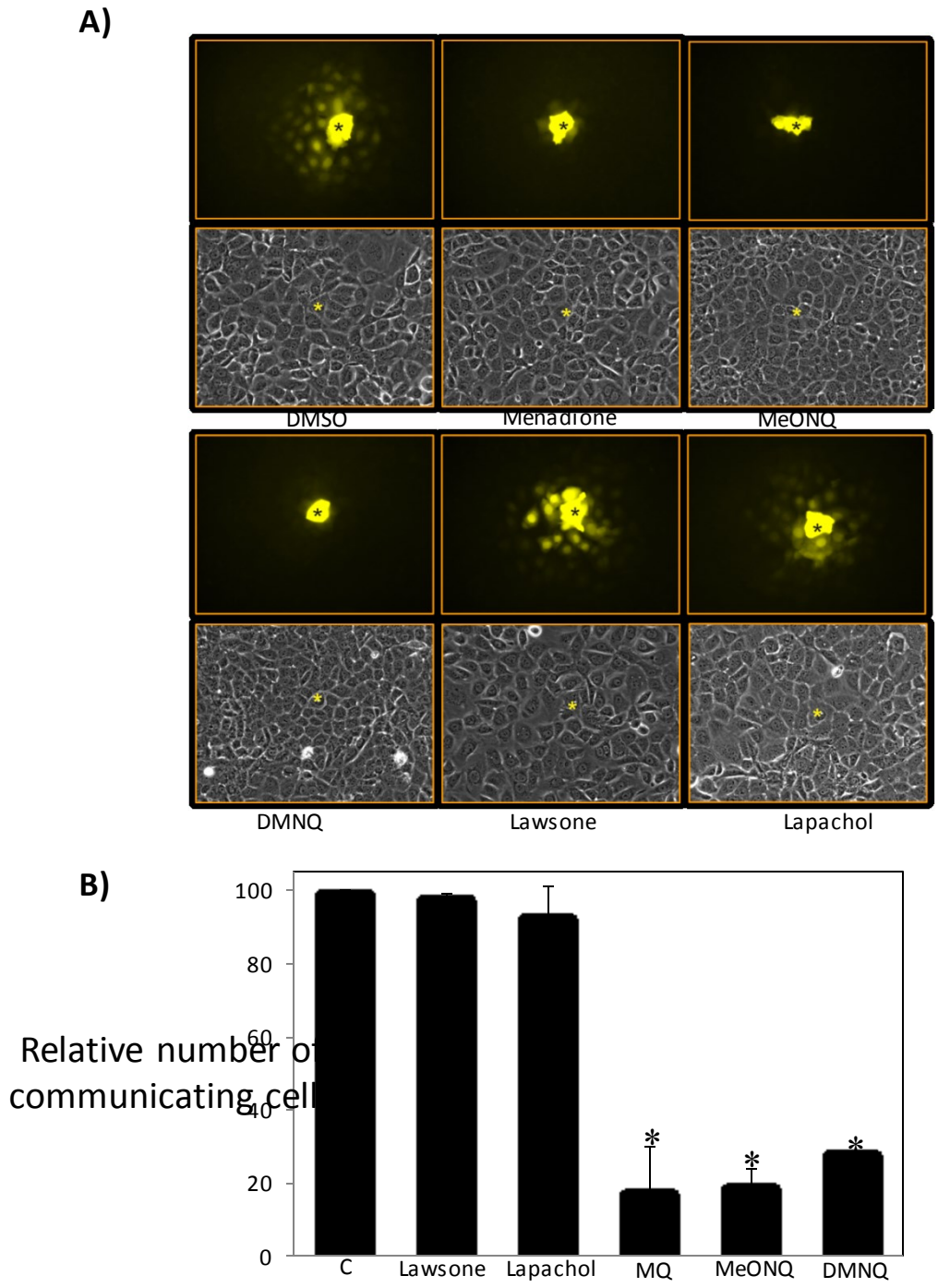

Figure 3.3 Gap junctional communication analysis using dye transfer assay. A) Phase contrast and fluorescent pictures of WB-F344 cells 1 minute after injection of Lucifer Yellow fluorescent dye. Treatment concentrations were 10 µM for 1 h; menadione, DMNQ, and

MeONQ lowered GJIC, as seen from the diffusion of the dye while lawsone and lapachol were similar to control. **B)** Quantitation of GJIC after exposure to 10 µM of naphthoquinone for 1h. Data are means of 3 (lawsone, lapachol: 2) independent experiments ± SD (lawsone, lapachol: ranges). "*" is significantly different from control (ANOVA, Tukey post-test). $P<0.05$ was chosen as level of significance.

3.3 Effect of naphthoquinones on gap junctional intercellular communication

Menadione has been investigated for its role in Cx43 phosphorylation and the consequent closure of gap junctions. Inhibition of protein tyrosine phosphatases negatively regulating EGFR activity results in the activation of ERK 1/2, kinases downstream of EGFR and known to phosphorylate Cx43 (Fig.3.4) (Abdelmohsen et al., 2004). We investigated this signaling cascade with the other naphthoquinones, starting with connexin 43 phosphorylation.

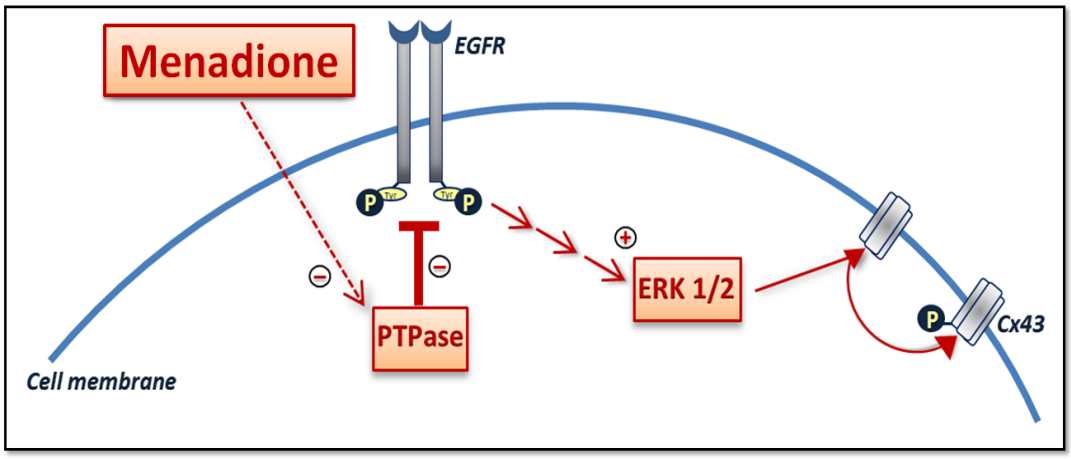

Figure 3.4 Mechanism of Cx43 phosphorylation. Exposure of WB-F344 cells to menadione resulted in extracellular signal–regulated kinases (ERK 1/2)-mediated Cx43 phosphorylation via the indirect activation of epidermal growth factor receptor (EGFR). Adapted from (Abdelmohsen et al., 2004).

3.3.1 Connexin 43 phosphorylation

Cx43 phosphorylation is observed in an SDS–polyacrylamide gel with a shift in protein electrophoretic mobility. Whereas Cx43 from lysates of untreated cells occurs in three bands (a lower unphosphorylated band (P0), singly phosphorylated band (P1), and doubly phosphorylated band (P2)), menadione treatment causes a shift in electropohoretic mobility. Menadione-induced phosphorylation of Cx43 was previously described to cause the amount of P0 to diminish and the hyperphosphorylated bands to appear (Klotz et al., 2002). To study this effect, WB-F344 cells were incubated with the 9 naphthoquinones for 30 minutes in serum-free media. The concentrations used were 30 μM and 50 μM. The concentrations were chosen based on established cytotoxicity data and previous published work. Treatment with menadione, MeONQ, DMNQ and 1,2-naphthoquinone induced a loss of P0 band intensity, whereas PN band amounts increased. The results from the other naphthoquinones were not conclusively different from DMSO treatment. Results are shown in Figure 3.5.

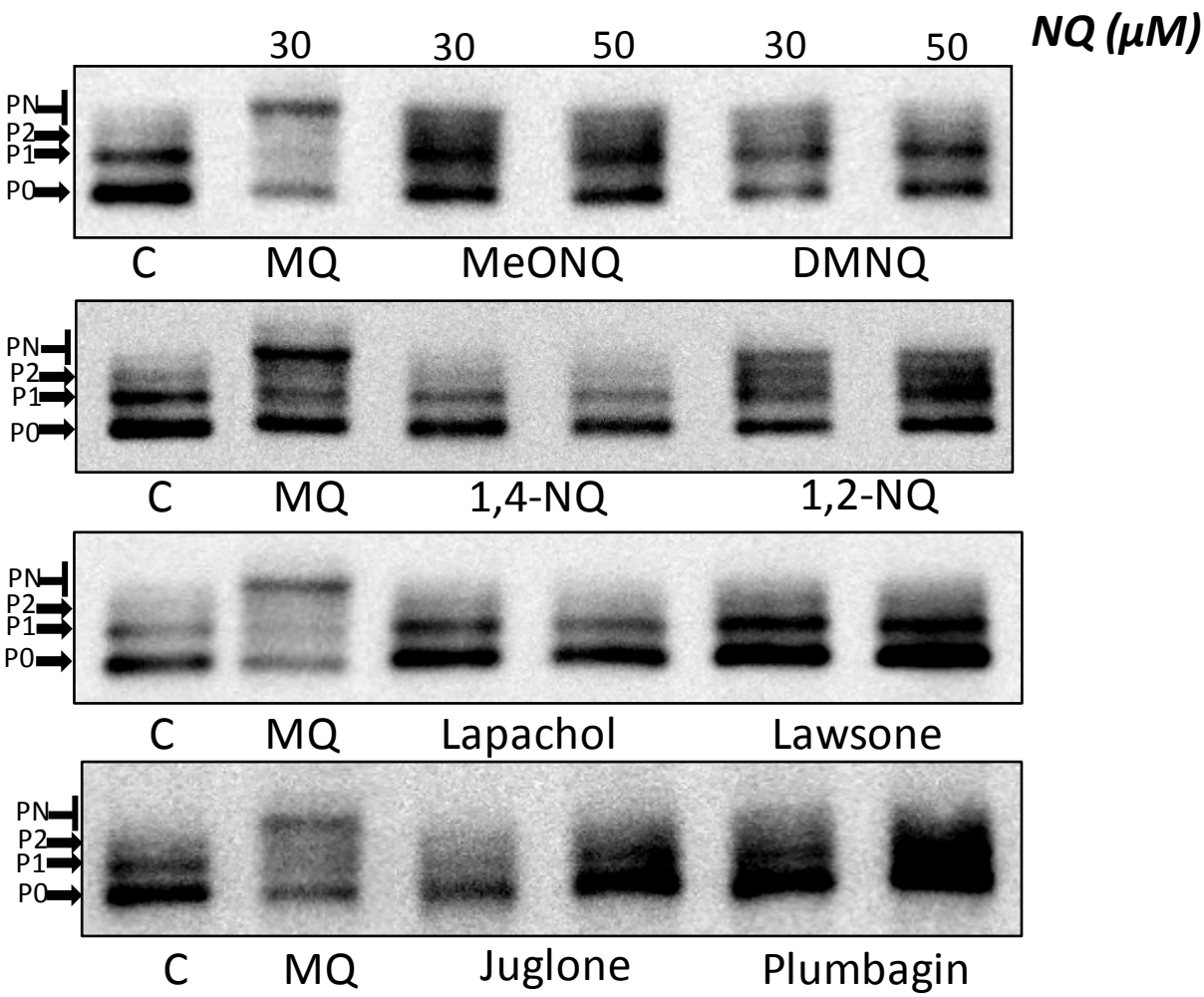

Figure 3.5 Naphthoquinone-induced Cx43 phosphorylation. WB-F344 cells were treated with DMSO and naphthoquinones at concentrations shown above for 30 minutes. An induction of Cx43 phosphorylation and electrophoretic mobility shift is visualized by the disappearance of unphosphorylated Cx43 (P0) and the appearance of hyperphosphorylated Cx43 (PN). Only treatments with MQ, DMNQ, MeONQ and 1,2 NQ resulted in enhanced Cx43 phosphorylation. Data are representative of 3 independent experiments.

3.3.2 Epidermal growth factor receptor (EGFR) activation

EGFR is a receptor tyrosine kinase that, upon binding to its ligands, autophosphorylates to trigger downstream kinases involved in many physiological functions regulating cell proliferation (Downward, Parker, & Waterfield, 1984). Two of the downstream kinases are extracellular signal-regulated kinases 1 and 2, both of which are known to attenuate GJIC through Cx43 phosphorylation (Johnstone et al., 2012). To evaluate EGFR activation, WB-F344 cells were incubated with naphthoquinones at 30 μM and 50 μM in serum-free media for 30 minutes. Except for lapachol and lawsone, all the other naphthoquinones employed were able to activate EGFR with different potency. MeONQ and DMNQ exhibited a lower extent of EGFR stimulation in comparison to other compounds. See Figure 3.6 for results.

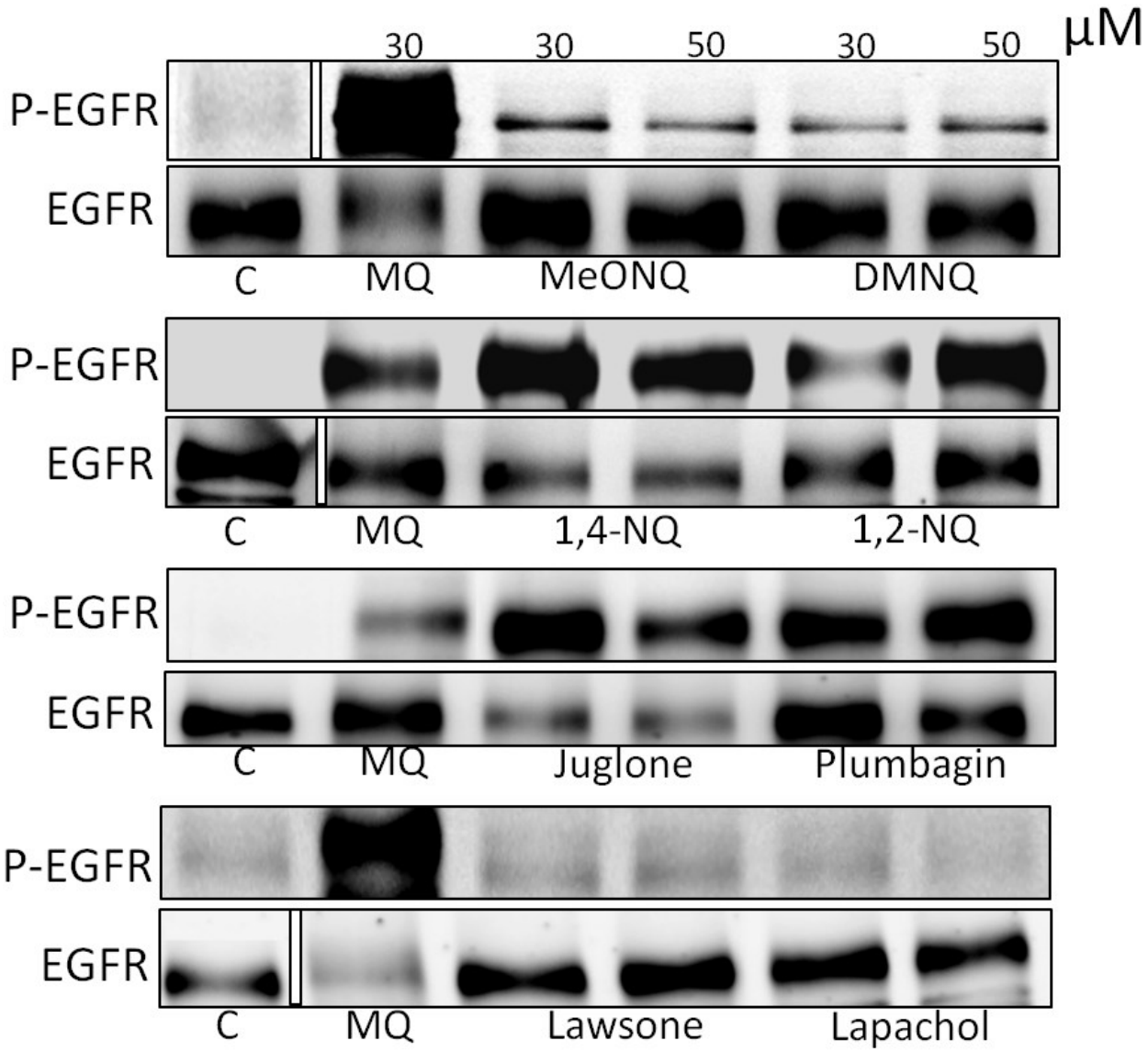

Figure 3.6 EGFR induction by naphthoquinones. Activation of epidermal growth factor receptor by various naphthoquinones was shown in WB-F344 cells that were treated with naphthoquinones at the concentrations shown for 30 minutes followed by Western blotting. Exposure to naphthoquinones led to the activation of EGFR, except for lawsone and lapachol, which were similar to the control (DMSO). Data are representative of 3 independent experiments (total EGFR: 2).

3.3.2 Extracellular signal–regulated kinase (ERK 1/2) activation

EGFR activation is responsible for the induction of different kinases that are involved in DNA synthesis and cell growth. Extracellular signal–regulated kinases (ERK 1/2) are kinases downstream of EGFR that are implicated in connexin 43 phosphorylation at serine 279 and S282 sites, prompting the closure of the gap junction channel (Pahujaa et al., 2007). To study ERK 1/2 activation, WB-F344 cells are treated with naphthoquinones for 30 minutes at 30 μM and 50 μM, all in serum-free media. Lapachol and lawsone were similar to the control, repeating the same pattern we observed previously. Interestingly, juglone and 1,4-naphthoquinone did not induce ERK 1/2 in opposition to their strong effect on EGFR. The remaining naphthoquinones readily promoted ERK1/2 phosphorylation. Results are shown in figure 3.7.

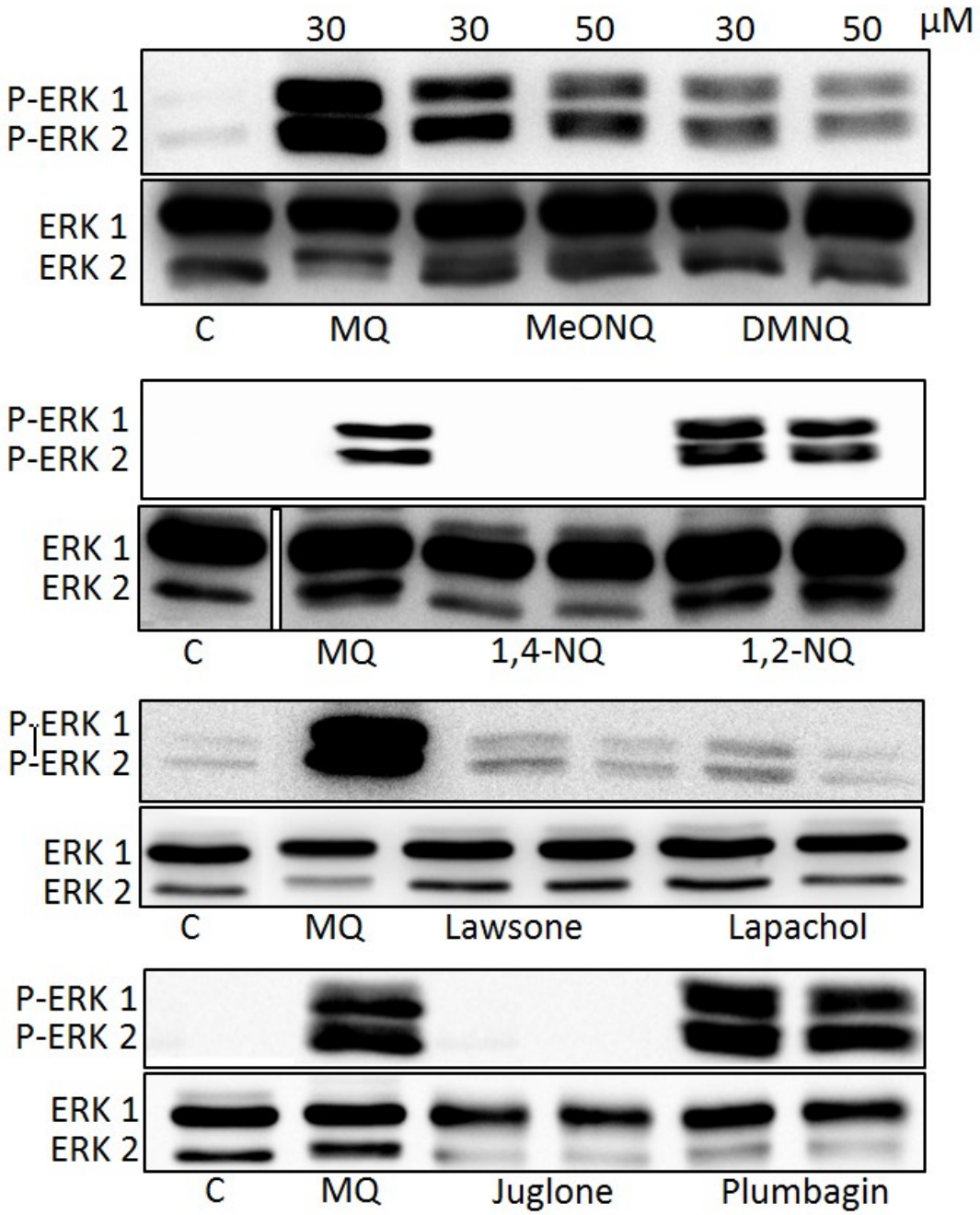

Figure 3.7 Activation of ERK 1/2 after treatment with naphthoquinones. WB-F344 cells were exposed to different concentrations of naphthoquinones for 30 minutes prior to being analyzed using Western Blotting and phospho-specific antibodies. ERK was activated by treatments with menadione, DMNQ, MeONQ, 1,2-NQ and plumbagin. Data are representative of 3 independent experiments (Total ERK: 2).

3.4 Time- and concentration-dependent activation of signaling induced by naphthoquinones

With a reported half-life of Cx43 between 60 and 90 minutes (Popolo, Morello, Sorrentino, & Pinto, 2013), consistently detecting phosphorylation changes in Cx43 proved elusive in our study. Furthermore, as previously illustrated at the start of this chapter, naphthoquinone toxicity prevented optimum study design. For those reasons, we opted to focus only on MeONQ and DMNQ. Both MeONQ and DMNQ were the least toxic naphthoquinones able to cause signaling activation. Additionally, DMNQ is known as an exclusive redox-recycler that does not attach to cellular nucleophiles, in contrast to menadione which triggers Cx43 via the alkylation of protein tyrosine phosphatases responsible for EGFR regulation (Abdelmohsen et al., 2004).

3.4.1 Time- and concentration-dependent induction of Cx43 phosphorylation

Different concentration and time points of MeONQ and DMNQ incubation with WB-F344 cells were performed to pinpoint a time interval and concentration persistently capable of causing Cx43 phosphorylation. To accomplish this task, 30 μM, 100 μM, and 300 μM of MeONQ and DMNQ were incubated with the cells for 5, 10, 15, 25, 30, 45, and 60 minutes. The exposure was done in serum-free media and cell lysates for Western blotting were collected every five minutes. The results presented in figure 3.8 suggest that the 20 minutes time point at 30 μM was the most consistent in terms of Cx43 phosphorylation for both MeONQ and DMNQ.

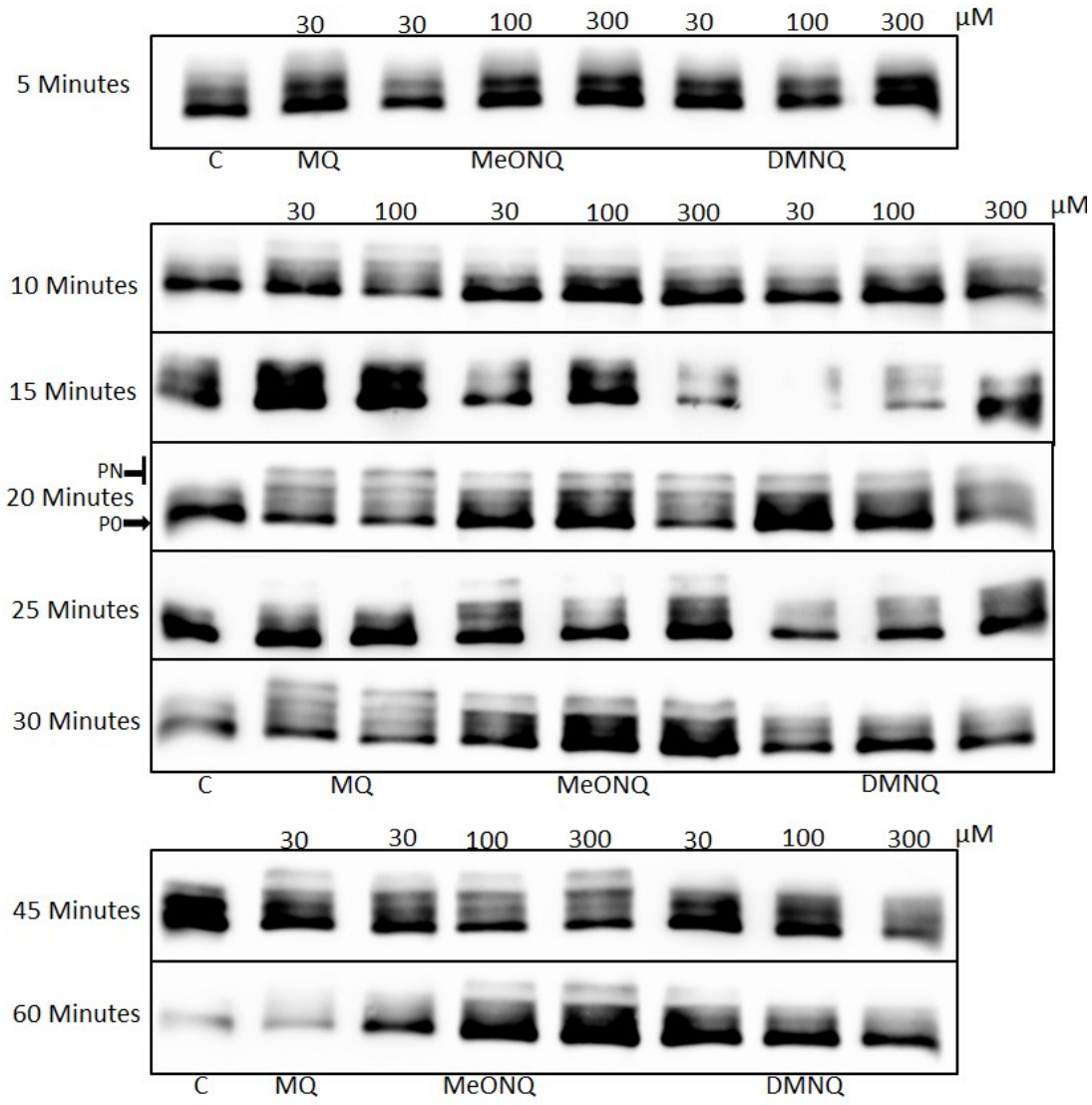

Figure 3.8 Time and concentration-dependent phosphorylation of Cx43. WB-F344 cells were treated with DMSO and naphthoquinones at the concentrations and for the times shown. Taking menadione as an example, we can see an induction of Cx43 phosphorylation and electrophoretic mobility shift manifested in appearance of hyperphosphrylated Cx43 (PN). Treatments with DMNQ and MeONQ at 30 µM resulted in Cx43 phosphorylation more persistently at the 20 minutes incubation. Data are representative of 3 independent experiments

3.4.2 Time- and concentration-dependent activation of EGFR (Tyr-1068)

Similar to Cx43 phosphorylation, we wanted to establish at what concentration and incubation time of MeONQ and DMNQ we see activation of EGFR, the hypothesized starting point of Cx43 phosphorylation. We treated WB-F344 cells with MeONQ and DMNQ at 30 μM, 100 μM, and 300 μM in serum-free media. The incubation was for 5, 10, 15, 25, 30, 45, and 60 minutes with samples collected at the time points previously mentioned for gel electrophoresis analysis. Menadione (MQ) was used as a positive control. EGFR was activated with MeONQ and DMNQ incubation for 20 minutes at all used concentrations, based on the previous results for Cx43 phosphorylation. Comparably, similar effects were observed with 25 and 30 minutes duration. See figure 3.9 for results.

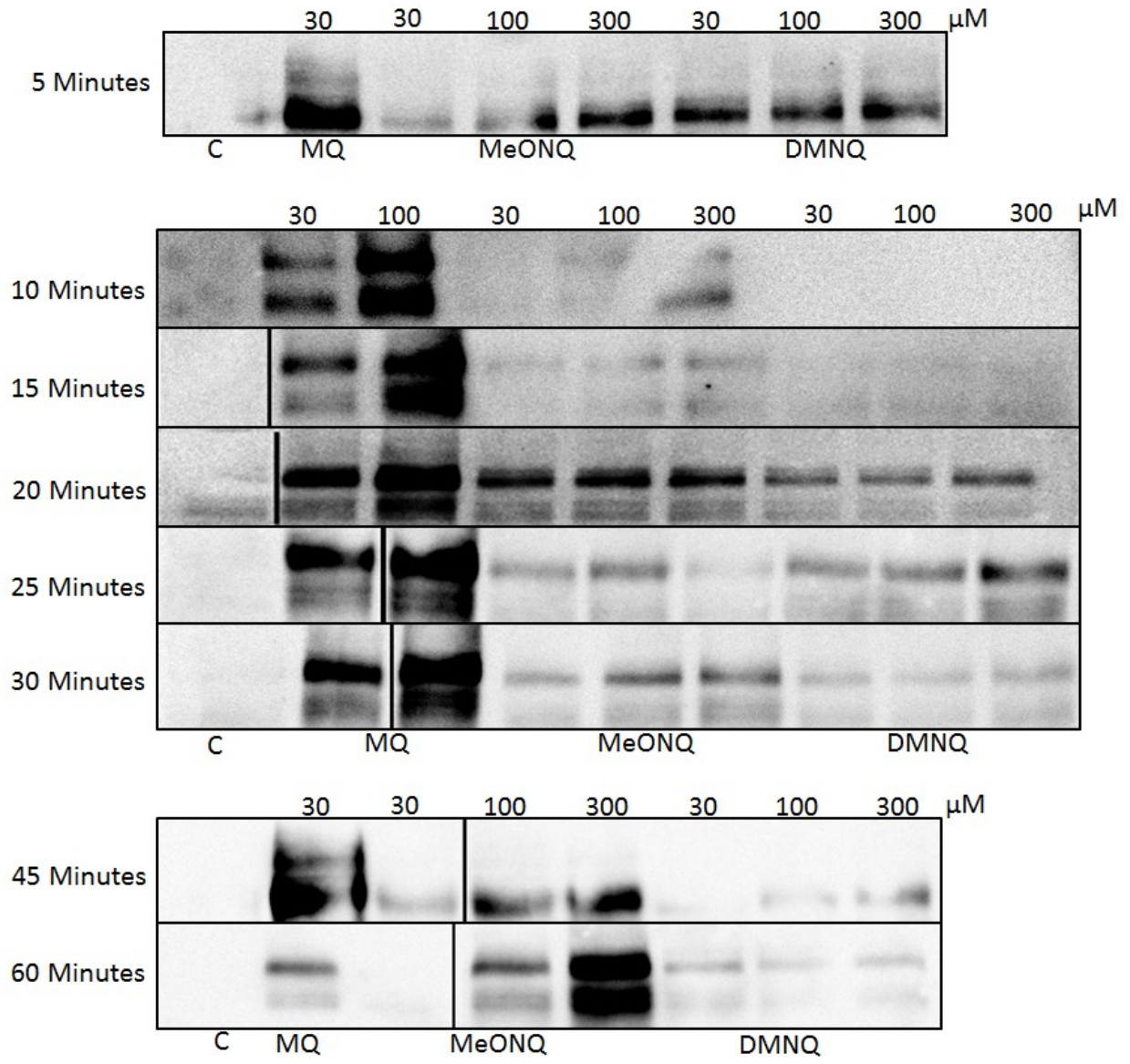

Figure 3.9 Time and concentration-dependent phosphorylation of EGFR (Tyr-1068). Activation of epidermal growth factor receptor by MeONQ and DMNQ is demonstrated in WB-F344 cells that were treated with the concentrations and time intervals shown, followed by Western blotting. MeONQ and DMNQ treatment for 20 minutes resulted in EGFR activation across all concentrations used. Similar results observed with 25 and 30 minutes incubations. Data are representative of 3 independent experiments.

3.4.3 Time and concentration-dependent activation of ERK 1/2

A similar approach to establish the most consistent concentrations and time point where we see an activation of EGFR and phosphorylation of Cx43 was performed for ERK 1/2 activation. WB-F344 cells were treated with MeONQ and DMNQ for 5, 10, 15, 25, 30, 45, and 60 minutes, and samples for Western Blot analysis were collected each 5 minutes. The treatment was done in serum-free media at 30 μM, 100 μM, and 300 μM. ERK 1/2 showed optimum induction after 20 minutes incubation. Activation of ERK 1/2 was also detected at other time points with varying degrees of intensity. Results are show in figure 3.10.

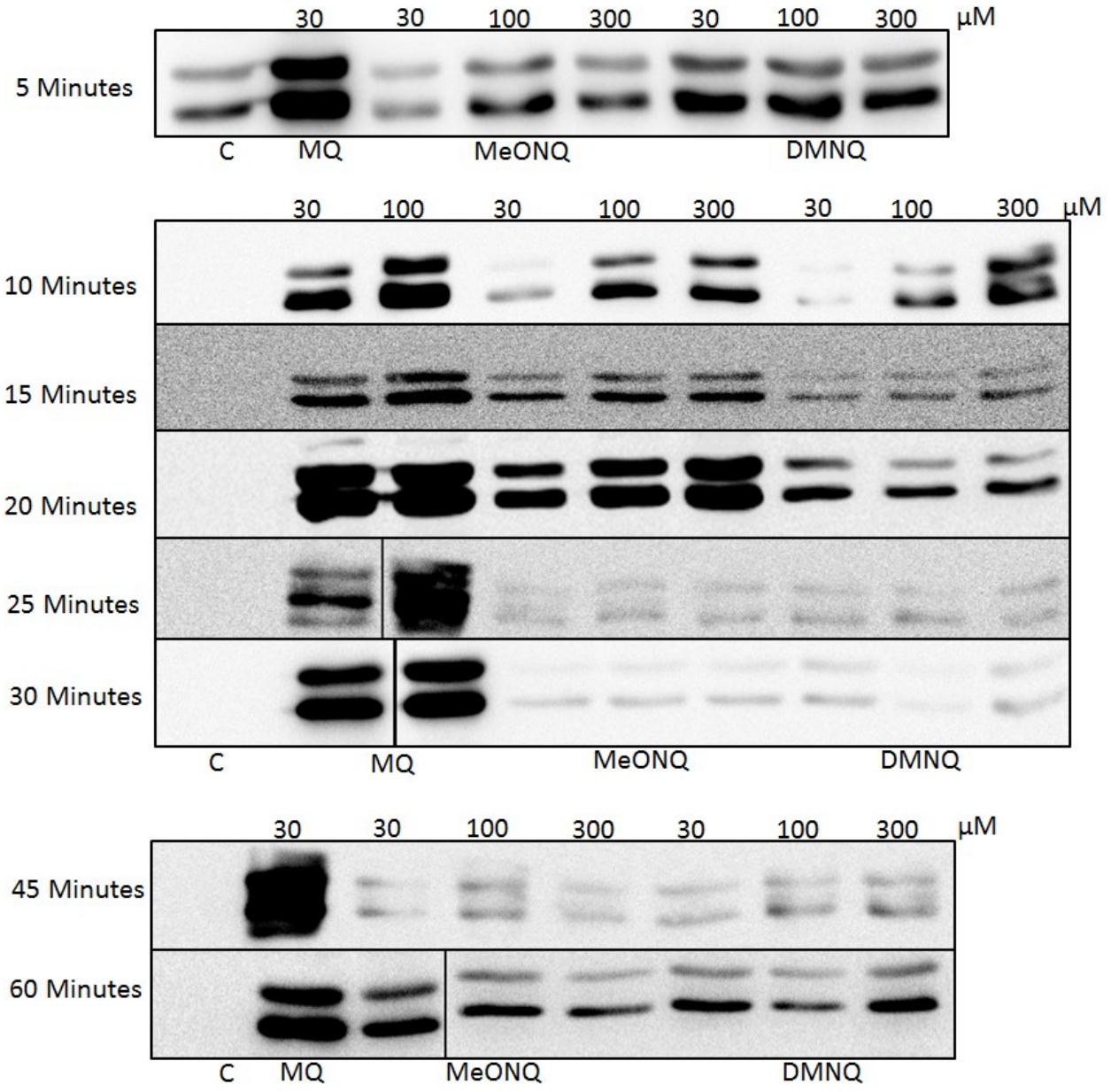

Figure 3.9 Time and concentration-dependent induction of ERK 1/2. Induction of Extracellular signal–regulated kinases (ERK 1/2) by MeONQ and DMNQ is demonstrated in WB-F344 cells that were treated with concentrations and incubation times shown, followed by Western blotting. MeONQ and DMNQ exposure for 20 minutes resulted in ERK 1/2 induction at 30 µM, 100 µM, and 300 µM treatments. Data are representative of 3 independent experiments.

3.5 EGFR and ERK 1/2 inhibition: effect on naphthoquinone-induced Cx43 phosphorylation

Too elucidate the mechanism of connexin 43 phosphorylation by MeONQ and DMNQ, inhibition of EGFR and of MEK 1/2, the kinases upstream of ERK 1/2, was achieved with tyrphostin AG1478 and with U0126, respectively. Tyrphostin AG1478 is a powerful and specific inhibitor of EGFR through the interference with EGFR intrinsic tyrosine kinase activity (del Carmen Medina, Vazquez-Prado, & Garcia-Sainz, 2000). U0126 is a potent selective inhibitor of ERK 1/2 activation by MEK 1/2 and was incorporated in the experiment to test for a role of ERK 1/2 in MeONQ and DMNQ-induced Cx43 phosphorylation (Marampon et al., 2011). By using these inhibitors we can understand the involvement of EGFR activated signaling cascade in the attenuation of gap junctional intercellular communication. Based on the time and concentration dependent analysis of Cx43 phosphorylation, 30 μM concentrations of naphthoquinones and 20 minutes incubation were chosen as the optimal setting to conduct the inhibitors assay.

3.5.1 Connexin 43 phosphorylation in the presence of EGFR or ERK 1/2 inhibitors

To assess EFGR and ERK involvement in MeONQ and DMNQ induced Cx43 phosphorylation, WB-F344 cells were preincubated in serum-free media containing 10 μM of EGFR inhibitor (AG1478) or MEK 1/2 inhibitor for 30 minutes. After the preincubation period expired, cells were washed with PBS followed immediately co-incubation of 10 μM of the inhibitors with 30 μM of MeONQ and DMNQ for 20 minutes prior to collecting the cells for gel electrophoresis analysis. Both MeONQ and DMNQ caused Cx43 phosphorylation that was not seen after using either one of the inhibitors. Moreover, MeONQ and DMNQ induced a significant EGFR and

ERK 1/2 activation only in the cells that were not treated with AG1478 or U0126. MeONQ result are shown in figure 3.10; DMNQ in figure 3.11.

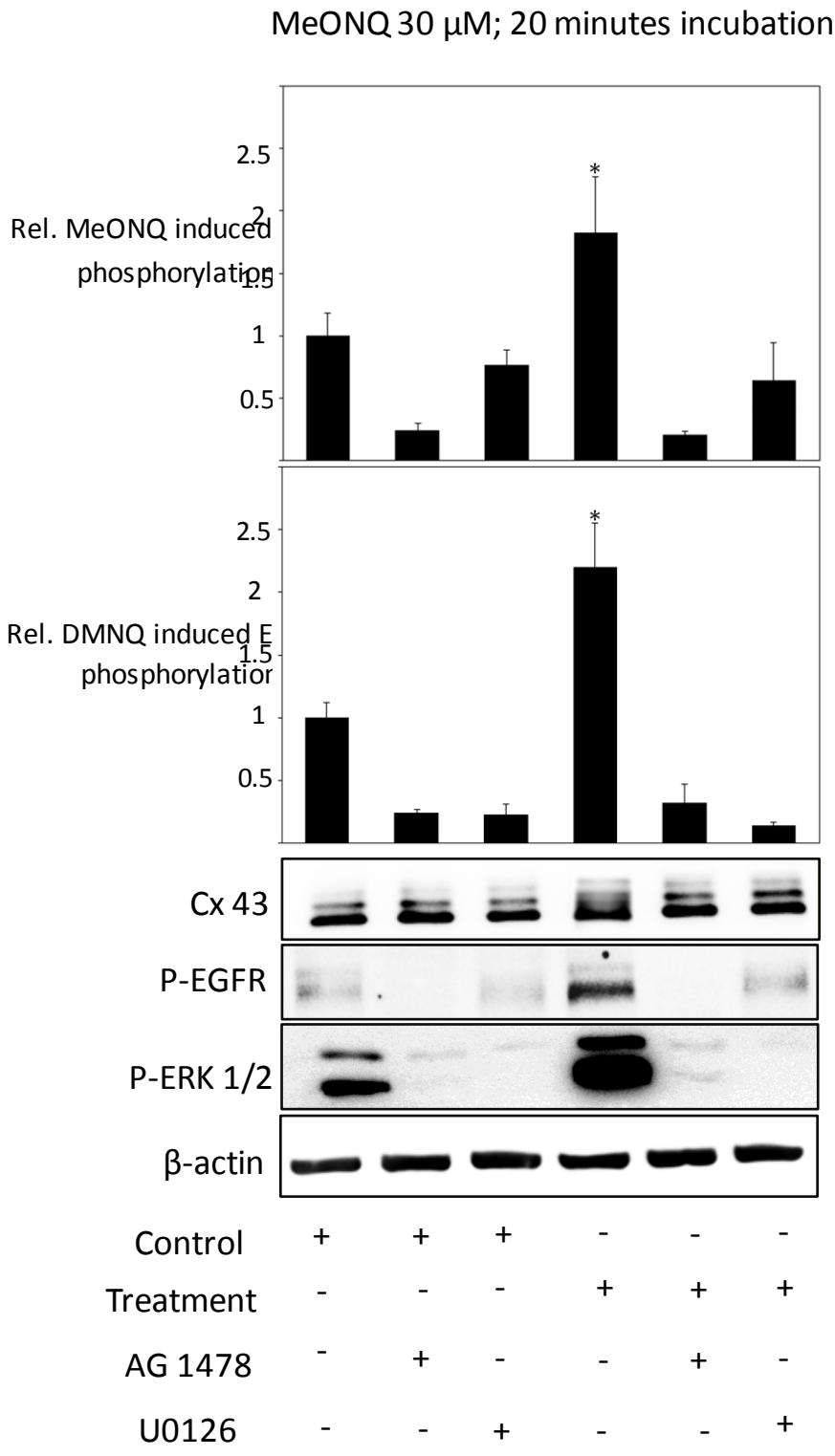

Figure 3.10 Effect of EGFR and MEK inhibitors on MeONQ-triggered Cx43 phosphorylation. Treatment with 30 μM for 20 minutes led to Cx43 phosphorylation accompanied by significant activation of EGFR and ERK in the absence of their inhibitors. In contrast, MeONQ treatment in the presence of EGFR and DMNQ inhibitors did not exhibit observable Cx43 phosphorylation or induction of the EGFR signaling cascade. Densitometry data are normalized to β-actin level and are presented as means of 3 independent experiments ± SE. "*" is significantly different from the other controls and treatments with inhibitors (ANOVA, Tukey post-test). $P<0.05$ was chosen as level of significance.

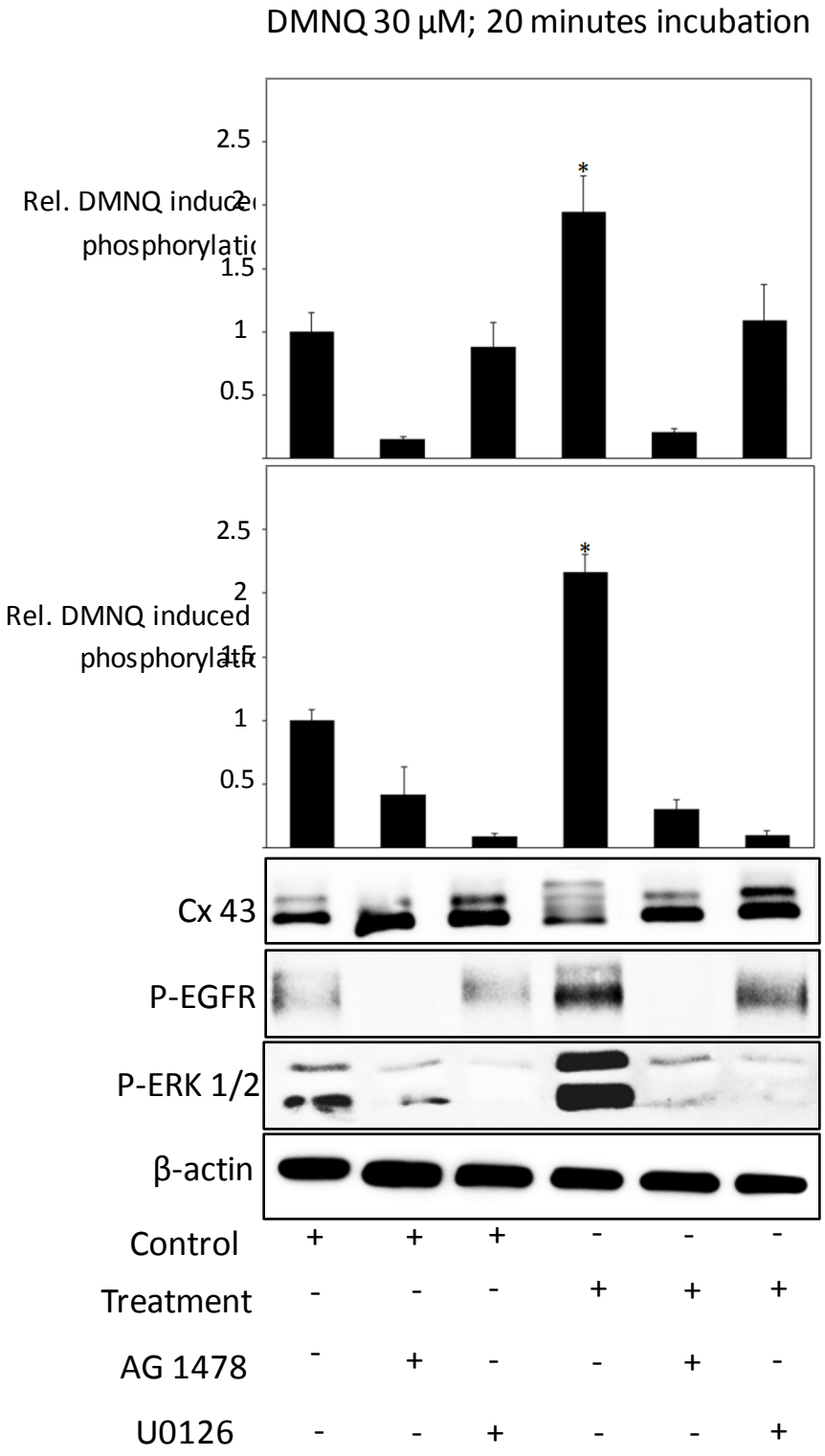
DMNQ 30 µM; 20 minutes incubation
Rel. DMNQ induced phosphorylation
2.5
2
1.5
1
0.5
*
Rel. DMNQ induced phosphorylation
2.5
2
1.5
1
0.5
*
Cx 43
P-EGFR
P-ERK 1/2
β-actin
Control + + + - - -
Treatment - - - + + +
AG 1478 - + - - + -
U0126 - - + - - +

Figure 3.10 Effect of EGFR and MEK inhibitors on DMNQ-induced Cx43 phosphorylation. Treatment for 20 minutes with DMNQ at 30 μM correlated with Cx43 phosphorylation. In addition, significant activation of EGFR and ERK in the absence of their inhibitors was detected. On the other hand, EGFR and MEK inhibitors prevented the activation of EGFR signal transduction and the expected result of Cx43 phosphorylation. Densitometry data are normalized to β-actin level and are presented as means of 3 independent experiments ± SE. "*" is significantly different from the other vehicle controls and treatments co-incubated with inhibitors except for DMNQ and U0126 co-incubated samples in the EGFR test (ANOVA, Tukey post-test). $P<0.05$ was chosen as level of significance.

CHAPTER 4

DISCUSSION

4.1 General discussion

Gap junctional intercellular communication (GJIC) is a pivotal regulator mechanism that ensures the ability of the tissue to maintain homeostasis by the transfer of nutrients and signaling molecules between adjacent cells. Gap junctions are channels made of a transmembrane family of proteins called connexin (Goodenough & Paul, 2009). Various physiological functions such as normal heart contractility, neuronal signaling transduction, normal hearing function, and the healing of skin injury are dependent on normal function of GJIC (Laird, 2006). Moreover, tumors regularly show a low basal level of connexin inside them, such state can be reversed be re-introducing the connexin protein inside again which results in better distribution of anticancer agents via the bystander effect (Sanson et al., 2002).

The connexin family is presented in the mammalian body with 21 isoforms that include connexin 43, the number 43 denotes the protein molecular weight, which is the most abundantly expressed connexin, and as such, the one that drew the interest of research focusing on understanding the nature of GJIC. Many factors have been implicated in modulating GJIC level, and these factors include: (1) pH level within the cell, (2) the concentration of readily available calcium ions, (3) translational alteration, and (4) posttranslational modification that is primarily achieve through connexin phosphorylation. (Lampe & Lau, 2000).

Connexin phosphorylation, and in particular Cx43, was investigated heavily as a main tool for the cell to control the opening or closure of the gap junction channel. This posttranslational modulation is performed with the help of protein kinases that target the carboxylic cytoplasmic tail of Cx43. In the project we focused on one of these kinases, namely the extracellular signal-

regulated kinases (ERK1/2), which is initiated as a result of the activation of the epidermal growth factor receptor (EGFR), the starting point of the cascade leading to Cx43 phosphorylation (Solan & Lampe, 2005). Refer to figure 3.4 for more details.

Menadione, a member of the naphthoquinone family, has been found to induce Cx43 phosphorylation via the alkylation of protein kinase phosphates that negatively control EGFR activity, which in turn enable ERK 1/2 (kinase down stream of EGFR) to phosphorylate Cx43 and consequently close the gap junction channel (Abdelmohsen et al., 2004). Nine synthetic and natural naphthoquinones were selected in this project to simulate the effect previously observed with menadione on GJIC and to enhance our understanding of the underlying mechanism implicated in modifying GJIC levels. The selected naphthoquinones were 1,2-naphthoquinone, 1,4-naphthoquinone, 2-methoxy-1,4-naphthoquinone (MeONQ), 2,3-dimethoxy-1,4-naphthoquinone (DMNQ), plumbagin, juglone, lawsone and lapachol. MeONQ, DMNQ, and the positive control menadione demonstrated a significant attenuation of GJIC that is clearly visualized and quantified in the microinjection assay, where low numbers of cells were able to receive florescent dye injected into a neighboring cell following exposure, contrary to lapachol and lawson, both of which did not hinder the dye diffusion. The dye transfer assay could not be used to analyse the rest of the compounds due to their high toxicity to the cells.

To dissect the hypothesized pathway involved in decreasing the levels of GJIC, we tested the effect of the utilized naphthoquinones on EGFR activation, ERK 1/2 induction, and finally Cx43 phosphorylation. In term of Cx43 phosphorylation, menadione, MeONQ, DMNQ, and 1,2-naphthoquinones treatment brought about a detectable phosphorylation of Cx43 which is

detected on western blots as an increase in the amount of hyperphosphorylated Cx43 protein. Excluding lapachol and lawsone, all the naphthoquinones showed different levels of EGGR induction. Lapachol and lawson displayed no effect on ERK 1/2 activation. While the other naphthoquinones, beside juglone and 1,4-naphthoquinones were potent in their phosphorylation of ERK 1/2. We could not find a clear explanation for the aforementioned results. We selected MeONQ and DMNQ to carry out the remaining objectives of this project based on their low toxicity in comparison to the other naphthoquinones. Additionally, DMNQ is known as an exclusive redox-recycler, contrary to menadione, which is both a redox-recycler and alkylating agent (Bosworth et al., 2009). Performing extensive time- and concentration dependent analysis of MeONQ and DMNQ effect on the signaling cascade hypothesized to be a culprit in decreasing the levels of GJIC found the ideal treatment to be 20 minutes at 30 μM concentration. Utilizing EGFR and ERK inhibitors, AG1478 and U0126, MeONQ and DMNQ showed a significant activation of both EGFR and ERK and resulted in the build of the hyperphosphorylated band of Cx43 only in the absence of either one of the inhibitors. This finding suggests that EGFR signaling cascade and the collateral Cx43 phosphorylation is the likely pathway in the attenuation of GJIC by MeONQ and DMNQ.

Clinically, maintaining a healthy level of GJIC is important in the positive response of many tumor types to anti-cancer agents. We established in this study that EGFR and its downstream kinases ERK 1/2 are responsible for Cx43 phosphorylation and the closure of gap junction channels. Employing inhibitors of either EGFR or ERK 1/2 prevents the decrease in GJIC and enhance the toxic effect of chemicals on cancerous cells through the bystander effect.

Developing specific EGFR and ERK inhibitors with minimum side effects and using them in an adjuvant therapy is a promising new approach to treat cancer.

4.2 Conclusion

Understanding how GJIC is regulated and maintained is essential in the discovery of new treatments aiming to restore homeostasis and normal cell-to-cell interaction. In this study we found that synthetic and natural naphthoquinones, specifically 2-methoxy-1,4-naphthoquinone (MeONQ) and 2,3-Dimethoxy-1,4-naphthoquinone (DMNQ) were able to attenuate the levels of GJIC. The decrease in GJIC was found to be a result of Connexin 43 phosphorylation that was mediated via the induction of epidermal growth factor receptor (EGFR) and its downstream extracellular signal-regulated kinases (ERK 1/2). In conclusion, Redox-cycling naphthoquinones (with exclusive redox- cyclers, such as DMNQ, and alkylating/redox-cycling naphthoquinones, such as MQ and MNQ) stimulate connexin phosphorylation and a loss of GJIC.

4.2 Future directions

1- Utilize *in vitro* and *in vivo* models to correlate between the ability of naphthoquinones to produce reactive oxygen species and the decrease in GJIC

2- Elucidate the involvement of alkylation and redox recycling in the naphthoquinones activation of EGFR

3- Investigate different kinases besides ERK 1/2 that cause the phosphorylation of Cx43

4- Use immunohistochemistry techniques to enhance our understanding of the posttranslational modification of Cx43

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