Fibroblast growth factor-2 protects neonatal rat cardiac myocytes from doxorubicin-induced damage via protein kinase C-dependent effects on efflux drug transporters

by

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Dedicated to my grandmother, mother and father

TABLE OF CONTENT

ABSTRACT

Introduction: Therapeutic agents like doxorubicin, an anthracycline antibiotic drug, are widely used in cancer chemotherapy. The use of doxorubicin is limited however by an increased risk of cardiac damage as a side effect, and an increased cancer cell drug resistance mediated by efflux drug transporters. Strategies are needed to protect the heart and still allow the benefits of drug treatment. “Basic” fibroblast growth factor-2 (FGF-2) is a multi-functional protein. It is angiogenic and cardioprotective against ischemia-reperfusion injury. FGF-2 can also regulate cancer cell drug resistance or sensitivity, however, so far, there is no evidence that FGF-2 protects against doxorubicin-induced cardiac damage through effects on efflux drug transporter levels or function.

Aims: To investigate whether: (1) FGF-2 can increase resistance to doxorubicin-induced neonatal rat cardiac myocyte damage; and if so whether (2) an effect on efflux drug transporters might contribute to this cardioprotection by FGF-2.

Methods: Neonatal rat cardiac myocyte cultures were treated with doxorubicin in the absence or presence of pre-treatment with FGF-2. To assess cell damage: (i) culture medium was tested for lactate dehydrogenase (LDH) activity as an indication of plasma membrane disruption; (ii) cells were stained with fluorescent apoptosis and necrosis biomarkers as well as (iii) terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and acridine orange to assess DNA fragmentation or compaction. The role of FGF receptor (FGFR) or protein kinase C (PKC) was addressed through use of inhibitors including SU5402, or chelerythrine as well as bisindomaleimide. Multidrug resistance gene 1a and 1b (MDR1a, 1b), multidrug resistance gene 2 (MDR2) and multidrug

resistance-related protein 1 (MRP1) gene expression, as well as the function of MDRs and MRPs protein products were assessed by real-time reverse transcriptase-polymerase chain reaction (qPCR), as well as retention/extrusion of (fluorescent) doxorubicin/calcein in cardiac myocytes, respectively. Efflux drug transporter inhibitors, including 20 μM cyclosporine A (CsA), 2 μM verapamil and 1 μM Tariquidar (XR9576) were used to asssess for a direct effect of FGF-2 on transporter function. Fluorescence-activated cell sorting (FACS) was used to measure fluorescent doxorubicin/calcein levels inside treated cardiac myocytes.

Results: Doxorubicin increased the incidence of programmed cell death, DNA damage, and lysosome and LDH activity, while decreasing cell number at 24 hours. FGF-2 prevented the detrimental effects of doxorubicin. In turn, the protective effects of FGF-2 were blocked in the presence of FGFR or PKC inhibitors. FGF-2 treatment significantly increased MDR1a, MDR1b, MDR2, MRP1 RNA levels by qPCR, and protein levels as assessed by function, and specifically extrusion of doxorubicin/calcein, in the presence of doxorubicin when compared to doxorubicin treatment alone. Furthermore, inhibition of efflux drug transporters with CsA and Tariquidar (XR9576) significantly reduced the ability of FGF-2 to protect against doxorubicin-induced damage; the beneficial effect of FGF-2 was completely blocked by pretreatment with verapamil.

Conclusion(s): These data indicate for the first time that exogenous FGF-2 can increase resistance to doxorubicin-induced neonatal rat cardiac myocyte damage, and implicate PKC and regulation of efflux transporter protein levels and/or function in the mechanism.

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LIST OF TABLES

LIST OF FIGURES

CHAPTER I: INTRODUCTION

CHAPTER II: HYPOTHESIS AND OBJECTIVES

CHAPTER III: MATERIALS AND METHODS

CHAPTER IV: RESULTS

ABBREVIATIONS

ABC transporters	ATP-binding cassette transporters
ABCB1	ATP-binding cassette sub-family B member 1
ABCB4	ATP-binding cassette sub-family B member 4
ABCC1	ATP-binding cassette sub-family C member 1
ABCC2	ATP-binding cassette sub-family C member 2
AKR	aldehyde-type reductase
Akt	protein kinase B
AM	acetomethoxy
Ang II	angiotensin-II
ANOVA	analysis of variance
ATP	adenosine-5'-triphosphate
B2M	beta-2-microglobulin
BCRP	breast cancer drug resistance protein
BIM-1	bisindolylmaleimide I
BSA	bovine serum albumin
CBR1	carbonyl reductase 1
Chel	chelerythrine
CK2	casein kinase 2
CsA	cyclosporine A
CT	cycle threshold
DAG	diacylglycerol
DNA	deoxyribonucleic acid

DOX	doxorubicin
EC50	effective concentration 50
EGF	epidermal growth factor
EPO	erythropoietin
ERK1/2	extracellular signal-related kinases 1 and 2
FACS	fluorescence activated cell sorting
FGF	fibroblast growth factor
FGF-2	fibroblast growth factor 2
FGFR	FGF receptor
FITC	fluorescein isothiocyanate
Hi-FGF-2	high molecular weight FGF-2
Ig	immunoglobulin
IGF-1	insulin like growth factor-1
IL-1β	interleukin-1β
JNKs	c-Jun N-terminal kinases
kDa	kilodalton
LDH	lactate dehydrogenase
Lo-FGF-2	low molecular weight FGF-2
MCF-7	Michigan Cancer Foundation-7
MDR1a	multidrug resistance gene 1a
MDR1b	multidrug resistance gene 1b
MDR2	multidrug resistance gene 2
miR	microRNA

MAPK	mitogen activated protein kinase
MRP-1	multidrug resistance related protein 1
NAD^+	nicotinamide adenine dinucleotide
$NADP^+$	nicotinamide adenine dinucleotide phosphate
nM	nanomolar
PBS	phosphate-buffered saline
P-gp	p-glycoprotein
PKC	protein kinase C
PKCε	protein kinase C epsilon
PLC	phospholipase C
PI3K	phosphatidylinositol-3-OH kinase
qPCR	quantitative real time polymerase chain reaction
RNA	ribonucleic acid
ROS	reactive oxygen species
SCF	stem cell factor
topo II	topoisomerase II
TUNEL	terminal transferase dUTP nick end labeling

CHAPTER I: INTRODUCTION

Part 1. Cardiovascular Diseases

Cardiovascular disease, collectively referring to diseases involving the myocardium and/or blood vessels, is the leading cause of death in North America [1]. In Canada, cardiac disease is the number one killer of women [2]. There are many causes of cardiac damage that can lead to heart failure including hypertension, coronary heart disease, vascular injury, atherosclerosis, infection, type II diabetes and life style choices (like smoking and stress), but some, like aging and genetics, cannot be avoided [3-7]. In addition, therapeutic agents used in the treatment of other diseases, like cancer, can be cardiotoxic and result in injury and/or heart failure, thereby limiting their usefulness [8-10]. Anti-cancer anthracycline drugs such as doxorubicin are associated with an increased and cumulative risk of cardiac damage, thus strategies are needed for protecting the heart from the causes of heart damage [11].

Life style choices, such as following a low fat or low cholesterol diet, appropriate exercise and good habits (like no smoking and less stress) could help sustain a healthy heart status and increase the heart resistance to diseases [12, 13]. Clinical and pharmacological intervention including calcium channel blockers, angiotensin-receptor blockers (ARBs), and ACE inhibitors for hypertension and heart failure, have significantly increased survival and life quality of patients [14, 15]. Hormones and growth factors like erythropoietin (EPO), insulin like growth factor-1 (IGF-1), and fibroblast growth factors (FGFs), as well as microRNA (miR) like miR-494, are

increasingly under investigation by the medical research community as potentially new biological approaches to interfere with cardiac damage by anti-cancer drugs [16-18].

Part 2. Doxorubicin

The anthracycline antibiotic, doxorubicin, is a metabolite of *Streptomyces peucetius var. caesius* that is widely used in cancer chemotherapy for leukemias, Hodgkin's lymphoma, multiple myeloma, as well as cancers of the breast, stomach, lung, ovaries, and others [19-25]. Doxorubicin is among the most effective anti-cancer drugs, but its metabolites are responsible for doxorubicin-associated cardiotoxicity [26, 27]. Doxorubicin can also be exported directly from cells without being metabolized [28]. Thus, understanding doxorubicin metabolism and its mechanism for inducing injury may provide additional targets for therapeutic intervention to maintain drug effectiveness as a chemotherapeutic agent but also to reduce or eliminate damaging and potentially lethal side effects.

I.2.1 Doxorubicin Metabolism

Metabolites of doxorubicin like doxorubicinol and reactive oxygen species (ROS) cause oxidative stress and are reported to contribute to its toxicity [28, 29]. There are four major pathways of doxorubicin metabolism (Figure 1.1):

(1) One-electron reduction of doxorubicin to form doxorubicin-semiquinone:

Oxidoreductase catalyzes the transfer of electrons from doxorubicin to an electron acceptor using NAD^+ and $NADP^+$ as cofactors to form doxorubicin-semiquinone radicals, for example, NADH dehydrogenases, NADPH dehydrogenases, and nitric oxide synthases [30-33]. However, re-oxidation of the doxorubicin-semiquinone radical back to

doxorubicin leads to oxidative damage by formation of ROS, including hydrogen peroxide (H_2O_2) [29]. In addition, lipid peroxidation is mediated by the release of iron in the process of re-oxidation of the doxorubicin-semiquinone radical back to doxorubicin [30]. Some consider that it's the oxidative stress caused by doxorubicin-semiquinone, and not doxorubicin itself, that is responsible for doxorubicin's chemotherapeutic effects and adverse cardiotoxicity [26, 27].

(2) Two-electron reduction of doxorubicin to doxorubicinol

It has been reported that the major metabolic pathway of doxorubicin involves enzymic reduction of a carbonyl group in the side chain of doxorubicin, yielding a secondary alcohol metabolite doxorubicinol [28, 29]. Aldehyde-type reductase(s) (AKR1A) of the aldo-keto reductase family is the predominant enzyme linked to reduction in the heart, and carbonyl reductase 1 (CBR1) is predominantly employed in the liver [34, 35]. It has been reported that doxorubicinol is responsible for the chemotherapeutic effects of doxorubicin as well as the adverse cardiotoxicity [29].

(3) Deglycosidation

Doxorubicin can also be reduced enzymatically to deoxyaglycone, or hydrolyzed to hydroxyaglycone [27]. The enzymes involved in these relatively minor metabolic pathways are less well characterized [36].

(4) Removal by efflux of drugs

The simple influx and efflux of drugs out of the cell without being metabolized accounts for almost half (~50%) of the doxorubicin that is eliminated from the human body during cancer treatment [28]. This activity is driven by a type of ATP dependent protein that can transport doxorubicin without metabolism [37]. This pathway that leads to the extrusion or transport of doxorubicin outside of the cell without causing any significant damage obviously decreases the anti-cancer efficiency of the drug.

It must be stressed that although ~50% of the doxorubicin is transported out of the cell without being metabolized, the retained doxorubicin is still significant and sufficient to damage the cells. As such, doxorubicin is considered an efficient and effective anti-cancer drug when compared to others [38, 39]. Nonetheless, given the role of doxorubicin transport as well as metabolism in determining a concentration at which differential damage of one cell type versus another might occur, there is a benefit in understanding these processes in an effort to improve outcomes (both in terms of the cancer and heart cells) with the use of this drug.

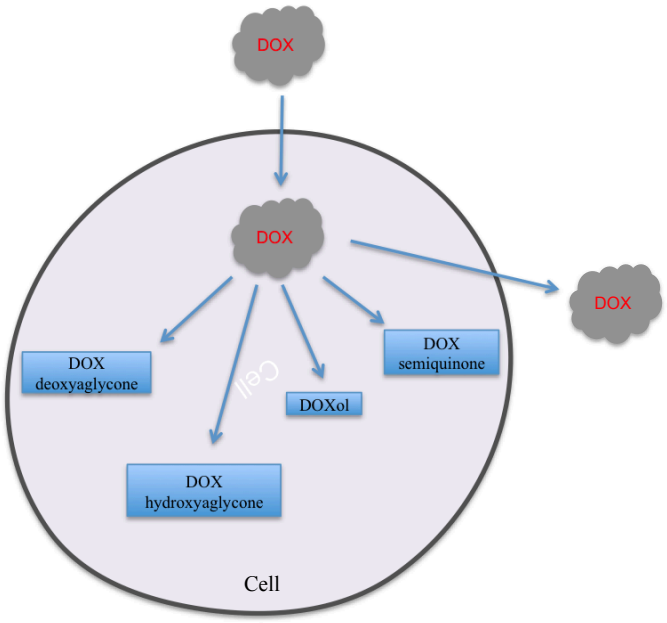

Figure 1. 1 - Schematic illustrating major metabolic routes linked to clearance of doxorubicin

(1) One-electron reduction of doxorubicin (DOX) to form doxorubicin-semiquinone; (2) two-electron reduction of doxorubicin to doxorubicinol (DOXol), and (3) doxorubicin deglycosidation. (4) Approximately 50% of doxorubicin is cleared from cells in an unmetabolized state. This figure is based on the PharmGKB doxorubicin pathway (pharmacokinetcs) diagram from Stanford University (Permission was given to reproduce this diagram).

I.2.2 Mechanisms of doxorubicin anti-tumor activity

Several mechanisms have been proposed to explain doxorubicin's anti-tumor activity [40]. Unfortunately, the mechanisms responsible for killing cancer cells are also the mechanisms that damage cardiac myocytes. Understanding these mechanisms would provide us with different perspectives and strategies to increase drug efficiency and prevention of adverse cardiotoxicity. There are three major mechanisms associated with cell toxicity of doxorubicin (Figure 1.2):

(1) Intercalating into the DNA and RNA structure and interfering with function. Inhibits DNA and RNA synthesis by intercalating between base pairs of the DNA/RNA strand, thus preventing the replication (DNA) and synthesis (RNA and, indirectly, protein) in rapidly growing cancer cells [41].

(2) Inhibits the topoisomerase II (topo II) enzyme. This will prevent the relaxation of supercoiled DNA and block DNA replication and transcription. Also, topo II inhibitors prevent topo II from turning over which is needed for dissociation of topo II from its nucleic acid substrate [42].

(3) Oxidative stress. Doxorubicin creates iron-mediated free oxygen radicals that can damage the DNA and cell membranes [43].

Doxorubicin is cytotoxic and has been reported to induce programmed cell death, including apoptosis and/or necrosis in different cell types [44-46]. Apoptosis is a major type of cell damage caused by doxorubicin [47]. It is characterized by DNA fragmentation, shrinking of cytoplasm, blebbing of the plasma membrane without loss of

integrity, mitochondria becoming leaky due to pore formation involving proteins of the bcl-2 family, and formation of membrane bound vesicles (apoptotic bodies) [47]. Necrosis is another major type of cell death induced by doxorubicin and can cause massive inflammatory response, characterized by swelling of the cytoplasm and mitochondria, as well as loss of membrane integrity and cell lysis [48].

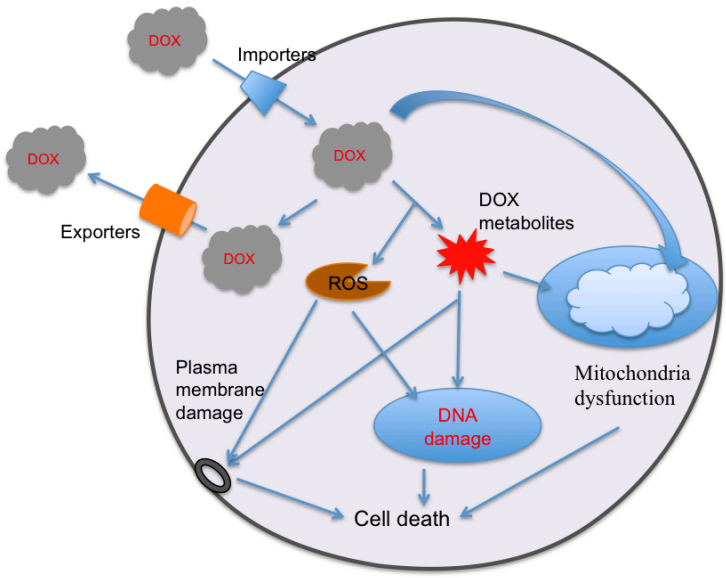

Figure 1. 2 - Two major mechanisms of doxorubicin-induced cell damage

DNA damage mediated by doxorubicin inhibits topoisomerase II activity and/or intercalates with DNA. Generation of free radicals during doxorubicin metabolism can induce both mitochondria dysfunction and plasma membrane damage. Abbreviations: doxorubicin, DOX; reactive oxygen species, ROS. This figure is based on the PharmGKB doxorubicin pathway (pharmacodynamics) diagram from Stanford University (Permission was given to reproduce this diagram).

I.2.3 Limitation of doxorubicin for cancer chemotherapy

Doxorubicin is an effective anti-cancer drug and has a broad spectrum of anti-cancer activity [49]. There are, however, two major obstacles that limit its effectiveness in a clinical setting, specifically, severe cardiac side effects and an increased cancer cell drug resistance [50]. These are discussed below.

I.2.3.1 Increased doxorubicin cardiotoxicity (side effects)

Doxorubicin has severe adverse side-effects, including vomiting, nausea, bowel infection, neutropenia, hair loss and an increased and cumulative risk of cardiac damage that can lead to death [51-55]. When the cumulative dose of doxorubicin reaches 550 mg/m², the risk of developing cardiac side effects, including heart failure, dilated cardiomyopathy, and even death, dramatically increased. It is estimated that approximately 1 in 10 patients treated with doxorubicin or its derivatives will develop cardiac complications up to 10 years after the last chemotherapy session [51]. Due to these side effects and its red color, doxorubicin has earned the nickname "red devil" or "red death".

Cardiac myocyte death by apoptosis and necrosis is a primary mechanism of doxorubicin-induced cardiomyopathy; other types of cell death, such as autophagy and senescence/aging, may also participate in this process [44]. The clinical effects of doxorubicin, observed as transient electrocardiographic changes and cardiomyopathy, have been modeled in rat cardiac cells, including neonatal cardiac myocytes [56-60]. Specifically, concentrations of 0.1-1.0 μM doxorubicin are reported to cause neonatal rat cardiac myocyte mitochondrial swelling and disruption of the plasma membrane, which are features of injury leading to cell death. Detection of normally intracellular LDH

activity outside the cell, as in the plasma or in the medium when cardiac myocytes are isolated and maintained in culture, is an indicator of membrane disruption and damage [58].

Strategies are needed to reduce the risk of heart damage while still allowing the benefits of doxorubicin treatment in terms of anti-cancer activity. Pharmocological approaches like the use of dexrazoxane, reduces the number of metal ions complexed with doxorubicin to decrease the formation of superoxide radicals, thus decreasing the toxicity of doxorubicin [61]. A nutritional approach of using vitamin C was able to decrease 48% of doxorubicin-induced release of reactive oxygen species and creatine kinase, as well as decrease 30% of P53 activity, thus offering some protection from doxorubicin-induced carditoxicity [62]. In addition, there is a number of factors produced by the heart that are reported to exert cytoprotection and cardioprotection to cardiac injury, such as hormones and growth factors [63, 64]. Thus, it is possible that endogenous resistance to cardiac injury, as a result of hormones, growth factors or other factors produced by heart, may already provide some protection from the cardiotoxicity of drugs like doxorubicin. For example, endogenous neuregulin offers some protection to the myocardium, while anthracycline trastuzumab partly blocked this protection and induces cardiotoxicity [65]. Endogenous levels of protection may not be optimal, however, in the context of chemotherapy. Thus, characterizing these endogenous cardioprotective factors, including how they function in an injury setting, may point to therapeutic strategies including supplementation with exogenous factors, to boost resistance against doxorubicin-related cardiotoxicity.

I.2.3.2 Increased cancer cell drug resistance

In addition to cardiotoxicity, cancer cell resistance is another obstacle that limits the effectiveness of chemotherapeutic agents like doxorubicin [66]. Overcoming the resistance of cancer cells to doxorubicin would increase the effectiveness of chemotherapy. There are a variety of mechanisms of cancer drug resistance including:

(1) Increased efflux, thereby reducing the drug concentration in the cancer cell [67];

(2) Enzymatic drug inactivation, deactivation or modification [68];

(3) Decreased permeability that prevents drugs from entering the cell [67];

(4) Altered binding-sites to prevent the interaction of cancer cells with drugs [69, 70];

(5) Compensation for the drug effect via use of alternate metabolic pathways [69];

In the case of doxorubicin, multidrug resistance due to an increase in efflux drug transporters such as multidrug resistance protein (MDRs) and multidrug resistance related protein-1 (MRPs), is a well-established cause [71-73]. These drug transporters will now be introduced.

Part 3. Efflux Drug Transporters

Efflux drug transporters function as pumps to move various molecules across extra- and intra-cellular membranes [74]. The expression of efflux drug transporters is one of the normal cellular "self-defense" systems against xenobiotics, including doxorubicin [75, 76]. ATP-binding cassette (ABC) efflux drug transporters consist of two distinct domains, an ATP binding site domain located in the cytoplasm and a transmembrane domain (Figure 1.3). ATP binding and hydrolysis facilitates conformational change of the transmembrane domain of the transporters, facilitating opening and efflux of substrates

[77].

Intracellular doxorubicin accumulation is a complex process including doxorubicin influx into the cell, retention and distribution in the cell, and efflux from the cell. At any given time, the net uptake (accumulation) of doxorubicin in the cell is the difference between the amount of influx and efflux. Efflux drug transporters can mediate doxorubicin removal, thus decreasing intracellular net uptake and thereby cause cancer cells to become more resistant to doxorubicin.

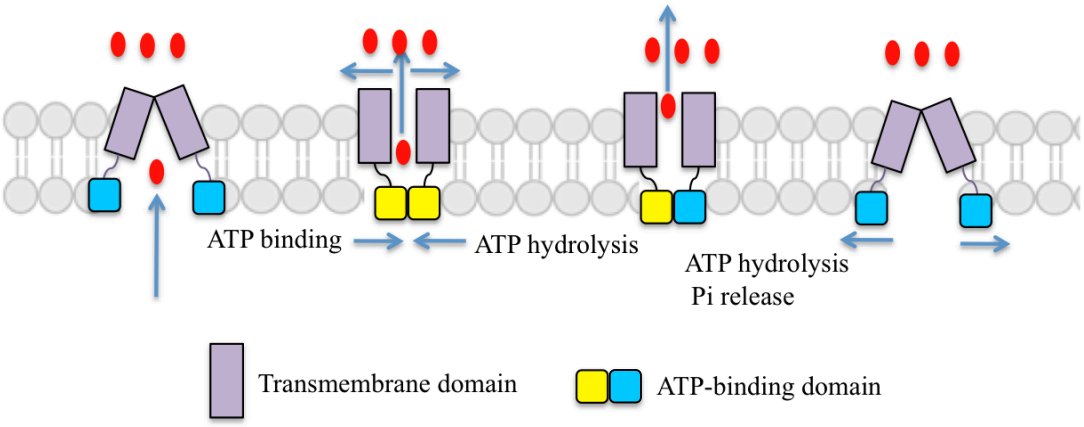

Figure 1. 3 - ATP-binding cassette (ABC) efflux transporters

ABC efflux transporters export xenobiotics, including doxorubicin, out of cells using ATP as an energy source. ABC efflux drug transporters consist of two distinct domains, an ATP binding site domain located in the cytoplasm and a second transmembrane domain. ATP binding and hydrolysis facilitates a conformational change in the transmembrane domain to open and allow efflux of substrates. This diagram was based on work from Alexanderaloy and Stargonzales and was adapted from Dong, J.; G. Yang; H.S. Mchaourab. 2005. Science 308, 1023-1028. Permission was given to use this work for any purpose, without any conditions, unless such conditions are required by law.

I.3.1 Major efflux drug transporters

The major **human, rat and mouse** efflux drug transporters that are expressed in both heart and cancer cells and have been linked to doxorubicin removal or retention are described below:

(1) Multidrug resistance protein 1 (MDR1, ABCB1)

Multidrug resistance protein 1 (MDR1) or ATP-binding cassette sub-family B member 1 (ABCB1), is encoded by the ABCB1 gene [78]. It is a well-known efflux ABC-transporter that transports a wide variety of substrate (hydrophobic, neutral, and positively charged drugs) from the inside to the outside of cells [79]. MDR-1 is extensively distributed and expressed in intestinal epithelium, capillary endothelial cells, hepatocytes, as well as in heart cells [80, 81]. The levels of the MDR-1a and 1b among the three MDR mRNAs has been reported for 13 mouse tissues: liver, kidney, stomach, duodenum, jejunum, ileum, colon, heart, lung, brain, testis, ovary, and placenta [82]. MDR1a RNA is predominant in colon (~100%), but only accounts for 9% of MDR RNA in the heart. By contrast, MDR1b expression is higher in ovary in the female (~100%), but is also relatively high (45%) in the heart [72, 82]. In some cancers, including breast and bladder, the over-expression of MDR-1 is correlated with increased resistance to chemotherapeutic drugs that cause decreased survival and poor prognosis [83, 84].

(2) Multidrug resistance protein 2 (MDR2, ABCB4)

Multidrug resistance protein 2 (MDR2) is also a member of the ABCB transporters family, and in humans is encoded by the ATP-binding cassette sub-family B member 4 (ABCB4) gene [85]. MDR2 is a membrane protein and functions as an efflux drug,

including doxorubicin, transporter and contributes to drug resistance [86, 87]. In terms of RNA levels from 13 mouse tissues for the three MDRs that have been reported, MDR2 is predominantly expressed in liver, but it is also relatively highly expressed in heart (38.5%) [72, 82].

(3) Multidrug resistance-associated protein 1 (MRP1, ABCC1)

MRP1 is the first member of the ATP-binding cassette sub-family C member (ABCC1). It was initially found as a multi-specific organic anion transporter, and transports cysteinyl leukotrienes, glucuronides and sulfate conjugates of steroid hormones and bile salts [88-90]. Later on, MRP1 was discovered as an abundant efflux drug transporter that contributes to drug resistance of cancer cells. For example, MRP1 are components of the multifactorial multidrug resistance phenotype of lung cancer [91].

(4) Multidrug resistance-associated protein 2 (MRP2, ABCC2)

Multidrug resistance-associated protein 2 (MRP2) is a protein that in humans is encoded by the ATP-binding cassette sub-family C member 2 (ABCC2) gene. MRP2 is a member of the ABCC/MRP subfamily, and is known to be expressed on the apical side of the hepatocyte, and the apical membrane of proximal renal tubule endothelial cells [92]. MRP2 transports bile acid, as well as anti-cancer drugs, including doxorubicin and vinblastine [93]. As such, MRP2 has been linked to the development of anti-cancer drug resistance in mammalian cells [94].

I.3.2 Efflux drug transporter modulators/inhibitors

Drug efflux is a significant contributor to multidrug resistance in cancer cells, and as such current research is mostly aimed at modulating or blocking specific efflux mechanisms, in an attempt to overcome the resistance due to MDRs or MRPs overexpression [95]. In 1981, it was reported that treatment with verapamil can reverse drug resistance, and since then, efflux drug transporter inhibitors have been intensively studied as potential drug resistance reversers [96]. Three generations of efflux drug transporter inhibitors have been developed and, including verapamil, are reviewed briefly below:

(1) First generation drugs: cyclosporine A and verapamil

In the beginning, agents were not specifically developed for inhibiting efflux drug transporters. These agents were used in the clinic for other pharmacological effects, and may have relatively low affinity for drug transporters [95]. Examples of first generation efflux drug transporter inhibitors include cyclosporine A (CsA), a competitive inhibitor, and verapamil, a non-competitive inhibitor. CsA is a non-steroidal agent used clinically for immunosuppression, and was developed as an inhibitor for MDR1, MRP2 and a modulator for MRP1 [97, 98]. Verapamil is an effective L-type calcium channel blocker, that has been used clinically for cardiovascular disease, such as in the treatment of hypertension, angina pectoris, and cardiac arrythmia [99]. Verapamil was subsequently identified as an efflux drug inhibitor of MDR1, MRP2 and modulator of MRP1 [100-102].

(2) Second generation: dexverapamil or dexniguldipine

Due to the broad pharmacological properties of the first generation, the second generation of agents were developed to exhibit higher affinity and specificity of inhibition with less potential broad/side effects, such as dexverapamil or dexniguldipine [103]. Dexverapamil can competitively inhibit the efflux drug transporter MDR1 with decreased calcium antagonistic activity and toxicity, compared to verapamil. Besides higher specificity, dexniguldipine-HCl can accelerate dissociation of tritiated vinblastine from MDR1, while CsA does not alter the kinetics of tritiated vinblastine dissociation [104].

(3) Third generation: tariquidar (XR9576)

A third generation of P-glycoprotein (P-gp) inhibitors has now emerged with even higher affinity for MDRs and MRPs at nanomolar concentrations, as well as less broad/side effects [105]. This includes the compound tariquidar (XR9576), which has passed phase II clinical trials successfully, and is now going into a phase III clinical trial [106].

Although several generations of efflux drug modulators/transporters have been developed, the specificity and mechanisms of the modulating effects are still not well understood [107]. Clinical trials of third generation efflux drug transporter inhibitors have not all ended successfully, having triggered severe side effects such as cancer development [106]. Thus, the task of discovering other modulators of drug transporters has attracted attention [108, 109]. Efforts has been made to try to discover endogenous modulators that may work as transcription factors to regulate drug transporter or manipulate drug influx or efflux, through the use of hormones, growth factors and other agents [110, 111].

I.3.3 Efflux drug transporters in the heart

MDRs, MRPs and other efflux ABC transporters are also expressed in the myocardium in human and rat heart; this includes relatively high levels of MDR-1, MRP1 and MDR2, and relatively low levels of MRP2 expression [112, 113]. A review of the recent literature suggests that ABC transporters have a role in controlling distribution of xenobiotics to the heart, thus protecting this organ [113]. For example, MRP1 may protect the heart by mediating the efflux of toxic products of oxidative stress from mitochondria and cardiac myocytes [114]. As such, increased efflux drug transporter levels that lead to decreased drug uptake in the heart would dramatically improve heart resistance to anti-cancer drugs. Thus, identification of effective modulators/inhibitors of efflux drug transport may provide a new perspective of rescuing or protecting a heart from cardiac injury induced by drugs.

Part 4. Fibroblast Growth Factor 2 and Cardioprotection

Efforts have been made to increase myocardial resistance to acute and chronic cardiac damage from anti-cancer drug side effects. Possible avenues include identification of pharmacological agents that might (a) interfere with (or protect from) side effects of anti-cancer drugs, or (b) induce regenerative and/or an angiogenic response in a damaged heart [17]. It is reported that erythropoietin, a modified anthracycline *N*-Benzyladriamycin-14-valerate (AD 198) [115, 116], and supplementation with the antioxidant L-carnitine [117], can protect against doxorubicin-induced cardiomyopathy. Additional strategies like regulating growth factor levels have also been reported as protective against doxorubicin-induced damage, including treatment with insulin-like

growth factor 1 (IGF-1) and fibroblast growth factors (FGFs) [118-120]. Thus, these growth factors have the potential to be developed as therapeutic agents to protect against doxorubicin-induced cardiac damage.

I.4.1 Fibroblast growth factor -2

Fibroblast growth factors, or FGFs (FGF-1 to FGF-23), are a family of growth factors involved in angiogenesis, wound healing, embryonic development and in proliferation and differentiation of wide variety of cells [121-124]. The FGF proteins share a heparin-binding domain and the ability to interact with cell-surface-associated heparan sulfate proteoglycans, and this binding is essential for FGF signal transduction via their receptors [125]. FGFs bind predominantly to one of four tyrosine kinase FGF receptors (FGFR1-4) and activate a series of signal transduction pathways and cellular events [125], including regulation of myocardial and vascular cell survival, growth and differentiation, under physiological conditions and in response to injury.

FGF-2 is also known as “basic” FGF (bFGF). In humans it is produced as a 18 kilodalton (kDa) low molecular weight (Lo-) FGF-2, and 20-34 kDa high molecular weight (Hi-) FGF-2 isoforms that are transcribed from the same FGF-2 mRNA, but with different start codons, methionine (AUG) and leucine (CUG), respectively (Figure 1.4). FGF-2 is produced by most cells and tissues, including heart and slow skeletal muscles [126]. In heart, FGF-2 is produced by both cardiac fibroblasts and myocytes, where it is released on contraction as well as after cell injury [17]. FGF-2 will bind to the extracellular matrix/heparan sulfate proteoglycans, where it is available to work in both an autocrine and paracrine manner [127, 128].

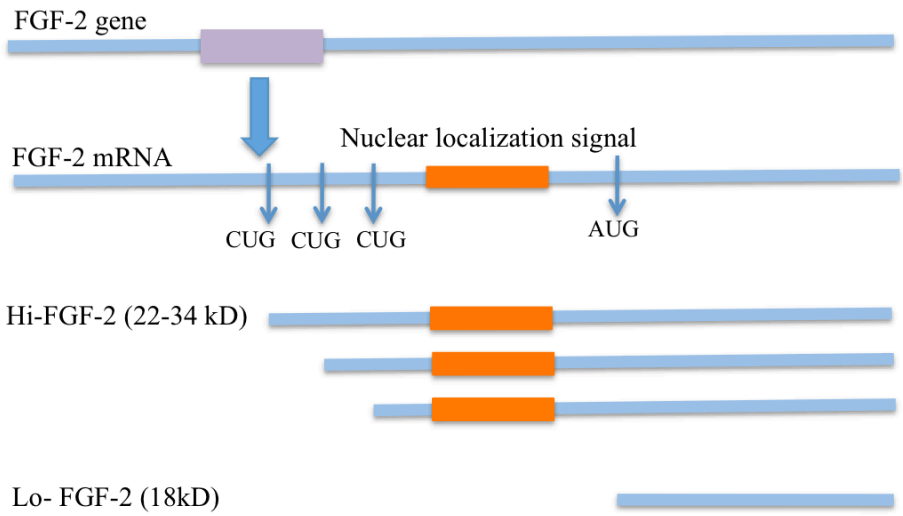

Figure 1. 4 – Human High (Hi-) and Low (Lo-) molecular weight FGF-2 isoforms

Hi-FGF-2 and Lo-FGF-2 are translated from the same FGF-2 mRNA with a different start codon, CUG and AUG, respectively. Hi-FGF-2 contains a nuclear localization signal that enables nuclear transportation [129, 130].

There are several mechanisms proposed to explain FGF-2 release, given its lack of classic export signal sequence: (1) released by dying cells during tissue injury [17]; (2) plasma membrane vesicle shedding [17]; (3) Na^+/K^+ ATPase pump also involved in the process of exporting FGF-2 in several cell lines [17]; (4) cardiac myocytes release FGF-2 on contraction on a beat-to-beat basis, and via stretching in fibroblasts [17]; (5) cardiac fibroblasts can export Hi-FGF-2 in response to pro-hypertrophic stimuli, such as angiotensin II [129]; (6) another secretory pathway of Hi-FGF-2 requiring activated caspase-1 [129]

After release, FGF-2 is retained by the extracellular matrix in the heart, where it provides an environment to potentially support the growth of cells, and confer some resistance to cardiac injury [131, 132]. Hi-FGF-2 isoforms are hypertrophic and can induce chromatin compaction (apoptosis-like damage) [17, 133]. By contrast, Lo-FGF-2 is a mitogen for embryonic development and a potent survival agent for many cell types after birth [17, 126, 134-141]. FGF-2 regulates cell proliferation, migration, and angiogenesis both in vitro and in vivo [142-144], FGF-2 exerts direct pre-conditioning and post-conditioning like cardioprotection from acute ischemia and reperfuiosn injury, independently of its angiogenic ability [17]. In addition, FGF-2 has the potential to induce cardiac regeneration, such as upregulate stem cell factor (SCF), a cytokine that binds to the c-Kit receptor (CD117), and a potent homing signal for mesenchymal cells going to areas of cardiac injury [145]. Consistent with many of these properties, FGF-2 deficient mice are associated with decreased endothelial proliferation and are more susceptible to injury [141, 146]. The known direct acute cardioprotection properties besides angiogenesis *in vitro* and *in vivo* is particularly relevant to this thesis [17]. In addition, FGF-2 gene

expression is regulated by many stress stimuli, including hypoxia, ischemia, as well as in response to angiotensin-II (Ang II) and adrenergic stimulation [17, 147]. FGF-2 can also increase its own gene expression in isolated cardiac myocytes [17]. Stimulated FGF-2 levels contribute to cardioprotective effects in response to damage/injury. Thus, the cardioprotective effects of FGF-2 will be further explored below with details of the signaling pathway involved in cardioprotection.

I.4.2 Signaling pathways of FGF-2 and cardioprotection

The biological activities of extracellular FGF-2, including cardioprotection, are mediated by binding to one or more high-affinity FGF receptors (FGFR1-4) to recruit and phosphorylate other signaling molecules culminating in the activation of major signal transduction pathways.

I.4.2.1 FGF receptors

There are five members of the FGF receptor family. FGFR (1-4) consist of an extracellular ligand binding domain, a transmembrane helix domain and an intracellular tyrosine kinase domain, while FGFR5 lacks the tyrosine kinase domain [148, 149]. The extracellular ligand-binding domain of FGFR (1-4) is composed of up to three immunoglobulin (Ig)-like domains and an “acid box” between immunoglobulin I and immunoglobulin II that form the binding site for FGF-2 [17, 126]. This “acid box” is a short stretch of acid amino acids that has auto-inhibitory functions. Interference with ligand binding or inhibition of tyrosine kinase activity will interrupt FGF-induced signaling.

I.4.2.2 Intracellular signaling pathways

Signal transduction pathways that can be recruited by FGF-2/FGFR binding includes all three branches of the mitogen activated protein kinase (MAPK pathway), c-Jun N-terminal kinases (JNKs), extracellular signal-related kinases 1 and 2 (ERK1/2), and p38, as well as the phospholipase C (PLC)-protein kinase C (PKC), and Src-associated pathways [17, 126, 131, 150-152]. FGF-2/FGFR can also activate casein kinase 2 (CK2) through the interaction of the beta-subunit of CK2 in the nucleus to induce a mitogenic response of cells [138, 150, 153-155]. In addition, activation of phosphatidylinositol-3-OH kinase-AKT (PI3K-Akt) by FGF-2 also stimulates cell survival and growth, as well as cardiac valve interstitial cell repair [156-159].

I.4.2.3 Cardioprotective signaling pathways

The protective effects of FGF-2 have been linked to PKC-dependent cascades, the ERK as well as the PI3K-Akt signaling pathways [160, 161]. In addiotion, other signaling pathways like nitric oxide and the nitric oxide synthases are involved in preconditioning cardioprotection by FGF-2 [162].

PKC is a family of serine/threonine kinases that have emerged as important regulators of cardiac contraction, hypertrophy, and signaling pathways that influence ischemia/reperfusion injury [163]. There are multiple PKC isoforms and some of the isoforms have specific actions in the heart [163] (Table 1). This specific activation and action offers potential development of PKC-targeted therapeutics [163]. For example, PKCα is the predominant isoform in most cardiac myocytes. The expression and activity of PKCα increases in models of cardiac injury, hypertrophy or heart failure [163]. The

expression and activity of PKCβ are increased in end-stage heart failure, while PKCδ is activated or increased in many models of cardiac ischemia or hypertrophy [163]. PKCε is also activated or increased in models of hypertrophy, but recent studies have focused on the cardioprotective effects of PKCε in ischemia pre-conditioning or ischemia reperfusion injuries [163, 164].

Table 1: List of PKC isoforms and mechanisms of activation

Conventional	PKC-α	Requires DAG, Ca^{2+}, and phospholipids for activation
	PKC-β	
	PKC-γ	
Novel	PKC-δ	Require DAG but not Ca^{2+} for activation
	PKC-δ1	
	PKC-δ2	
	PKC-δ3	
	PKC-ε	
	PKC-η	
	PKC-θ	
Atypical	PKC-ι	Require neither DAG nor Ca^{2+} for activation
	PKC-τ	
	PKC-N1	
	PKC-N2	
	PKC-N3	

Ischemic preconditioning and protection from ischemia-reperfusion by FGF-2 are both sensitive to PKC activation, and especially to the episolon (ε) isoform [17]. PKCε is the principal, but not the only PKC isozyme to be expressed in the rat heart [165]. PKCε is a central contributor to cardiac injury resistance by modifying targets at the plasma membrane, mitochondria, and other subcellular sites [154, 166]. PKCε is also involved in the phosphorylation of connexin-43 within gap junctions at the intercalated discs *in vitro*

and in the adult perfused heart [159, 167]. This modification was shown to contribute to an injury resistant cardiac phenotype [159, 167]. Lo-FGF-2 overexpression in transgenic mouse hearts resulted in increased levels of membrane associated and total PKCα and PKCε activity, which would be expected to elicit cardiac protection [132]. Chelerythrine, a PKC inhibitor, can interact with the catalytic domain of PKC, and is a competitive inhibitor with respect to the phosphate acceptor (histone IIIS) and a non-competitive inhibitor with respect to ATP. Chelerythrine blocks the cardioprotective effects of FGF-2 from ischemia-reperfusion damage in isolated rat heart [155]. Bisindolylmaleimide I (BIM-1), an ATP-competitive PKC inhibitor, can suppress FGF-mediated activation of Erk MAP kinase in chondrocytes, further supporting the involvement of PKC in FGF-2 biological activities [168].

Activation of mitogen-activated protein kinase kinase (MEK) with consequent phosphorylation of extracellular signal-regulated kinases (ERKs) has been reported to mediate a pro-survival and mitogenesis phenotype that contributes to FGF-2-induced protection [169, 170]. In addition, cardioprotection induced by overexpression of cardiac specific FGF-2 in a transgenic mouse model was reported to be dependent on the ERK signaling pathway [171]. Cardiac specific overexpression of FGF-2 in a transgenic mouse resulted in increased recovery of contractile function and decreased infarct size after ischemia-reperfusion injury [171]. Inhibition of the MEK-ERK pathway with U-0126 (a MEK1 and MEK2 inhibitor) reversed the protective effects of FGF-2 against the injury [171]. In addition, western blot analysis of FGF-2 transgenic and wild type mouse hearts during early ischemia or reperfusion injury revealed signaling alterations in ERK activation [171].

In the myocardium, FGF-2 is upregulated in response to ischemia-reperfusion injury at both the transcriptional and translational level [126]. Exposure to a short period of ischemia triggers regulation of FGF-2 gene expression and/or post-translational modification resulting in an increased level of endogenous FGF-2 that, presumably, can offer protection in the event of a second ischemic event or perfusion injury [17, 150, 154]. Efforts have been made to identify possible mediators that trigger this "pre-conditioning" from FGF-2 [17]. Exogenous administration of FGF-2 can also confer protection from cardiac injury, including from H_2O_2–induced damage to neonatal rat cardiac myocyte cultures and ischemia-reperfusion injury in a Langendorff isolated heart model [17, 172]. However, the effect of FGF-2 with regard to doxorubicin-induced damage of cardiac myocytes has not been reported.

I.4.3 FGF-2 and efflux drug transporters

As suggested earlier, there is an effort to identify factors other than efflux drug transporter inhibitors that can regulate drug transporter expression or function for the purpose of modulating drug resistance of different cell types. Recent examples include epidermal growth factor (EGF) and insulin-like growth factor II (IGF-II) increase the levels and function of another type of efflux drug transporter, breast cancer resistance protein (BCRP) that specifically expressed in breast cancer cells, resulting in increased efflux activity. On the other hand, tumor necrosis factor α and interleukin-1β (IL-1β) decreases MDR-1 level and function, resulting in decreased efflux activity in placental trophoblasts and as a consequence increases fetal susceptibility to toxic xenobiotics [173].

Interestingly, FGF-2 has also emerged as a growth factor that is capable of modifying the sensitivity of normal and tumor cells to anti-cancer drugs [174-176]. FGF-2 can induce both drug resistance and drug sensitization in different cell types treated with a variety of cytotoxic agents. For example, recent advances suggest that expression of FGF-2 in tumor cells is involved with the loss of response to chemotherapy *in vivo* [177]. FGF-2 is also capable of modifying the sensitivity of cells to anti-cancer drugs, resulting in either increased drug resistance or sensitivity by affecting efflux ATP-binding cassette (ABC) drug transporters, including MDR-1 and MRP-1 [175, 177, 178]. In addition, FGF-2 has also been reported to enhance MDR-1 expression in human colorectal (HCT-15, SW620-14) cancer cells and human breast (MCF-7/MDR, MDA-MB-231/MDR) cancer cells [175]. Thus, the manipulation of FGF-2 activity in this model to increase the effectiveness of chemotherapeutic agents may have important clinical implications in cancer cells [177].

Increased drug resistance to anti-cancer drugs might be beneficial to the heart by offering cardioprotection, but at the cost of promoting cancer by decreasing its susceptibility to chemotherapy. This of course might vary between cancer types or stages of development. For example, this could reflect type and/or number of FGFRs as well as the major signaling pathways at play in the cell. Thus, an understanding of the differential cellular trafficking and biological activities of the multiple FGF-2 isoforms may help to determine the circumstances under which FGF-2 acts to inhibit versus potentiate drug action. A first step before addressing effects in cancer cells is to establish a beneficial effect of FGF-2 in terms of protection of cardiac cells from a chemotherapeutic agent like doxorubicin, and a role for efflux transport in the mechanism of cardioprotection. **To**

date, however, there is no report on the cardioprotective effects of FGF-2 against doxorubicin-induced injury in cardiac myocytes involving efflux drug transporters.

Thus, the effects of exogenous FGF-2 on cardiac myocyte survival and on efflux drug transporter levels or function in the context of doxorubicin treatment, as well as the link between these two aspects were pursued as the subject of my project and thesis.

CHAPTER II. HYPOTHESIS AND OBJECTIVES

Rationale

Doxorubicin is an effective anti-cancer drug used in chemotherapy, however, the cardiac side effects that can lead to death complicate and limit its clinical application [179]. FGF-2 protects cardiac myocytes from ischemia-reperfusion injury and PKC, especially PKCε, has been implicated in the signaling pathway [154]. There is no report, however, as to whether exogenous FGF-2 treatment can offer protection against doxorubicin-induced damage in cardiac myocytes, including neonatal rat cardiac myocyte cultures.

Besides cardiac side effects, drug resistance is another obstacle that limits its application to chemotherapy. It has been reported that about 50% of doxorubicin is extruded by efflux drug transporters without metabolism in cancer cells. FGF-2 has, however, been linked with the increased drug resistance of tumor cells to anti-cancer drugs by increasing efflux ABC drug transporters, including MDR and MRP1 [175, 177, 178]. By contrast, neonatal rat cardiac myocytes also express relatively high levels of MDR1b that has been reported to contribute to MDR-1-based drug extrusion in the heart that is beneficial [180]. There is no evidence, however, of whether FGF-2 can regulate drug transporters in the presence of doxorubicin in cardiac myocytes, including in neonatal rat cardiac myocyte cultures.

Hypothesis

FGF-2 protects neonatal rat cardiac myocytes from doxorubicin damage via protein kinase C- dependent effects on efflux transporters.

Objectives

1. To establish a "model" of doxorubicin-induced injury in neonatal rat cardiac myocyte cultures.

The effect of doxorubicin will be assessed: (a) on LDH release as an indicator of plasma membrane damage; (b) on DNA fragmentation; (c) on fluorescent markers of cell death, annexin-V for apoptosis and ethidium homodimer III for necrosis; and (d) on lysosome activity.

2: To identify the half maximal effective concentration (EC50) of FGF-2 in preventing doxorubicin-induced LDH release at 24 hours.

Neonatal rat cardiac myocytes will be pretreated with 0.2 nM, 1 nM, 2.5 nM, 10 nM and 20 nM FGF-2 before doxorubicin treatment for 24 hours and culture medium will be collected for assessment of LDH activity.

3. To assess the ability of exogenous FGF-2 (10 nM) treatment to protect against doxorubicin-induced injury in neonatal rat cardiac myocytes.

(a) Endogenous FGF-2 RNA levels with doxorubicin treatment will be assessed; then the ability of exogenous FGF-2 treatment to protect against doxorubicin-induced injury will be assessed (b) on LDH release as an indicator of plasma membrane damage, (c) on DNA fragmentation and (d) on fluorescent markers of cell death, annexin-V for apoptosis and ethidium homodimer III for necrosis.

4: To investigate a role for FGFR on any beneficial effects of FGF-2 observed in relation to doxorubicin-induced injury, using an FGFR inhibitor.

A FGF receptor tyrosine kinase inhibitor, SU5402, will be added before FGF-2 and doxorubicin, and culture medium will be collected for the LDH activity assay.

5. To investigate a role for PKC activation on any beneficial effects of FGF-2 observed in relation to doxorubicin-induced injury, using multiple PKC inhibitors.

Two different PKC inhibitors, chelerythrine and BIM-1 will be used for pre-treatment and inhibition. Culture medium will then be collected and tested for LDH activity and compared to doxorubicin alone or pre-treatment with FGF-2 before doxorubicin.

6. To test the ability of FGF-2 to regulate multi-drug resistance gene RNA levels in neonatal rat cardiac myocyte cultures.

Total RNA will be isolated from neonatal rat cardiac myocyte cultures and efflux drug transporter (MDR1a, MDR1b, MDR2, MRP1, MRP2) RNA levels will be assessed by qPCR for: (a) the ability of doxorubicin and FGF-2 to regulate multi-drug resistance gene RNA levels; and (b) assessing whether the regulation of FGF-2 on multi-drug resistance gene RNA is PKC dependent (using PKC inhibitors).

7: To test whether FGF-2 has positive effects on efflux drug transporter levels/function measured by doxorubicin retention/extrusion in neonatal rat cardiac myocytes.

The ability of FGF-2 to regulate efflux drug transport levels/function (retention or extrusion) will be assessed by fluorescence activated cell sorting (FACS) on doxorubicin autofluorescence intensity level. Retention of doxorubicin will be assessed to measure the effect of FGF-2 and doxorubicin on efflux transporter activity. As positive controls, neonatal rat cardiac myocytes will be pretreated with 20 μM CsA, an inhibitor of MDR1 and MRP2 transporters, or 2 μM verapamil, which blocks MDR1 in neonatal rat cardiac myocytes.

8. To test whether FGF-2 has positive effects on efflux transporter levels/function measured by calcein retention/extrusion in neonatal rat cardiac myocyte cultures.

The ability of FGF-2 to regulate efflux drug transport levels/function (retention or extrusion) will be assessed by FACS after pre-loading neonatal rat cardiac myocytes with fluorescent calcein. The rentention/extrusion of calcein provides an average calcein fluorescence intensity level that can be compared between each untreated and treated groups of cells.

9. To test whether the positive effects of FGF-2 on efflux drug transport contributes to the increased resistance of neonatal rat cardiac myocytes to doxorubicin-induced plasma membrane damage.

The possible relationship between the stimulation in efflux transporter and increase in resistance to doxorubicin-induced cell injury with FGF-2, as measured by LDH release, will be examined by the addition of the transport inhibitors CsA, verapamil or XR9576 to

neonatal rat cardiac myocyte cultures before treatment without or with FGF-2 and/or doxorubicin.

A diagram of the hypothesis and experimental design is shown in Figure 2.1. Each objective and observations made, and how they relate to the hypothesis, as well as the consequences of the observations made and resulting claims, will be discussed in Chapter IV.

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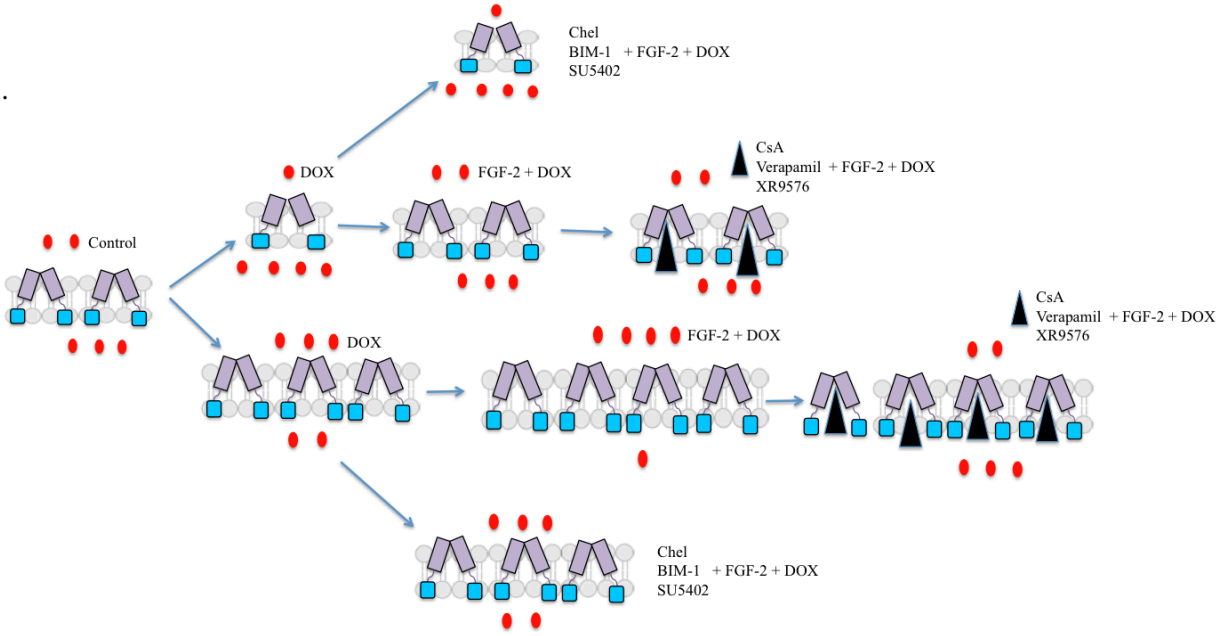


Figure 2.1 - Hypothesis and Objective

FGF-2 protects cardiac myocytes from doxorubicin damage via protein kinase C-dependent effects on efflux transporters. Abbreviations: cyclosporine A, CsA; doxorubicin, DOX.

CHAPTER III. MATERIALS AND METHODS

Animals

All animals were housed and treated according to standards and guidelines set by the Canadian Council for Animal Care. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011). The protocol for primary culture of neonatal rat ventricular myocytes was approved by the Bannatyne Campus Protocol Management and Review Committee at the University of Manitoba.

Neonatal rat cardiac myocyte primary cultures

One-day-old Sprague-Dawley rat neonates were decapitated and heart ventricles were isolated by excision and digested with collagenase (5180 units), trypsin (2590 units) and DNase (15960 units, Worthington Biochemical Corporation, Lakewood, NJ, USA) at 37 ^{o}C for 10 minutes each digestion and repeating 6 times. Cells were fractionated by centrifugation (2,000 xg using an Avanti JE, Beckman Coulter, Ontario, Canada) on a Percoll (GE Healthcare, SE-75184, Uppsala Sweden) gradient with an upper layer and lower layer, 55% and 45% of the total volume, respectively.

Myocytes (lower layer) were counted using a hemocytometer and plated on collagen-coated culture plates at a density of 1.3 million cells/60-mm diameter plate or 0.45 million/35-mm plate with coverslips in medium consisting of Ham's F-10 (Sigma-Aldrich, Oakville, ON, Canada) supplemented with 10% fetal bovine serum (FBS), 10% horse serum (GIBCO, Life Technologies, Burlington, ON, Canada), and 1% Penstrep (GIBCO, Life Technologies, Burlington, ON, Canada) for 24 hours. Culture plates with

or without coverslips were coated with 0.25% w/v rat tail collagen, type I, (BD Sciences, Mississauga, ON, Canada), and then dried under ultraviolet light in a culture hood with constant air flow for 12 hours.

Doxorubicin and growth factor treatments

Cells were maintained for 24 hours in defined medium consisting of DMEM/F-12 (GIBCO, Life technologies, Burlington, ON, Canada), supplemented with 0.5% FBS, 0.66% Albumin (Sigma-Aldrich, Oakville, ON, Canada), 1% Penstrep, 0.02% Ascorbic Acid (Sigma-Aldrich, Oakville, ON, Canada), and 1% Insulin-Transferrin-Selenium (GIBCO) for 24 hours. For doxorubicin or FGF-2 treatment alone, cultures were refed with medium supplemented with either 0.5 μM doxorubicin (Sigma-Aldrich) or recombinant rat FGF-2 (10 nM) for 6 or 24 hours. For pretreatment with FGF-2, cultures were refed and treated with FGF-2 for 30 minutes and then supplemented with 0.5 μM doxorubicin treatment for up to 24 hours. For FGFR and PKC inhibition, 20 μM SU5402 and 5 μM chelerythrine or 20 nM bisindolylmaleimide (BIM-1) were added for 15 minutes before without or with FGF-2 and doxorubicin. For treatment with efflux drug transporter inhibitors, cyclosporine A (CsA, 20 μM), verapamil (2 μM) or XR 9576 (Tariquidar, 1 μM) were added 15 minutes before with or without FGF-2 and doxorubicin (Figure 3.1).

RNA isolation and quantitative real-time reverse transcriptase-polymerase chain reaction (qPCR)

Total RNA from neonatal rat cardiac myocytes was isolated using the RNeasy Plus Mini Kit (Qiagen Inc, Mississauga, Ontario, Canada) and assessed for quality on 1% agarose

gel (Bio-Rad, Mississauga, Ontario, Canada) with ethidium bromide (0.4%, Sigma-Aldrich) staining. Cell cultures were washed three times with phosphate-buffered saline (PBS) (GIBCO, Life technologies, Burlington, ON, Canada). Five hundred µl Buffer RLT Plus with 1% beta-mercaptoethanol (Sigma-Aldrich, Oakville, ON, Canada) was added to cell culture dishes to disrupt the cells. The lysate was collected with a rubber policeman and then transferred by pipette into a QIAshredder spin column placed in a 2 ml collection tube, and centrifuged for 2 minutes at maximum speed (>10,000 xg). The QIAshredder is a unique biopolymer shredding system in a micro-centrifuge spin-column format. It homogenizes cell or tissue lysates to reduce viscosity. Homogenization shears the high-molecular weight genomic DNA and other high-molecular-weight cellular components to create a homogenous lysate. The homogenized lysate was then transferred to a genomic DNA Eliminator spin column placed in a 2 ml collection tube, and centrifuged for 30 seconds at maximum speed to eliminate genomic DNA. The column was discarded, and the flow-through was saved. Five hundred µl of 70% ethanol was added to the flow through and mixed well. The sample, including any precipitate that may have formed, was then transferred to an RNeasy spin column placed in a 2 ml collection tube and centrifuged for 15 seconds at maximum speed. The RNeasy spin column can bind to mRNA larger than 200 nucleotides. The flow through was discarded, and the RNeasy spin column was washed one time with 700 µl of buffer RW1 and two times with Buffer RPE to wash away the contaminants. The RNeasy spin column was placed in a new collection tube and spun for 1minute to eliminate any possible carryover of Buffer RPE. The RNeasy spin column was placed in a new 1.5 ml collection tube and 25 µl RNase-free water added directly to the spin column membrane. The column was

then spun for 1 minute at maximum speed to elute RNA. The RNA concentration was determined with a Nanodrop 200 spectrophotometer (Thermo Scientific, Ontario, Canada); an absorbance A260/280 ratio for the RNA sample between 1.8-2.2 was considered an indication that the RNA was "good quality". In addition, 2 µl RNA was mixed with RNA loading dye (10%) and separated by electrophoresis in a 1% agarose gel and stained with ethidium bromide. Three sharp bands corresponding to 28S, 18S and 5S RNA provided a further indication of RNA quality.

Total RNA (1 µg) was converted to cDNA using the QuantiTect Reverse Transcription Kit (Qiagen). One µg total RNA template, plus 2 µl gDNA Wipeout Buffer, up to 14 µl total volume with RNase-free water was incubated at 42 ^{o}C for 2 minutes to eliminate any genomic DNA. Then 1 µl Quantiscript Reverse Transcriptase (Comprising of Quantiscript Reverse Transcriptase and an RNase inhibitor), 1 µl RT primer Mix (oligo-dT and random primers) and 4 µl Quantiscript RT Buffer (contains dNTPs) were added to total of 20 µl. Mixed samples were then were incubated for 45minutes at 42^{o}C for the reverse transcript reaction and 3 minutes at 95^{o}C to inactivate Quantiscript Reverse Transcriptase.

PCR amplifications were performed in triplicate in 20 µl using the SYBR green PCR Master Mix Kit (10 µl; A&B Applied Biosystems, Warrington, UK), with 33 ng of template cDNA and 1 mM each of forward and reverse primers (Invitrogen) in an ABI 7500 fast Real-Time PCR System; cycle conditions were: incubation 95 ^{o}C/10 minute, then 40 cycles with denaturation 95 ^{o}C/15 seconds, annealing 60 ^{o}C/30 seconds, and extension 72 ^{o}C/1 minute. RNA levels were analyzed using the comparative cycle

threshold (CT) method. The average CT values were determined for both the Target gene and housekeeping gene Beta-2-microglobulin (B2M) A in each sample from the treated and untreated (control) groups (n=3 per group). The average delta CT value (DCT) was determined for each group by subtracting the average CT value for the housekeeping gene from the average CT value for the target gene. The DDCT value was then determined by subtracting the DCT value for the untreated group from the DCT value for the treated group. The relative quantity was determined for each group using the RQ Study feature within the 7500 fast Real Time PCR System Sequence Detection Software, version 2.0.5 (A&B Applied Biosystems).

Table 2: Rat Primer Sequence (5'-3') of mRNA for qPCR

Primers	Forward Sequence	Reverse Sequence
B2M	GACCGTGATCTTTCTGGTGCTT	TTCCCATTCTCCGGTGGA
MRP1	AAGGAGTCCAGTCCTCAGG	AGAGGTCACTGCTCTTCAGG
MDR1b	GAAATAATGCTTATGAATCCCAAAG	GGTTTCATGGTCGTCGTCTCTTGA
MDR1a	AGCGGTCAGTGTGCTCACA	CTTGGCATATATGTCTGTAGCA
MDR2	AAGAATTTGAAGTTGAGCTAAGTGA	TGGTTTCCACATCCAGCCTAT
MRP2	GAAGGCATTGACCCTATCT	CCACTGAGAATCTCATTCATG
FGF-2	TCTTCCTGCGCATCCATCCAGA	CAGTGCCACATACCAACTGGAG

Immunohistochemistry

Neonatal rat cardiac myocytes were plated in 35 mm dishes with coverslips coated with 1 ml 0.05% collagen. At each experimental end time point, coverslips with cells were

rinsed 3 times with PBS. Freshly prepared 4% paraformaldehyde (Fisher Scientific, Ottawa, Ontario, Canada) was stored at 4 ^{o}C to keep it cold before addition to each dish containing a coverslip and incubated for 15 minutes at 4 ^{o}C for fixation. Cells on coverslips were rinsed 3 times with PBS, and 0.1% Triton/PBS were added to each dish and incubated for 15 minutes at 4 ^{o}C in order to disrupt the membrane to have higher permeability to antibodies or staining solutions. Cells on coverslips were rinsed 5 times with PBS after permeabilization. Primary antibodies were diluted in 1% bovine serum albumin (BSA)/PBS solution and added to completely cover the coverslips in order for antibody binding with the target protein structure. Cells on coverslips were covered with aluminum foil for protection from light and left overnight at 4 ^{o}C. Cells on coverslips were rinsed 4 times with PBS. A seconday antibody with a fluorescent tag (Texas-Red for red color or fluorescein (FITC) for green color) were diluted in 1% BSA in PBS solution and added to cover the cells on coverslips for 1 hour at room temperature for binding with the primary antibody. Plates were covered with aluminum foil to protect from light. Cells on coverslips were washed 4 times with PBS over 5 minutes and mounted on a clean slide using Prolong (Gibco) with DAPI (blue) to stain nuclei. Pictures were taken using a confocal laser scanning microscope (ZEISS, LSM5, PASCAL) and the Axionvision Rel. 4.8 program was used for further analysis or measurement.

Cell death quantification

Cell death quantification was assessed with the LDH assay (In Vitro Toxicology Assay Kit, Lactic Dehydrogenase based, Sigma), TUNEL (Terminal Transferase dUTP Nick End Labeling) kit (Roche, Mississauga, ON, Canada), Apoptosis & Necrosis & Healthy

Cells Quantification kit (Sigma) and ethidium bromide/Acridine Orange staining (Sigma) (Figure 3.1).

(1) LDH assay

The LDH assay was performed with an In Vitro Toxicology Assay Kit, Lactic Dehydrogenase based (Sigma). The assay is based on the reduction of NAD by LDH. The resulting reduced NAD (NADH) is utilized in the stoichiometric conversion of a tetrazolium dye. One of the LDH control group cultures was treated with 1/10 volume of LDH assay Lysis Solution per well and incubated for 45 minutes to lyse cells to release large amount of LDH for a positive control. Cell culture medium was collected and centrifuged to pellet the debris before using the LDH release assay. Fifty µl medium from the LDH control group, 50 µl medium from each control and treatment group, and a blank sample with all the solutions but without any cells, were added to a 96-well culture dishes. A negative control containing only culture medium was used as the blank sample. One hundred µl Lactate Dehydrogenase Assay Mixture (by mixing equal volume of LDH Assay Substrate Solution, LDH Assay dye solution, and 1x LDH Assay Co-factor Preparation) was then added to each well. Culture dishes were then covered with aluminum foil for protection from light and incubated at room temperature for 30 minutes. The reaction was terminated by the addition of 15 µl 1M hydrochloric acid to each well. Absorbance was measured spectrophotometrically at a wavelengh of 490 nm. The background absorbance of the multiwall plates was measured at 690 nm and this value was subtracted from the primary wavelength measurement (490 nm). The amount of LDH release from the treatment and control groups were then measured in comparison

(2) TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) - In situ Cell Death Detection Kit, TMR red

Neonatal rat cardiac myocytes were plated in 35 mm dishes with coverslips coated with 1 ml of 0.05% Collagen. Cell damage was induced with doxorubicin treatment. At each experimental end time point, coverslips with cells were rinsed 3 times with PBS. Four percent paraformaldehyde (Fisher Scientific, Ottawa, Ontario, Canada) was stored at 4 ^{o}C before addition to each dish containing a coverslip and incubated for 15 minutes at 4 ^{o}C for fixation. Cells on coverslips were rinsed 3 times with PBS, and 0.1% Triton/PBS was added to each dish and incubated for 15 minutes at 4 ^{o}C in order to disrupt the membrane for higher permeability to the staining solutions. Cells on coverslips were rinsed 5 times with PBS again. Fifty µl of TUNEL reaction mixture was prepared and added to each coverslip with cells, in a ratio of Enzyme solution (Terminal deoxynucleotidyl transferase): Label solution (Nucleotide mixture in reaction buffer) = 1:9. Coverslips with cells were then incubated in a humidified atmosphere for 60 minutes at 37 ^{o}C in the dark. Cells on coverslips were rinsed 3 times with PBS. Pictures of the slides were taken using a confocal laser scanning microscope (ZEISS LSM5 PASCAL) and the Axionvision Rel. 4.8 program was used for further analysis and measurement.

(3) Apoptosis, necrosis and "healthy" cell quantification

Evidence of programmed cell death was also assessed using the Apoptosis & Necrosis Quantification Kit (Biotium Inc., CA, USA). In apoptotic cell death, phosphatidylserine is translocated from the inner to the outer surface of the cell for phagocytic cell recognition [181]. The human anticoagulant, annexin V, is a 35 kD Ca^{2+}-dependent phospholipid protein with a high affinity for phosphatidylserine. Annexin V labeled with

fluorescein isothiocyanate (FITC, $\lambda_{abs}/\lambda_{em}$ = 492/ 514 nm) that can identify apoptotic cells in green by binding to phosphatidylserine exposed on the outer membrane [182]. In necrotic cell death, both internal organelle and plasma membrane integrity are lost, resulting in spilling of cytosolic and organellar contents into the surrounding environment [183]. Ethidium homodimer III is a highly positively charged nucleic acid probe, which is impermeant to live cells or apoptotic cells, but stains necrotic cells with red fluorescence ($\lambda_{abs}/\lambda_{em}$ = 528/617 nm) [184]. Ethidium Homodimer III has high affinity for DNA and high fluorescence quantum yield. Hoechst 33342 is a cell membrane-permeant, minor groove-binding DNA stain that emits bright blue fluorescence upon binding to DNA ($\lambda_{abs}/\lambda_{em}$ = 350/461 nm). It has been used for staining the nuclei of cells [184].

Neonatal rat cardiac myocytes were plated in 35 mm dishes with coverslips coated with 1 ml 0.05% collagen. Cell damage was induced using doxorubicin treatment. For microscope viewing, cells on coverslips were washed twice with 1X Binding Buffer. Five µl FITC-Annexin V (binding to the pSer exposed on the outer membrane), 5 µl EthD-III (impermeable for apoptotic cells but can bind to DNA of necrotic cells) and 5 µl Hoechest 33342 (binding to DNA) were added into 100 µl 1X Binding buffer for staining the cells on coverslips. Cells were washed two times with 1X Binding Buffer. Cells on coverslips were fixed with 2% paraformaldehyde in 1X Binding Buffer. Cells were then washed 2 times with 1X Binding Buffer and mounted onto a slide with 1X Binding Buffer. Pictures were taken and analyzed using a confocal laser scanning microscope (ZEISS LSM5 PASCAL) with the Axionvision Rel. 4.8 program.

For flow cytometry, cells were washed with PBS twice and detached from the culture plate by 1 ml Accutase (Invitrogen). Four ml culture medium was added to stop the accutase reaction. The total 5 ml solution was collected and centrifuged at 600 xg for 5 minutes. Supernatants were discarded and pellets were resuspended with 100 µl 1X Binding Buffer. Five µl FITC-Annexin V, 5 µl EthD-III and 5 µl Hoechst 33342 solutions were added to each 100 µl 1X Binding Buffer. An additional three samples, one for each of the staining dyes (FITC-Annexin V, ethidium homodimer III or Hoechst 33342) control was also included. All samples were incubated at room temperature for 15 minutes in the dark. Four hundred µl 1X Binding Buffer was then added to each tube. All cells were analyzed by FACS according to the manufacturer's instructions (MoFloXDP, Beckman Coulter, Mississauga, Canada) within 1 hour of staining. Data were analyzed using the Summit v.5.2 program (Beckman). Gating for the analysis was based on determinations for untreated cells, as well as annexin V and/or ethidium homodimer III positive cells.

(4) Acridine orange staining

Acridine orange is a slightly cationic, lipophilic, weak base capable of permeating cell and organelle membrane structure. Acridine orange can also be protonated and trapped on the low pH side of the membrane barrier leading to its accumulation in acidic organelle structures, such as lysosomes. Proton pump driven lysosomal acidity generates a significant pH gradient resulting in the efficient concentration of acridine orange within the lysosome organelles. The effectiveness of this acridine orange concentrating process is sufficient to create intra-lysosomal concentrations leading to precipitation of the

acridine orange into aggregated granules. These oligomeric structures exhibit a red shift (640 nm) compared to the monomeric acridine orange (525 nm).

Neonatal rat cardiac myocytes were plated in 35 mm dishes with coverslips coated with 1 ml 0.05% collagen. Cell damage was induced using doxorubicin treatment for 6 and 24 hours. Cells were washed three times with PBS, and then stained with 100 µl acridine orange staining solution (2 mg/ml acridine orange in double distilled water). Pictures of the slides were taken within 20 minutes of staining with a confocal laser scanning microscope (ZEISS LSM5 PASCAL) and the Axionvision Rel. 4.8 program was used for further analysis and measurement.

Efflux drug transporter function quantification

(1) Doxorubicin autofluorescence quantification

Doxorubicin is a substrate for efflux drug transporters and has a fluorescent hydroxy-substituted anthraquinone chromophore structure that enables us to detect an average fluorescence intensity level that can be compared between each groups [185].

Cells were processed according to growth factor and doxorubicin treatment regimen, and a positive control treated with CsA (20 µM), verapamil (2 µM) were included. Cells were then pelleted and resuspended with DMEM/F-12 (without phenol red) and analyzed by FACS according to the manufacturer's instructions (MoFloXDP, Beckman Coulter, Mississauga, Canada) within 1 hour of staining. Data were analyzed using the Summit v.5.2 program (Beckman). Gating for the analysis was based on determinations for no treatment (doxorubicin) cells.

(2) Multi-Drug Resistance Assay Kit (Calcein AM quantification)

For studying MDR proteins (including P-gp and MRP) modulators, neonatal rat cardiac myocyte cultures were assessed with a Multi-Drug Resistance Assay Kit (Calcein AM) according to the manufacturer's instructions (Cayman Chemical Company, Ann Arbor, Michigan, USA). Cells were treated with or without FGF-2 and doxorubicin, and a positive controls reflecting treatment with CsA (1:1000, 20 μM), verapamil (2 μM) or XR 9576 (Tariquidar) were included. To load cells with calcein, cultures were treated with cell-permeable non-fluorescent substrate calcein AM solution 25 minutes prior to harvesting. Cells were then pelleted and resuspended in 400 μl of Assay Buffer and analyzed with a flow cytometer. Following intracellular esterase activity that removes the acetomethoxy (AM) group, calcein AM is converted to fluorescent calcein, which is retained in the cytoplasm [186]. Cells were pelleted and resuspended with Assay Buffer.

All cells were analyzed by FACS according to manufacturer's instructions (MoFloXDP) within 1 hour of staining. Data were analyzed using the Summit v.5.2 program (Beckman). Gating for the analysis was based on determinations for calcein AM positive cells.

Statistical analysis

For single comparisons, paired t-tests were applied, and two-way analysis of variance (ANOVA) with a post-hoc Bonferonni test or one-way ANOVA with a post-hoc Tukey test were used for multiple (treatments and time) and single group (treatments) analyses, respectively. Mean values were considered significantly different if $p<0.05$. Unless stated otherwise, all studies were done in triplicate (n=3). In figures, comparisons made relative

to a 'control' and arbitrarily set to 1.0 or 100%, are represented as * $p<0.05$, ** $p<0.01$ and *** $p<0.001$. For comparisons made between treatment groups (and not arbitrarily set to 1.0 or 100%), these are represented as $^{\#}$ $p<0.05$, $^{\#\#}$ $p<0.01$ and $^{\#\#\#}$ $p<0.001$.

A summary of the methods used is illustrated schematically in Figure 3.1.

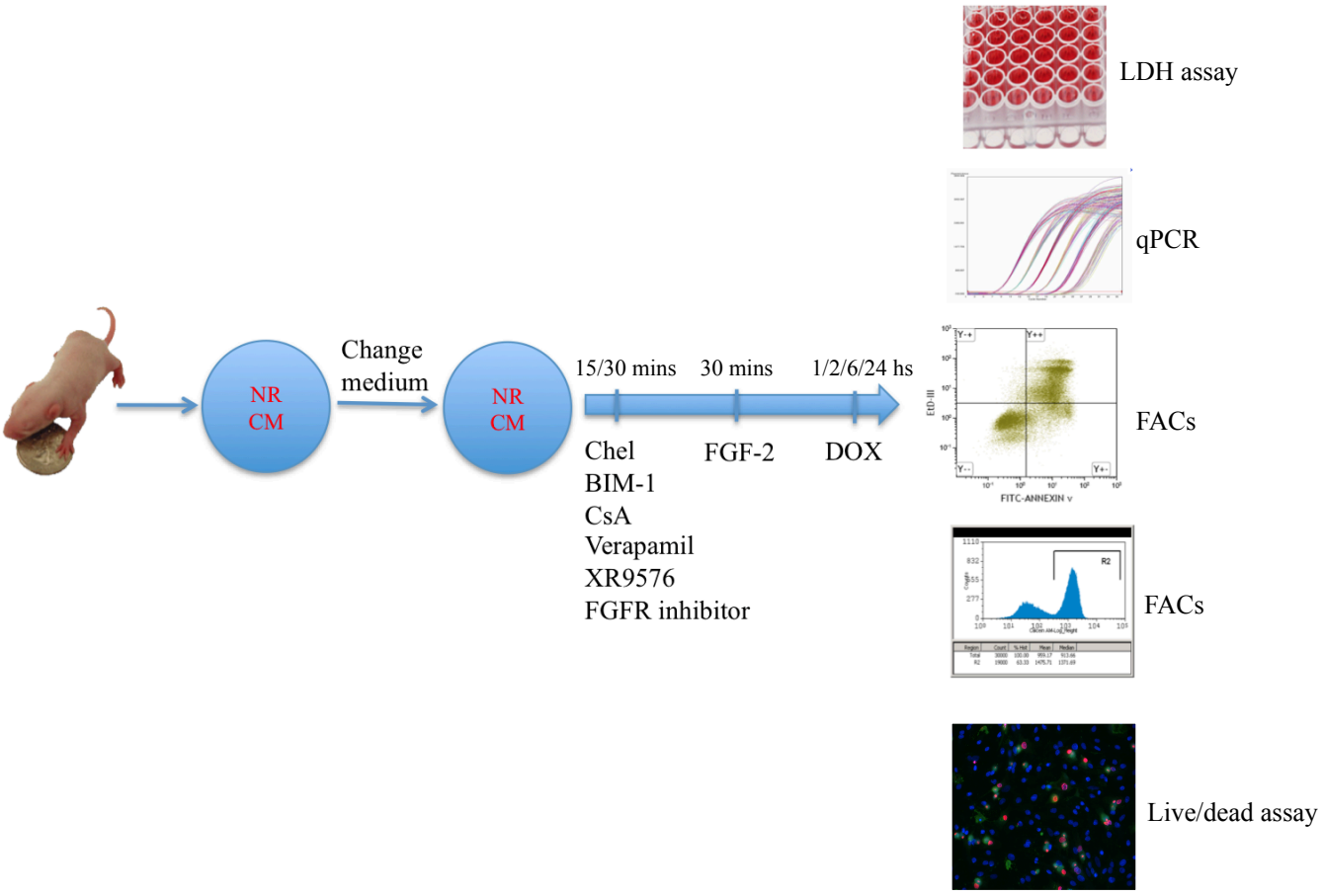


Figure 3. 1 - Methods and Treatment Regimen

Abbreviations: Chelerythrine, Chel; bisindolylmaleimide I, BIM-1; cyclosporine A, CsA; doxorubicin, DOX; real time polymerase chain reaction, qPCR; fluorescence activated cell sorting, FACS.

CHAPTER IV: RESULTS

4.1 Establish a "model" of doxorubicin-induced injury in neonatal rat cardiac myocyte cultures

Rationale

Primary cell cultures have the advantage over whole organism studies of being less expensive, often more convenient, and certainly offer greater versatility, particularly when it comes to applying a broad spectrum of experimental approaches [187]. Neonatal rat cardiac myocyte cultures have been used as a model in many studies looking at morphological, biochemical and electrophysiological characteristics of the heart; this includes the study of transport and toxicity of drugs [188-191]. More importantly, doxorubicin is cytotoxic and has been reported to induce cell death, transient electrocardiographic changes and cardiomyopathy in neonatal rat cardiac myocyte cultures [56-60]. The protocol used for isolation and culture of neonatal rat cardiac mycoytes has been used successfully and produces a high yield (40 million cells/36 neonatal rat hearts) and high quality functional cells that was used in many publications in our lab [192, 193]. In addition, some studies have shown that neonatal rat cardiac myocyte primary cultures maintain a similar stable contractile profile during hypoxia-reoxygenation compared with *in vivo* hearts during ischemia-reperfusion [194]. This contrasts with adult cardiac myocyte cultures, where the profile is different [194].

Observations suggest that a concentration of between 0.1-1.0 μM doxorubicin is sufficient to induce plasma membrane damage in neonatal rat cardiac myocytes, as indicated by the presence of LDH in the medium [58]. LDH is usually stored in

cytoplasm inside of the cells, however, when cell membrane integrity is disrupted, LDH leaks from the inside to the outside of the cells. In this case, LDH was released from the cell to the culture medium that is measurable by the LDH assay. A doxorubicin concentration in the range of 0.1-1.0 μM has been used in multiple studies to demonstrate cytotoxic effects as well as in studies to look at prevention of these effects [195-197]. Thus, a concentration of 0.5 μM doxorubicin was pursued initially.

Approaches

To establish a "model" of doxorubicin-induced injury in neonatal rat cardiac myocyte cultures, the effect of 0.5 μM doxorubicin was assessed: (a) on LDH release as an indicator of plasma membrane damage; (b) on DNA fragmentation as indicated by TUNEL; (c) on fluorescent markers of cell death (annexin-V for apoptosis and ethidium homodimer III for necrosis); and (d) on lysosome activity.

Results

4.1a 0.5 μM doxorubicin treatment causes plasma membrane damage in neonatal rat cardiac mycoyte cultures

Neonatal rat cardiac myocyte cultures were plated at a density of 1.3-million cells/60-mm diameter plate and were refed with defined medium for 24 hours before treatment with 0.5 μM doxorubicin for 1, 2, 6 or 24 hours. The culture medium was collected at each time point and assessed by LDH assay as an indication of level of plasma membrane disruption.

There were no significant effects on LDH release in the presence or absence of doxorubicin at 1 and 2 hours doxorubicin treatment, however, there were significant increases in LDH activity detected after doxorubicin treatment at 6 and 24 hours compared to control (Figure 4.1A). Although a variation in the degree of response (LDH activity level) was noted between preparations, significant increases in LDH activity with 0.5 μM doxorubicin treatment for 24 hours was observed consistently compared to controls. This consistent increase in LDH with doxorubicin treatment, in spite of the variability in the degree of increase in LDH detected, may reflect differences between preparations. This may include differences in the status of cells or even survival, especially membrane integrity, following tissue digestion by enzymes, isolation via centrifugation through a Percoll gradient, and maintenance in low (0.5%) serum for 24 hours, prior to treatments. A sense that even the untreated control cells are to some extent damaged during isolation and/or culture might be reflected in total cell yield or density, but is perhaps best illustrated by the 2.6-fold increase in LDH activity observed in an untreated 'control' culture over 24 hours of assessment (Figure 4.1A). It has been reported that within the first 2-3 days, about 20–30% of neonatal rat cardiac myocytes were lost in the normal dense culture and can be removed by changing the medium [198].

4.1b 0.5 μM doxorubicin treatment induces DNA fragmentation in neonatal rat cardiac myocytes

Neonatal rat cardiac myocytes were plated at a density of 0.45-million/35-mm plate containing collagen-coated coverslips. Cultures were changed to defined medium for 24 hours before treatment with 0.5 μM doxorubicin for 1, 2, 6 and 24 hours. Cardiac

myocytes were fixed with 4% paraformaldehyde at each time point, and then assessed by TUNEL (red) staining to detect DNA fragmentation.

No TUNEL positive nuclear staining was observed in the control (untreated) group at any time point as well as in cultures treated with doxorubicin for 1 hour. TUNEL positive (red) nuclei cardiac myocytes were observed at 2 hours doxorubicin treatment, and the intensity of positive staining gradually got stronger over the 24 hour period of assessment (Figure 4.1B). The identity of cardiac myocytes was confirmed by α-actinin (green) staining of muscle fibers, and all cells were detected by DAPI (blue) nuclear DNA staining (Figure 4.1C).

The detection of low intensity TUNEL-positive nuclei as early as after 2 hours of doxorubicin treatment, suggests an early stage of DNA fragmentation with less DNA breaks. At 24 hours, the high intensity positive/staining indicates a late stage of DNA fragmentation. More than 85% of the TUNEL positive cardiac myocytes were observed, from as early as 2 hours. Thus, it appears that introduction of DNA breaks by doxorubicin occurs quite rapidly in the vast majority of cells, and that further DNA fragmentation accumulates with time of exposure facilitating more ready detection by TUNEL (Figure 4.1B).

4.1c 0.5 μM doxorubicin treatment is associated with both apoptosis and necrosis in neonatal rat cardiac myocyte cultures

Neonatal rat cardiac myocytes were plated at a density of 1.3-million cells/60-mm diameter collagen-coated culture plate. Cultures were then changed to defined medium for 24 hours prior to treatment with 0.5 μM doxorubicin for 6 and 24 hours, then cells were pelleted with Accutase and resuspended with 1x annexin-V binding buffer and stained for apoptosis (annexin-V, green and necrosis (ethidium homodimer III, red) markers by FACS. Cell nuclei were stained with Hoechst 33342 (blue) (Figure 4.1D). At 6 and 24 hours, damaged (positive) cells were assessed in categories: early apoptosis cells -stained green with blue nuclei; late apoptosis and/or necrosis - stained green with red nuclei; and early necrosis - red stained nuclei only.

Examples of each of the positive-staining cells were identified in control cultures. This presumably reflects a range of damage (even death) that would be expected from the mechanical and enzymic disaggregation of an organ (neonatal heart) as well as centrifugation that makes up the cell isolation process, in addition to culture/medium conditions (Figure 4.1D). No significant difference in the percentage of each stage of apoptosis or necrosis was observed at 6 hours after doxorubicin treatment when compared to control group. There was a significant increase in the relative percentage of cells positive for markers (apoptotic and/or necrotic) of programmed cell death (total) with doxorubicin treatment, when compared to the control group at 24 hours (Figure 4.1E). Based on the blue staining of the nuclei, a significant decrease in total cell number was observed at 24 hours with doxorubicin treatment, but not at 6 hours when compared to the control untreated group (Figure 4.1F).

Annexin-V staining is very sensitive and quite specific for early apoptotic cell death, but at the end stage of cell death, it is very difficult to distinguish between late apoptosis and necrosis. As a result, cells at late apoptosis or necrosis might be stained and thus positive for both annexin-V and ethidium homodimer III. It is also important to note that this method is dependent on the preparation and staining procedure. For example, disassociation of cardiac myocytes from collagen-coated plates may tear the cell surface marker, including annexin-V, which could cause false positive staining of cells [199]. Regardless, however, of whether we consider early or late stage of cell injury, the total damage of neonatal rat cardiac myocytes caused by doxorubicin is increased significantly compared to the control untreated cells.

4.1d 0.5 μM doxorubicin increases lysosome activity

Neonatal rat cardiac myocytes were plated at a density of 0.45-million/35-mm plate containing collagen-coated coverslips. Cultures were changed to defined medium for 24 hours before being treated with 0.5 μM doxorubicin for 6 and 24 hours. Lysosome activity was then assessed by acridine orange staining. “Orange” staining in the cytoplasm (lysosomes) was observed after doxorubicin treatment at 24 hours, but not in the control culture (Figure 4.1G).

Increased lysosome activity was reported to be linked to different types of cell death [200, 201] More specifically, rupture of the lysosomes has been reported as a determinant for plasma membrane disruption in tumor necrosis factor α-induced cell death [202]. Thus, the results of the current study support an increase in both lysosome activity and

membrane disruption (LDH release), as events associated with doxorubicin-induced injury in neonatal rat cardiac myocytes.

Conclusions

A "model" of 0.5 μM doxorubicin-induced injury has been established in neonatal rat cardiac myocyte cultures. Membrane disruption, DNA damage, cell surface markers of programmed cell death and lysosome activity are all detectable and/or measurable indicators of doxorubicin-induced injury in neonatal rat cardiac myocyte cultures.

While cell surface markers can be used to indentify individual cells that are damaged by doxorubicin, measurement of LDH activity in the culture medium is a more convenient method of assessing damage to a culture. More specifically, LDH release is consistently and significantly increased by doxorubicin at 24 hours. The assay also requires only a small volume of medium without interrupting the experimental treatment of the cells in culture. As a result this offers the opportunity to assay the medium of the same culture over time. Perhaps most importantly, besides a well estabished measurement of damage as indicated by LDH release, the assay allows the cells themselves to be assessed either *in vitro* or *in situ* (in living cells in culture) for gene expression or protein function. Thus, elements of damage and possible mechanism can be assessed from the same culture dish.

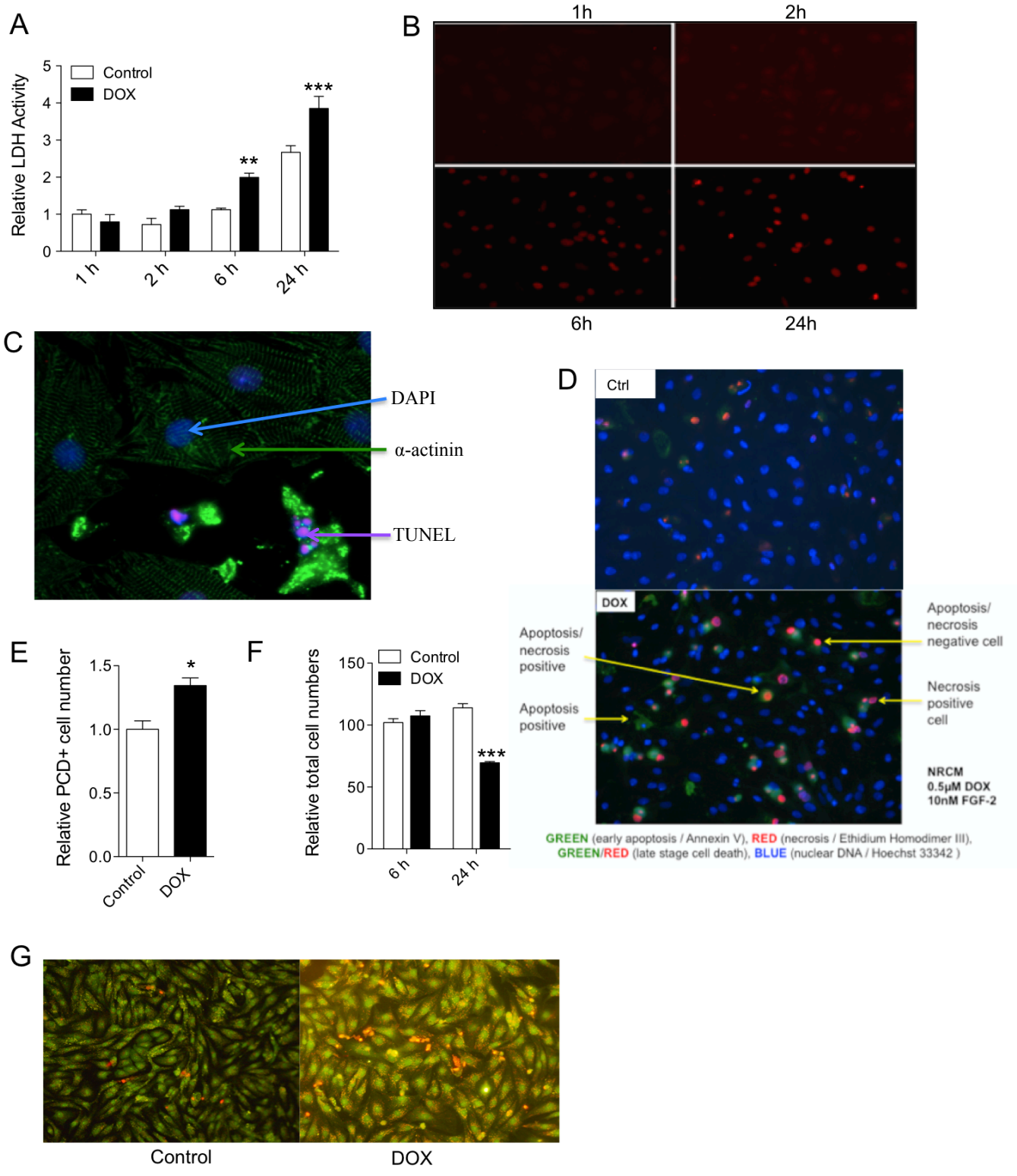

A
Relative LDH Activity
Control
DOX
1 h
2 h
6 h
24 h
B
1h
2h
6h
24h
C
DAPI
α-actinin
TUNEL
D
Ctrl
DOX
Apoptosis/ necrosis positive
Apoptosis positive
Apoptosis/ necrosis negative cell
Necrosis positive cell
NRCM
0.5μM DOX
10nM FGF-2
GREEN (early apoptosis / Annexin V), RED (necrosis / Ethidium Homodimer III),
GREEN/RED (late stage cell death), BLUE (nuclear DNA / Hoechst 33342)
E
Relative PCD+ cell number
Control
DOX
F
Relative total cell numbers
Control
DOX
6 h
24 h
G
Control
DOX

Figure 4. 1 - Doxorubicin induces damage in neonatal rat cardiac myocyte cultures

The effect of 0.5 μM doxorubicin (DOX) was assessed on: (A) LDH release as an indicator of plasma membrane damage by LDH assay; (B/C) on DNA fragmentation by TUNEL; (D/E) on fluorescent markers of programmed cell death, annexin-V for apoptosis and ethidium homodimer III for necrosis; (F) total cell number and (G) on lysosome activity by acridine orange staining.

Cardiomyocytes were treated without or with 0.5 μM doxorubicin for 1, 2, 6 and/or 24 hours (h). (A) Culture medium was assessed for LDH activity. (B/C) Cells were then fixed with 4% paraformaldehyde before staining with TUNEL reaction mix (Enzyme solution: Label solution=1:9) at 37 ^{0}C for 1 hour. To distinguish between cardiac myocyte and fibroblast, DAPI was used to stain nucleus and α-actinin antibody was used for stain cardiac myocyte muscle fibers. Pictures of the slides were taken using a fluorescence microscope (ZEISS LSM5 PASCAL) and the Axionvision Rel. 4.8 program was used for further analysis and measurement. (D/E) Cells were harvested, stained with programmed cell death (PCD) markers (annexin V and ethidium homodimer III) and the proportion of PCD positive (+) cells assessed by FACS. (F) Total cell number was counted at 6 and 24 hours by nuclei staining with Hoechst 33342. (G) Cells were harvested, stained with acridine orange and pictures were taken within 20 minutes using a fluorescence microscope described in (A). Results are expressed as the mean plus or minus standard error of the mean (+SEM), relative to the control (untreated) value, which is arbitrarily set to 1. Data were assessed by one-way ANOVA with the Tukey-Kramer post-test. A value of $p<0.05$ is considered statistically significant: $p<0.05$ */#; $p<0.01$, **/##; $p<0.001$, ***/###.

4.2: Assess the ability of exogenous FGF-2 (10 nM) treatment to protect against the doxorubicin-induced injury in neonatal rat cardiac myocytes.

Rationale

FGF-2 has been reported to be cardioprotective to cardiac myocyte injury both *in vitro* and *in vivo*. Addition of exogenous FGF-2 to neonatal rat cardiac myocyte cultures treated with hydrogen peroxide or starved for serum resulted in improved cell survival and decreased cardiac myocyte injury as evidenced by preservation of nuclear morphology and myofibrillar structure [126]. Administration of exogenous FGF-2 before or during ischemic injury in various heart ischemia/reperfusion models was shown to increase cardiac myocyte viability and/or functional recovery in the rat or mouse heart [126, 132, 150, 203]. Endogenous FGF-2 gene expression alone can be regulated by many stress stimuli and injury, including hypoxia, ischemia, as well as in response to Ang II and adrenergic stimulation to offer cardioprotection [147]. In addition, local FGF-2 synthesis at the transcriptional level through adrenergic stimulation, increased contraction with increased FGF-2 release and contributed to the cardioprotection during injury, as well as in the maintenance of a healthy myocardium [147].

It has been reported that 2 μM doxorubicin treatment results in a decrease of FGF-2 expression in neonatal rat cardiac myocyte cultures [120]. If so, any possible benefit endogenous FGF-2 might confer in terms of protection of the cardiac myocytes from doxorubicin-induced injury would be decreased or lost. Thus, supplementing with exogenous FGF-2 might be seen as replacing a “natural” or endogenous protective agent. Both the effects of doxorubicin on endogenous FGF-2 gene expression, as well as the potential protective effect of 10 nM FGF-2 against doxorubicin-induced damage were

pursued.

Approaches

To assess the (a) effect of doxorubicin on endogenous FGF-2 RNA levels in neonatal rat cardiac myocytes; (b) ability of exogenous FGF-2 (10 nM) to protect against doxorubicin-induced damage and release of LDH: (c) to determine the half maximal effective concentration (EC50) of FGF-2 in protecting from damage and LDH release; (d) ability of FGF-2 to protect against DNA fragmentation, and (e) levels of programmed cell death (detection of fluorescent markers of cell death, annexin-V for apoptosis and ethidium homodimer III for necrosis) in the neonatal rat cardiac myocyte cultures.

Results

4.2a 0.5 μM doxorubicin decreases endogenous FGF-2 mRNA levels

Neonatal rat cardiac myocyte cultures were plated at a density of 1.3-million cells/60-mm diameter plate and were refed with defined medium for 24 hours before treatment with 0.5 μM doxorubicin for 6 or 24 hours. Total RNA was isolated and FGF-2 mRNA levels were assessed by qPCR and normalized relative to endogeneous B2M mRNA levels. There was a significant 49% decrease in FGF-2 mRNA levels with doxorubicin treatment at 6 hours in the cardiac myocyte cultures. A similar significant decrease was also detected at 24 hours (Figure 4.2A). This decrease in FGF-2 mRNA levels with 0.5 μM doxorubicin treatment confirms a previous report of a negative effect with 2 μM doxorubicin, and extends this observation to include a lower doxorubicin dose [204].

4.2b FGF-2 increases resistance to doxorubicin-induced plasma membrane damage in neonatal rat cardiac myocyte cultures

Neonatal rat cardiac myocyte cultures were plated at a density of 1.3-million cells/60-mm diameter plate and were refed with defined medium for 24 hours before treatment without or with 0.5 μM doxorubicin and 10 nM FGF-2, or pre-treated with FGF-2 for 30 minutes prior to the addition of doxorubicin. The effect of FGF-2 pre-treatment on plasma membrane damage was also assessed by measuring LDH activity in the culture medium of cardiac myocytes treated without or with doxorubicin for 1, 2, 6 or 24 hours.

No significant effects on LDH release were observed with FGF-2 and/or doxorubicin treatment at 1 and 2 hours. A significant increase in LDH activity was detected with doxorubicin treatment at 6 and 24 hours, but these increases were not observed in cultures pre-treated with FGF-2 (Figure 4.2B). There was, however, no significant effect of FGF-2 treatment alone on LDH activity at 6 or 24 hours. This indicates exogenous administration of FGF-2 increases resistance of neonatal rat cardiac myocytes to doxorubicin-induced plasma membrane damage at 6 and 24 hours.

4.2c To identify the half maximal effective concentration (EC50) of FGF-2 in preventing doxorubicin-induced LDH release at 24 hours

Neonatal rat cardiac myocytes were pretreated with 0.2, 1.0, 2.5, 10 and 20 nM FGF-2 before doxorubicin for 24 hours and culture medium was collected for the LDH activity assay. EC50 of FGF-2 was calculated based on the standard curve of the FGF-2 concentration and LDH release. A dose-dependent effect on reducing LDH release was observed, and a EC50 of about 1.5 nM FGF-2 was determined. Thus, use of 10 nM FGF-

2, which was maximal (in the plateau range) under the culture conditions tested (Figure 4.2C), was used for subsequent studies.

4.2d FGF-2 increases resistance to doxorubicin-induced DNA fragmentation in neonatal rat cardiac myocytes

Neonatal rat cardiac myocyte primary cultures were repeated and cells were plated at a density of 0.45-million/35-mm plate containing collagen-coated coverslips. Cultures were changed to defined medium for 24 hours before treatment without or with 0.5 μM doxorubicin and 10 nM FGF-2, or pre-treatment with FGF-2 for 30 minutes before doxorubicin for 24 hours. Cardiac myocytes were fixed with 4% paraformaldehyde at each time point, and then assessed by TUNEL (red) staining to detect DNA fragmentation. The identification of cardiac myocytes was confirmed by α-actinin (green) staining of muscle fibers, and all the cells were detected by DAPI (blue) nuclear DNA staining. The α-actinin (green) and DAPI (blue) positive cells indicate cardiac myocytes and these were assessed for percentage of DNA fragmentation.

The intensity of the red (TUNEL) staining was lower in the presence of FGF-2 (pre-treatment) and doxorubicin treatment versus doxorubicin treatment alone at 24 hours. This suggests less DNA damage or breaks in the FGF-2 pre-treatment group as discussed in section 4.1b (Figure 4.2D).

4.2e FGF-2 increases resistance to doxorubicin-induced programmed cell death in neonatal rat cardiac myocytes

Neonatal rat cardiac myocyte cultures were plated at a density of 1.3-million cells/60-mm diameter plate and were refed with defined medium for 24 hours before treatment without or with 0.5 μM doxorubicin or 10 nM FGF-2, or pre-treatment with FGF-2 for 30 minutes before doxorubicin. The effect of FGF-2 pre-treatment on programmed cell death induced by doxorubicin was assessed by FACS at 24 hours, using a combination of staining with fluorescent markers for apoptosis (FITC-annexin-V) and necrosis (ethidium homodimer III) with FACS analysis (Figure 4.2E).

No effects of FGF-2 treatment alone were observed, however, a significant decrease in relative programmed cell death in cultures was detected with FGF-2 pre-treatment with doxorubicin compared to no pre-treatment and doxorubicin alone (Figure 4.2F). In addition, pre-treatment with FGF-2 was associated with significantly higher total cell numbers when compared to doxorubicin treatment alone; this was done by counting nuclei staining using Hoechest 33342 staining (Figure 4.2G). These observations are consistent with the ability of FGF-2 to increase resistance to doxorubicin-induced programmed cell death, including apoptosis and necrosis.

Conclusions

The ability of doxorubicin treatment to decrease endogenous FGF-2 mRNA levels in neonatal rat cardiac myocytes after (6 or) 24 hours was confirmed, and at a lower dose (0.5 μM) than reported previously [120]. Exogenous addition of FGF-2 can increase resistance to doxorubicin-induced injury, in regards to plasma membrane damage, DNA

fragmentation and cell survival. Although increased protection of cardiac myocytes from doxorubicin-induced damage by FGF-2 has not been reported previously, exogenous low molecular weight 18 kDa FGF-2 is known to affect sensitivity to anti-cancer drugs including doxorubicin [176, 205, 206].

Although the protective effect of FGF-2 against doxorubicin on total cell number is significant, it is not normalized by FGF-2 like programmed cell death (PCD) and LDH release compared to control. This might be due to the cell loss that occurs during the staining procedure. PCD and LDH measures total cells including cells that are compromised, including cells floating in the medium and/or not attached well to the coallgen-coated plate; perhaps in the process of lysis. However, most of the compromised cells (late stage apoptotic or necrotic cells) might be washed away during DAPI staining, and thus, only relatively healthy cells are collected in the DAPI assay.

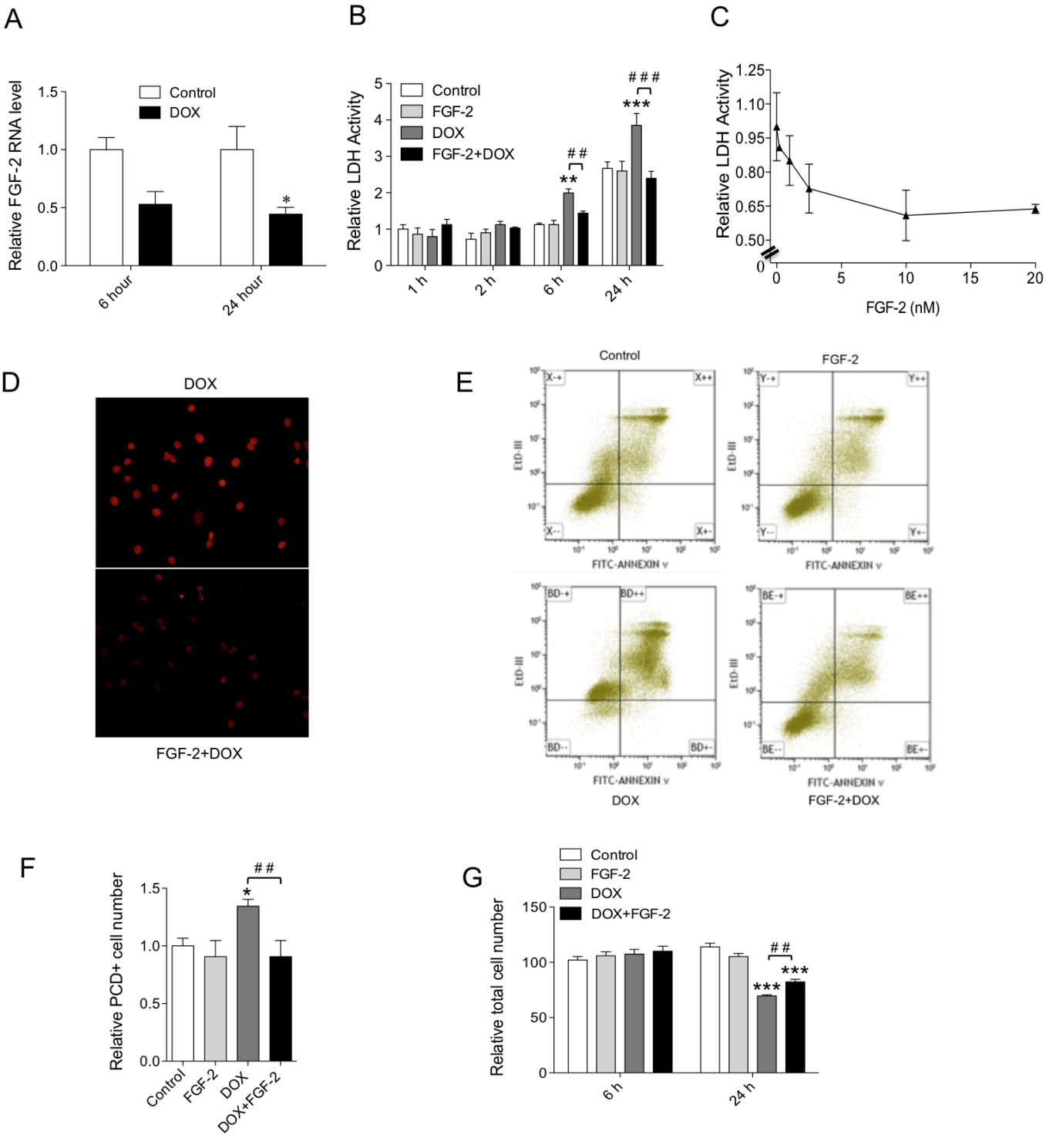

A
Relative FGF-2 RNA level
Control
DOX
6 hour
24 hour
B
Relative LDH Activity
Control
FGF-2
DOX
FGF-2+DOX
1 h
2 h
6 h
24 h
C
Relative LDH Activity
FGF-2 (nM)
D
DOX
FGF-2+DOX
E
Control
FGF-2
DOX
FGF-2+DOX
FITC-ANNEXIN v
F
Relative PCD+ cell number
Control
FGF-2
DOX
DOX+FGF-2
G
Relative total cell number
Control
FGF-2
DOX
DOX+FGF-2
6 h
24 h

Figure 4. 2 - Exogenous FGF-2 increases resistance of neonatal rat cardiac myocytes against doxorubicin-induced injury

Cardiomyocytes were treated with 10 nM FGF-2 or 0.5 µM doxorubicin (DOX), or pretreated with FGF-2 for 30 minutes before doxorubicin addition for 24 hours. (A) The effect of doxorubicin on FGF-2 mRNA expression in neonatal rat cardiac myocyte cultures was assessed by qPCR after 6 and 24 hours. (B) Culture medium was assessed for LDH activity. (C) EC50 for FGF-2 on preventing doxorubicin-induced LDH release at 24 hours. Cultures were treated with 0.2 - 20 nM FGF-2 before doxorubicin treatment, and culture medium was collected for LDH assessment. Dose dependent curves were drawn and analyzed with Graphpad Prism. (D) Cells were fixed with 4% paraformaldehyde before staining with TUNEL reaction mix (Enzyme solution: Label solution=1:9) at 37^0C for 1 hour. Pictures of the slides were taken using a fluorescence microscope (ZEISS LSM5 PASCAL) and the Axionvision Rel. 4.8 program was used for further analysis and measurement. (E/F) Cells were harvested, stained with programmed cell death (PCD) markers (annexin V and ethidium homodimer III) and the proportion of PCD positive (+) cells assessed by FACS. Gating for the analysis was based on determinations for untreated cells, as well as annexin V and ethidium homodimer III. Examples of plots for annexin-V and ethidium homodimer III are shown. (G) Total cell number was counted at 6 and 24 hours by nuclei staining with Hoechst 33342. The results are expressed as the mean+SEM, relative to the control (untreated) value, which is arbitrarily set to 1 or 100. Data were assessed by two-way ANOVA with the Bonferonni post-test. A value of $p<0.05$ is considered statistically significant: $p<0.05$ */#; $p<0.01$, **/##; $p<0.001$, ***/###.

4.3: To investigate a role for FGFR in the beneficial effects of FGF-2 observed in relation to doxorubicin-induced injury by using an FGFR inhibitor.

Rationale

FGF-2 binds predominantly to one of the four tyrosine kinase FGF receptors (FGFR1) and activates a series of signal transduction pathways and cellular events [125]. SU5402 is a cell-permeable, reversible, and ATP-competitive inhibitor of the tyrosine kinase activity of FGFR1 and also inhibits the FGF-induced tyrosine phosphorylation of ERK1 and ERK2. SU5402 is only a weak inhibitor of tyrosine phosphorylation of the PDGF receptor and does not inhibit phosphorylation of the insulin receptor and EGF receptor. Thus, SU5402 inhibits tyrosine kinase phosphorylation that would normally occur as a result of FGF-2/FGFR1 binding, and thus block the effect of triggering any downstream signaling pathways [167, 207].

Approaches

To investigate the involvement of FGFR signaling in the protective effect of FGF-2 against doxorubicin-induced injury, 20 μM SU5402 was added to neonatal rat cardiac myocyte cultures before FGF-2 and doxorubicin. Culture medium was then collected and LDH activity was assessed.

Results

The increased resistance of neonatal rat cardiac myocytes conferred by FGF-2 treatment against doxorubicin-induced LDH release was blocked by pretreatment with 20 μM SU5402 at 24 hours (Figure 4.3).

Conclusions

The effectiveness of protection offered by FGF-2 in this experiment presumably reflects variation in the functional activity of different FGF-2 preparations or loss of activity with age. However, the protection by FGF-2 was significant and blocked by the FGFR inhibitor SU5402.

This is consistent with the beneficial effect of FGF-2 being mediated by a mechanism involving FGFR signaling. This implicates any signaling pathway triggered by FGFR/tyrosine kinase activation and tyrosine phosphorylation that is present in neonatal rat cardiac myocytes.

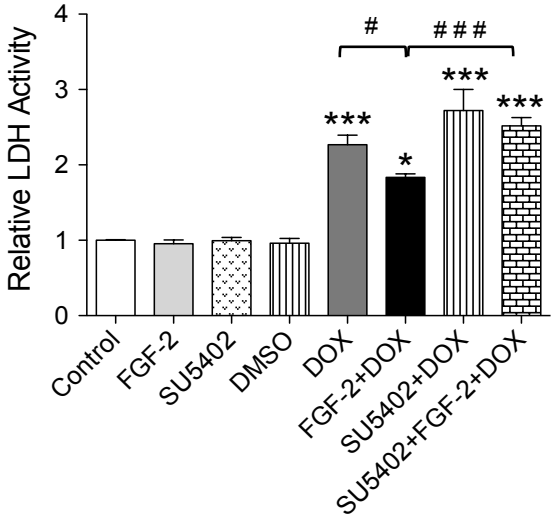


Figure 4. 3 - FGFR inhibitor SU 5402 interferes with the ability of FGF-2 to limit the release of LDH in response to doxorubicin treatment

Cultures were treated with FGF-2 or doxorubicin, or pretreated with FGF-2 for 30 minutes before doxorubicin (DOX), or pretreated with SU5204 or DMSO vehicle, for 30 minutes before FGF-2 and doxorubicin. Culture medium was assessed for LDH activity. Results are expressed as the mean plus or minus standard error of the mean (+SEM), relative to the control (untreated) value, which is arbitrarily set to 1. Data were assessed by one-way ANOVA with the Tukey-Kramer post-test. A value of $p<0.05$ is considered statistically significant: $p<0.05$ */#; $p<0.01$, $**^{/\#\#}$; $p<0.001$, $***^{/\#\#\#}$.

4.4: To investigate a role for PKC activation in any beneficial effect of FGF-2 observed in relation to doxorubicin-induced injury, using multiple PKC inhibitors.

Rationale

Increased resistance to doxorubicin-induced injury is observed with FGF-2 treatment of neonatal rat cardiac myocytes. The effects of FGF-2 on cardioprotection and ischemic preconditioning are associated with similar signal transduction pathways, and particularly PKCε [166]. Thus, involvement of PKC signaling was pursued as the prime candidate in any beneficial effects of FGF-2 in relation to doxorubicin-induced injury. Effective inhibition of PKC signaling pathways would provide further evidence of the direct cardioprotection of FGF-2 through the PKC signaling pathway. Chelerythrine, a PKC inhibitor, blocks the cardioprotective effects of FGF-2 from ischemia-reperfusion damage [155]. Bisindolylmaleimide I (BIM-1), another PKC inhibitor, can also suppress FGF-mediated activation of Erk MAP kinase in chondrocytes by preventing Shp2 association with the Frs2 and Gab1 adaptor proteins, further supporting involvement of PKC [168].

Approaches

Neonatal rat cardiac myocyte cultures were plated at a density of 1.3-million cells/60-mm diameter plate and were refed with defined medium for 24 hours. The involvement of PKC activation in the cytoprotection observed was pursued using independent cultures treated with PKC inhibitors, either 5 μM chelerythrine (Figure 4.4A) or 20 nM BIM-1 (Figure 4.4B), before co-treatment with 10 nM FGF-2, and subsequent addition of 0.5 μM doxorubicin. Cultures treated with doxorubicin or no pre-treatment were included as controls. Medium was then collected and tested for LDH activity and compared with doxorubicin alone or pre-treatment with FGF-2 before doxorubicin treatment.

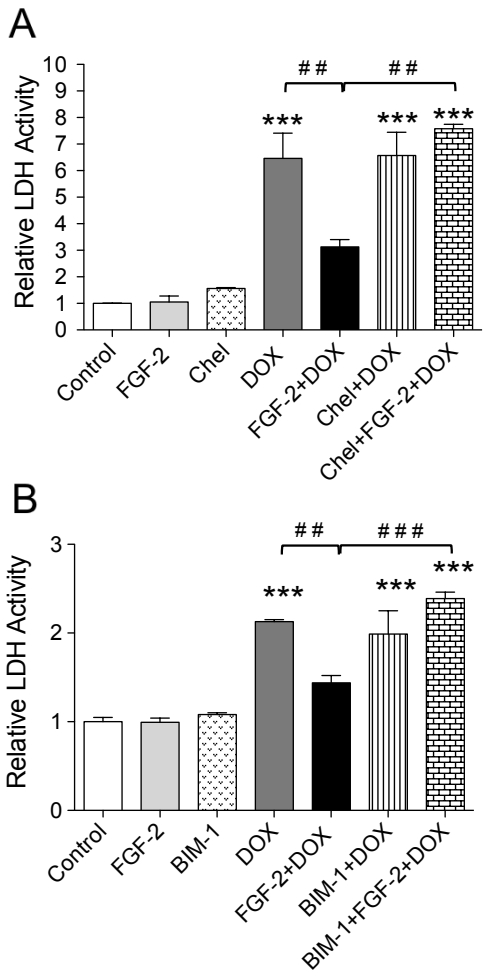
Results

There was no effect of FGF-2 on either chelerythrine or BIM-1 when used alone at 24 hours, however, there was a significant (2-6.5 fold) increase in LDH activity in the presence of doxorubicin. As expected, a significant decrease in LDH activity was observed with FGF-2 pre-treatment in the presence of doxorubicin at 24 hours compared to doxorubicin treatment alone. This decreased LDH activity associated with FGF-2 before treatment with doxorubicin, was blunted with either chelerythrine or BIM-1 pre-treatment (before FGF-2 and doxorubicin), such that LDH levels were not significantly different from cultures treated with doxorubicin alone.

Conclusions

The increased resistance of neonatal rat cardiac myocytes to doxorubicin-induced injury by FGF-2 is linked to the PKC signaling pathway. This is consistent with the involvement of PKC in the cardioprotective effects of FGF-2 seen in relation to ischemia/reperfusion injury [138]. This does not, of course, exclude other signaling pathways, including ERK and Akt. Previous studies showed that FGF-2 induces an increase in active MAPK at sarcolemmal and cytosolic sites [155]. The active sarcolemmal MAPK remained elevated, when the FGF-2-induced protection was prevented by chelerythrine [155]. This suggests the ERK-MAPK pathway may be upstream of the PKC signaling pathway [155]. There was no strong rationale to pursue the involvement of ERK and/or Akt in the context of FGF-2 protection from doxorubicin-induced injury, since the PKC inhibitors appear to block the increased resistance to injury completely.

Figure 4. 4 - PKC inhibitor, (A) chelerythrine and (B) BIM-1, both block the protective effects of FGF-2 against doxorubicin-induced LDH release



The increased resistance to doxorubicin induced LDH release seen with FGF-2 was inhibited by (A) chelerythrine (Chel) and (B) bisindolylmaleimide I (BIM-1) pretreatment in independent cultures. Cardiomyocytes were: left untreated (Control) or treated with 10 nM FGF-2, 5 μM Chel, 20 nM BIM-1, or 0.5 μM doxorubicin alone; or pretreated with FGF-2 for 30 minutes before doxorubicin for 24 hours (FGF-2+DOX), or Chel or BIM-1 for 15 minutes before addition of doxorubicin (Chel+DOX or BIM-1+DOX); or Chel or BIM-1 for 15 minutes before co-treatment with FGF-2 for 30 minutes before addition of doxorubicin (Chel+FGF-2+DOX or BIM-1+FGF-2+DOX). Culture medium was collected and the results for LDH activity. Results are expressed as the mean plus or minus standard error of the mean (+SEM), relative to the control (untreated) value, which is arbitrarily set to 1. Data were assessed by one-way ANOVA with the Tukey-Kramer post-test. A value of $p<0.05$ is considered statistically significant: $p<0.05$ */#; $p<0.01$, $^{**/\#\#}$; $p<0.001$, $^{***/\#\#\#}$.

4.5: To test the ability of FGF-2 to regulate multi-drug resistance gene mRNA levels in neonatal rat cardiac myocyte cultures

Rationale

Having established the concept of the ability of FGF-2 to increase the resistance of neonatal rat cardiac myocytes to doxorubicin-induced injury, further investigation into the mechanism(s) responsible was warranted. As stated in section I.2.1, "Doxorubicin metabolism", efflux of doxorubicin without conversion contributes to 50% of doxorubicin metabolism, and is the major reason for cancer cell drug resistance [28, 75]. Neonatal rat cardiac myocytes express high levels of MDR1b, MDR2 and MRP1, and relatively low MDR1a and MRP2 levels [72, 76]. It has been reported that MDR1b contributes to a P-gp/ABCB1-based drug extrusion in the heart [180]. Interestingly, FGF-2 has also emerged as a factor capable of modifying the sensitivity of normal and tumor cells to anti-cancer drugs, resulting in either increased drug resistance or sensitivity by affecting efflux ABC drug transporters, including MDR-1 and MRP1 [175, 177, 178]. This further increases the possibility of the ability of FGF-2 (as well as doxorubicin) to regulate efflux drug transporter level in cardiac myocytes.

Approaches

Total RNA was isolated from neonatal rat cardiac myocyte cultures and efflux drug transporter (MDR1a, MDR1b, MDR2, MRP1, MRP2) mRNA levels were assessed by qPCR to test: (a) the ability of doxorubicin and FGF-2 to regulate multi-drug resistance gene mRNA levels; and if regulation by FGF-2 in the presence of doxorubicin is detected (b) whether this regulation by FGF-2 on multi-drug resistance gene mRNA is PKC dependent.

Results

4.5a The ability of FGF-2 to regulate multi-drug resistance gene mRNA levels in neonatal rat cardiac myocyte cultures

Neonatal rat cardiac myocyte cultures were plated at a density of 1.3-million cells/60-mm diameter plate and were refed with defined medium for 24 hours before treatment without or with 0.5 μM doxorubicin or 10 nM FGF-2, or pre-treatment with FGF-2 for 30 minutes before doxorubicin. A possible effect of FGF-2 on efflux (drug) transporter gene mRNA (MDR1a, MDR1b, MDR2, MRP1, MRP2) expression in cardiac myocyte cultures was assessed by qPCR and compared to endogeneous B2M mRNA levels after 24 hours treatment (Figure 4.5A). Melting curve for all primers are shown in Figure 4.5B.

Doxorubicin administration induced a 10 and 9-fold increase in MDR1a and MDR1b RNA levels, respectively. By contrast, a 95% decrease in MDR2 and no effect on MRP1 transcripts were observed. An attempt was also made to assess MRP2 mRNA levels, however, although a low signal was detected with doxorubicin treatment, the 'untreated' control was below the level of detection relative to MDR1, MDR2 and MRP1 mRNAs. An increase in MDR1a mRNA levels was suggested that with FGF-2 treatment alone (significant by t-test) but, as with MDR1b and MRP1, the difference was not significant by one-way ANOVA. A significant 50% decrease in MDR2 mRNA levels was detected with FGF-2 administration (Figure 4.5A). MDR1a, MDR1b, MRP1 and MRP2 mRNA levels were all increased significantly with FGF-2 treatment in the presence of doxorubicin, when compared to doxorubicin treatment alone (Figure 4.5A); the increase in MDR2 mRNA levels was "just not significant" by one-way ANOVA, but very

significant by a t-test (Figure 4.5A).

MRP2 mRNA levels were relatively low in the neonatal cardiac myocyte cultures, and only detected after doxorubicin treatment, which is consistent with reports by others [76]; a further modest increase in mRNA was seen in the presence of FGF-2. By contrast, MDR1a and 1b, as well as MDR2 and MRP1 transcripts appear to be relatively more abundant, and RNA levels were increased by FGF-2 in the presence of doxorubicin.

These data suggest FGF-2 might facilitate the extrusion of doxorubicin via increase efflux drug transporters level. Increased transporter levels like MDR1a, 1b and MRP2 in response to doxorubicin treatment will facilitate extrusion of doxorubicin, which might contribute to doxorubicin resistance. FGF-2, when used with doxorubicin to treat neonatal cardiac myocytes, further increased the level of MDR1a, 1b, and MRP2 compared to cultures treated with doxorubicin alone, indicating a possibility of further extrusion of doxorubicin. Thus, MDR1a, 1b and MRP2 are relevant to doxorubicin efflux and the further increase in doxorubicin extrusion that is facilitated by FGF-2.

As for MDR2, doxorubicin alone decreases its level indicating a possible retention but not extrusion of doxorubicin as compared to the control (untreated). Pre-treatment with FGF-2 (before doxorubicin addition) decreases MDR2 levels as compared to the control (untreated), but notably increases MDR2 levels when compared to doxorubicin treatment alone. In fact, based on the RNA levels, MDR-2 might be involved in the extra extrusion/less retention of doxorubicin when FGF-2 was added before doxorubicin, and compared to doxorubicin alone.

In summary, when FGF-2 was added, all the transporters were increased and thus might participate in the protection conferred by FGF-2 against doxorubicin-induced neonatal rat cardiac myocyte injury.

4.5b Regulation of multi-drug resistance gene mRNA by FGF-2 is PKC dependent

To assess whether the increases in efflux (drug) transporter gene (MDR1a, MDR1b, MRP1, MRP2) mRNAs seen with FGF-2 in the presence of doxorubicin are PKC-dependent, cultures were treated without or with chelerythrine prior to treatment with FGF-2 and doxorubicin or doxorubicin alone for 24 hours. Relative MDR1b, MDR2, MRP1 and MRP2 mRNA levels were decreased significantly with treatment prior to treatment with FGF-2 and doxorubicin compared to prior treatment of doxorubicin alone, while MDR1a transcript levels were unaffected by chelerythrine inhibition (Fig. 4.5D). There was also no effect of chelerythrine alone on MDR1a and MDR1b mRNA levels, while those of MDR2 and MRP1 were decreased (Figure 4.5E). Chelerythrine alone significantly decreases MDR2 mRNA levels about 50% compared to Control, which indicates PKC might be involved in MDR2 expression or function. It has been reported that PKC is involved in expression and functinon of MDRs, depending on the isoforms of PKC and the cell type [208, 209]. For example, PKCα not γ is involved in upregulation of MDR in BC-19 cells [208, 209]. In addition, MDR serine 661, 667 and 671 residues can be phosphrylated *in vitro* by PKC to increase function activity, which supports a possible role for post-translational modification [210].

MDR1b, MDR2, MRP1 and MRP2 mRNA levels were increased by FGF-2 in the presence and absence of doxorubicin in cardiac myocytes, and this increase was blunted

by PKC inhibition. While MDR1a mRNA levels were increased by FGF-2 in the presence and absence of doxorubicin in cardiac myocytes, they were not significantly affected by PKC inhibition. Like PKC, both ERK and Akt are reported downstream targets of FGF-2 and FGFR interaction [211]. In this context, MDR1 production was increased via the Raf/MEK/ERK signaling cascade in breast cancer cells, and MRP1 expression was increased by the PI3K/PTEN/Akt pathway in prostate cancer cells [212, 213]. Different pathways may be involved with this increased efflux transporter by FGF-2 with doxorubicin treatment.

Conclusions

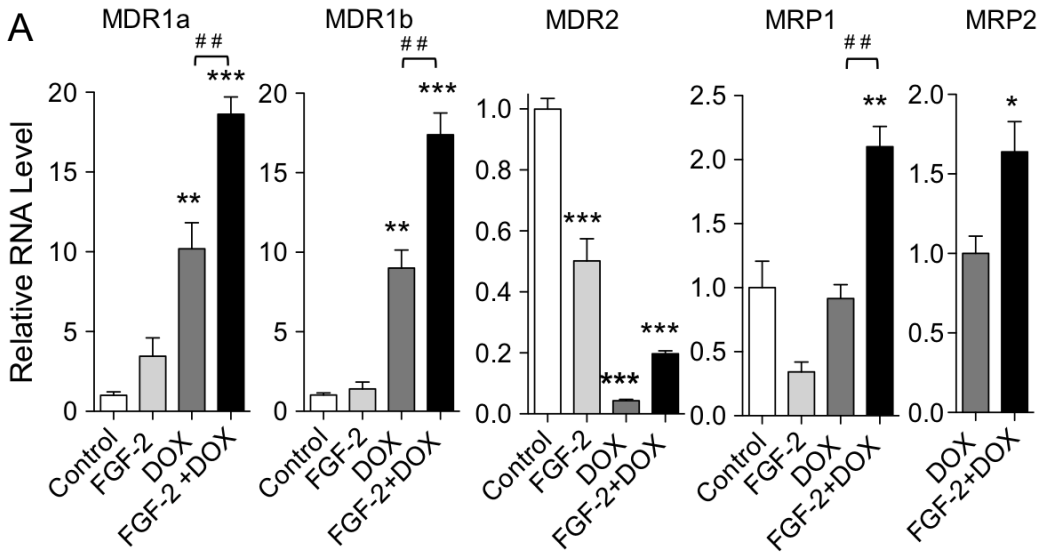
FGF-2 is able to increase multi-drug resistance gene (MDR1a, MDR1b, MDR2, MRP1, MRP2) mRNA levels in neonatal rat cardiac myocytes treated with doxorubicin, compared to those treated with doxorubicin alone. The results also indicate that this regulation by FGF-2 (in the presense of doxorubicin) of multi-drug resistance gene mRNAs (except MDR1a) is PKC dependent.

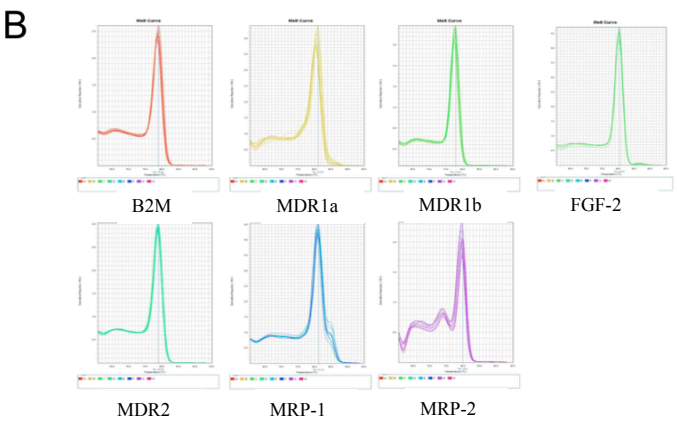
Additional evidence comes from our observations that the protection of FGF-2 against doxorubicin on LDH release from FGF-2 is PKC-dependent, or chelerythrine and BIM-1 sensitive, and that MDR1b, MRP1, MDR2 and less highly expressed MRP2 mRNA levels were also sensitive to chelerythrine pre-treatment. While these data support a role for MDRs and even MRPs in the FGF-2-related cytoprotective effects detected in the presence of DOX, it does not rule out possible participation of other efflux transporter proteins. In addition, a role in the removal of cardiotoxic substances would further emphasize the importance of PKC activation in cardioprotection by FGF-2, since this

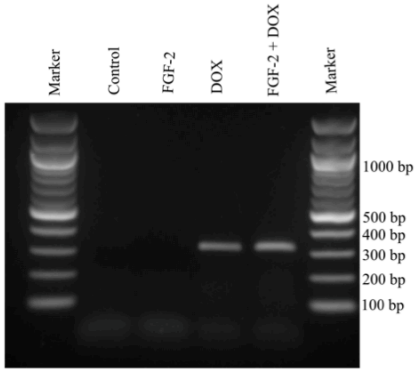
pathway has already been shown to signal increased resistance to ischemia-reperfusion injury with FGF-2 treatment [154]. Although the beneficial effect of FGF-2 on plasma membrane damage was reduced by PKC inhibition in this study, the results as suggested above indicate a role for additional pathways in the regulation of efflux transporters by FGF-2.

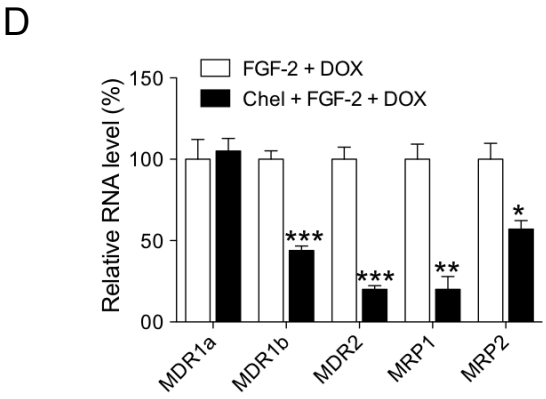
Although the increase in MDR1a transcript levels with FGF-2 in the presence of doxorubicin was not PKC dependent, PKC inhibition of other drug transporters still blunted the protective effects from FGF-2. Furthermore, MDR1a may not be as important, relatively, as MDR1b and MDR2 in the heart, based on distribution levels. MDR1a is expressed at relatively low levels in the heart (9%), compared to MDR1b (45%) and MDR2 (38.5%) [72, 82]. Thus, even decreases of the two more highly expressed efflux drug transporters (MDR1b and MDR2) by PKC would be expected to be significant.

As the melting curve for MRP2 suggested the possibility of a minor primer dimer (Figure 4.5B), agarose gel electrophoresis was done, and the presence of one major product of the expected size (318 bp) for MRP2 was detected (Figure 4.5C). MRP2 is expressed at relatively low levels in heart tissue compared to the other transporters tested [214], and was not detected in the control or FGF-2 treatment alone samples. The product was detected after DOX treatment (with and without FGF-2 treatment), but again at low levels.


A
MDR1a
MDR1b
MDR2
MRP1
MRP2
Relative RNA Level
Control
FGF-2
DOX
FGF-2+DOX


B
B2M
MDR1a
MDR1b
FGF-2
MDR2
MRP-1
MRP-2


C
Marker
Control
FGF-2
DOX
FGF-2 + DOX
Marker
1000 bp
500 bp
400 bp
300 bp
200 bp
100 bp


D
FGF-2 + DOX
Chel + FGF-2 + DOX
Relative RNA level (%)
MDR1a
MDR1b
MDR2
MRP1
MRP2

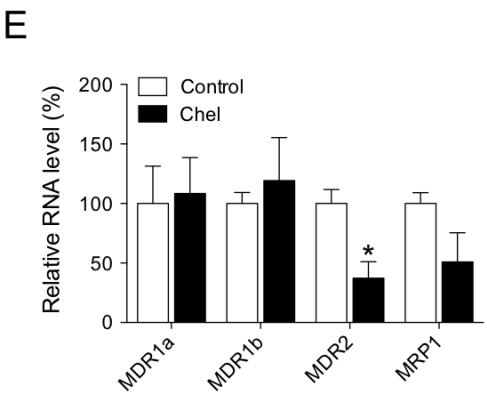

E
Control
Chel
Relative RNA level (%)
MDR1a
MDR1b
MDR2
MRP1

Figure 4. 5 - FGF-2 up-regulates MDR/MRP mRNA levels in the presence of doxorubicin (DOX) and a decrease in MDR/MRP mRNA levels are seen with chelerythrine pretreatment, except MDR-1a, in the presence of FGF-2 and doxorubicin.

(A) Cultures were treated with 10 nM FGF-2, 0.5 μM doxorubicin, or pretreated with FGF-2 for 30 minutes before treatment with DOX for 24 hours. MDR/MRP mRNA levels were assessed by qPCR. (B) Melting curve for all primers used for qPCR. (C) As the melting curve for MRP2 suggested the possibility of a minor primer dimer, agarose gel electrophoresis was done and the presence of one major product of the expected size (318 bp) for MRP2 was detected. MRP2 is expressed at relatively low levels in heart tissue compared to the other transporters tested, and was not detected in our control or FGF-2 treatment alone samples. The product was detected after doxorubicin treatment (with and without FGF-2 treatment), but again at low levels. (D) Cultures were treated with 10 nM FGF-2 and 0.5 μM doxorubicin without or with 5 μM chelerythrine (Chel) pretreatment, as well as (E) Chel treatment alone as a control. RNA was assessed by qPCR at 24 hours. The results are expressed as the mean plus or minus standard error of the mean (+SEM), relative to the control (untreated) value, which is arbitrarily set to 1. Data were assessed by one-way ANOVA with the Tukey-Kramer post-test except for MRP2, where a t-test was used. A value of $p<0.05$ is considered statistically significant: $p<0.05$ */#; $p<0.01$, **/##; $p<0.001$, ***/###.

4.6: To test if FGF-2 increases efflux drug transporter levels/function measured by doxorubicin retention/extrusion in neonatal rat cardiac myocytes

Rationale

The ability of FGF-2 to regulate multi-drug resistance gene mRNA levels in neonatal rat cardiac myocyte cultures suggested the ability to upregulate efflux drug transporter level/function. Doxorubicin has a fluorescent hydroxy-substituted anthraquinone chromophore structure that enables detection of an average fluorescence intensity level inside the cell that can be compared between untreated and treated groups [185] (Figure 4.6A). Decreased intensity indicates an increased efflux transporter level/function.

Approaches

The effect of FGF-2 on efflux drug transport levels/function (retention or extrusion) was measured by FACS of doxorubicin autofluorescence intensity levels. As positive controls, cardiac myocytes were pretreated with 20 μM CsA, an inhibitor of MDR1 and MRP2 transporters [215] or 2 μM verapamil, which blocks MDR1 in neonatal rat cardiac myocytes [188].

Results

No doxorubicin was detected in Control and FGF-2 groups of cardiac myocytes since no doxorubicin was added to the culture medium. There is a significant increase in fluorescence with doxorubicin treatment, while levels were decreased with FGF-2 pretreatment with doxorubicin when compared to doxorubicin treatment alone (Figure 4.6B). Treatment with CsA and verapamil was used as positive controls, and each resulted in a significant increase in doxorubicin concentration in cells (Figure 4.6B).

Standard curves of autofluorescence levels in relation to doxorubicin concentration were analyzed, and the median intracellular concentration of doxorubicin for each treatment was calculated using the software Graphpad Prism (Figure 4.6C). Examples of histograms of doxorubicin autofluorescence levels are shown (Figure 4.6D).

Conclusions

FGF-2 treatment is associated with decreased doxorubicin concentration in neonatal cardiac myocytes with doxorubicin treatment.

Although no phenol red medium was used, there is still a weak false positive signal from the autofluorescence backgroud of medium, however, this was not analyzed or included in our data. The autofluorescence intensity directly reflects the drug transporter activity on doxorubicin, however, there are limitations with this method. The effect of FGF-2 treatment alone cannot be compared to the control or any other test group, as there is no positive doxorubicin signal to detect. In addition, doxorubicin has not been reported as a substrate for any specific drug transporter, and our data showed that it had effects on all five transporters tested. Thus, the concentration of doxorubicin inside the cell indicates a balance of all drug transporters, including potentially any other efflux transporters that can be regulated by doxorubicin but are not shown/discussed here. Confirmation of the effect on efflux drug transporter by FGF-2 will be explored by inhibiting efflux drug transporter activity.

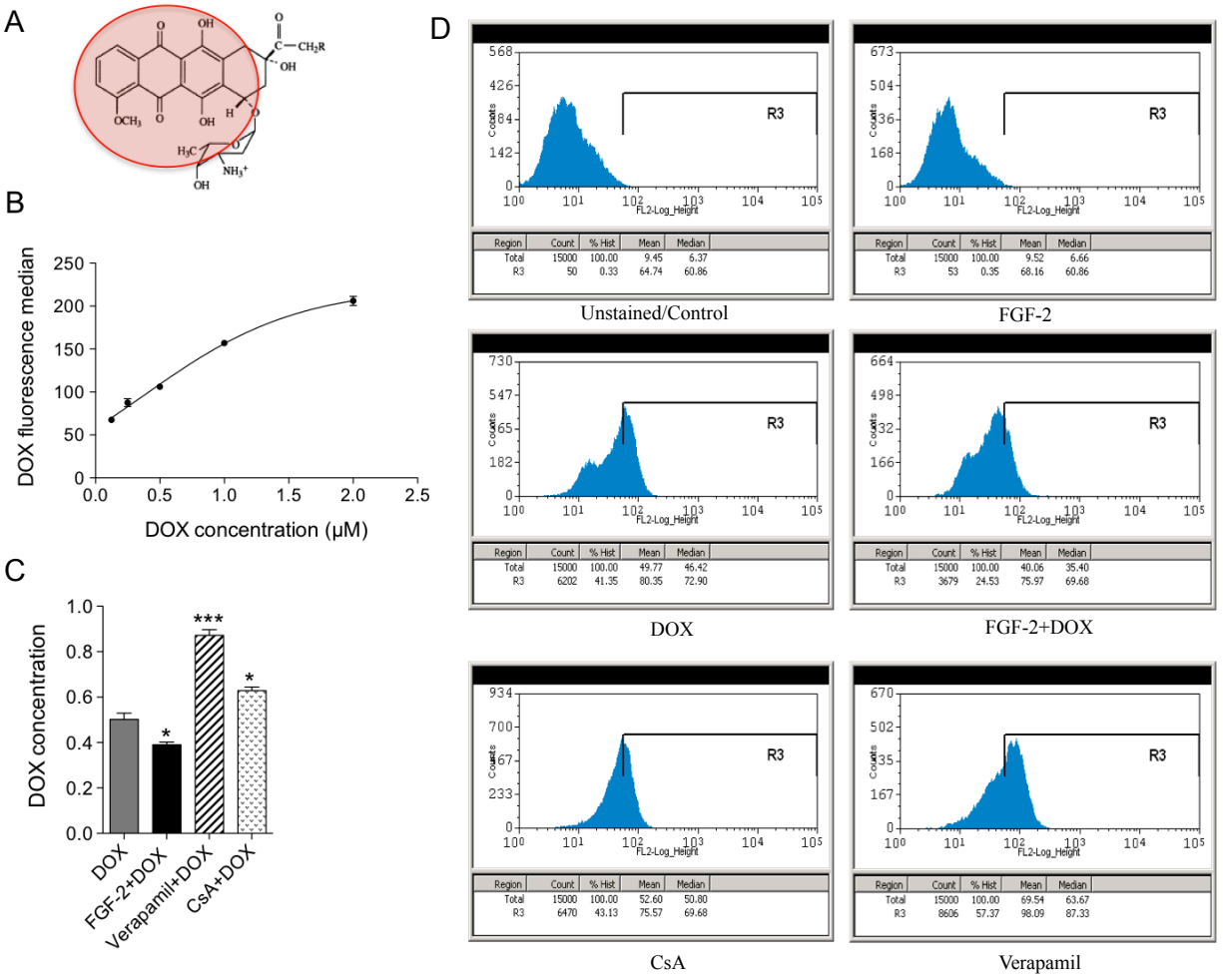

A
B
DOX fluorescence median
DOX concentration (μM)
C
DOX concentration
DOX
FGF-2+DOX
Verapamil+DOX
CsA+DOX
D
Unstained/Control
FGF-2
DOX
FGF-2+DOX
CsA
Verapamil

Figure 4. 6 - FGF-2 increases efflux drug transporter levels/function measured by doxorubicin retention/extrusion

Effects on intracellular doxorubicin (DOX) concentration by FGF-2 were also assessed by FACS using the autofluorescence property of doxorubicin (A). Briefly, cardiomyocytes were treated with doxorubicin and/or FGF-2, or 20 μM CsA or 2 μM verapamil as a positive control for efflux inhibition in medium (DMEM/F-12) without phenol red. Cells were pelleted and resuspended in the same medium for FACS analysis with λ_{Ex} and λ_{Em} of 488 and 585 nm as described. (B/C) The median intracellular concentration of doxorubicin for each treatment was calculated based on standard curve of autofluorescence levels in relation to doxorubicin concentration using the software Graphpad Prism. (D) Examples of histograms of doxorubicin autofluorescence levels are shown. The results in (B) are expressed as the mean plus or minus standard error of the mean (+SEM), relative to the doxorubicin concentration value. Data were assessed by one-way ANOVA with the Tukey-Kramer post-test. A value of $p<0.05$ is considered statistically significant: $p<0.05$ */#; $p<0.01$, **/##; $p<0.001$, ***/###.

4.7: To test whether FGF-2 increases on efflux transporter levels/function when measured by calcein retention/extrusion in neonatal rat cardiac myocytes

Rationale

Measuring doxorubicin autofluorescence intensity allowed a direct assessment of the effect of some treatments on doxorubicin transport, and more specifically indicated an increase in efflux drug transporter function/levels by FGF-2. This study was complemented, however, with the use of a cell-permeable non-fluorescent substrate calcein AM to "load" cardiac myocytes; calcein AM is converted to fluorescent calcein and remains in the cell cytoplasm following intracellular esterase activity that removes the acetomethoxy (AM) group, thereby converting the hydrophobic to a hydrophilic molecule [186] (Figure 4.7A). Calcein AM is a more specific substrate for the five transporters pursued. It also has the advantage of allowing the effect of FGF-2 alone to be assessed relative to other treatment groups and controls. Calcein AM assay has been used to detect drug interactions with multidrug resistance proteins in intact cells, such as anti-cancer drugs and its interaction with MDR-1 and MRP1 [216]. It can also be used as a model for drug-drug interactions, for screening drug transporter substrates and/or inhibitors, and also to determine *in vitro* drug resistance of cells, including samples from patients [217].

Approaches

Following treatment and loading of neonatal rat cardiac myocytes with calcein AM, the ability of FGF-2 to regulate efflux drug transport levels/function (retention or extrusion) was assessed by FACS of retained fluorescent calcein. The rentention/extrusion of calcein provides an average calcein fluorescence intensity level that can be compared

between each untreated and treated group. Gating for the analysis was based on determinations for untreated cells and calcein AM positive cells.

Results

Treatment with CsA, verapamil or XR9576 resulted in significant increases in calcein retention in the presence and absence of doxorubicin (Figure 4.7B). In contrast, a significant decrease in calcein (evidence of increased efflux/removal) was observed with FGF-2 treatment in the presence and absence of doxorubicin at 24 hours (Figure 4.7B). Examples of histograms of calcein levels are shown (Figure 4.7C).

FGF-2 alone decreases calcein levels and thus possibly increases calcein efflux, whereas it does not increase MDR/MRP expression. In regards to the calcein levels, it is a balance of all the transporters, including MDR, MRP and others. The data indicate that doxorubicin or FGF-2 alone can increase MDR1a, 1b levels but decrease MDR2 levels. This balance of the two transporters levels with one increasing and the other decreasing may account for the observation that calcein levels are not changed significantly compared to control in the neonatal cardiac myocyte cultures.

Conclusions

FGF-2 treatment is associated with increased calcein removal from neonatal cardiac myocytes in the presence or absence of doxorubicin treatment. The efflux pump inhibitors CsA, verapamil, XR9576, used as positive controls, all promoted calcein retention in the cell.

Three generations of efflux drug transporter inhibitors have been developed, thus,

manipulating efflux drug transporters to modulate the concentration of anti-cancer drugs such as doxorubicin offers a new prospect to clinical approaches for breaking through its limitations.

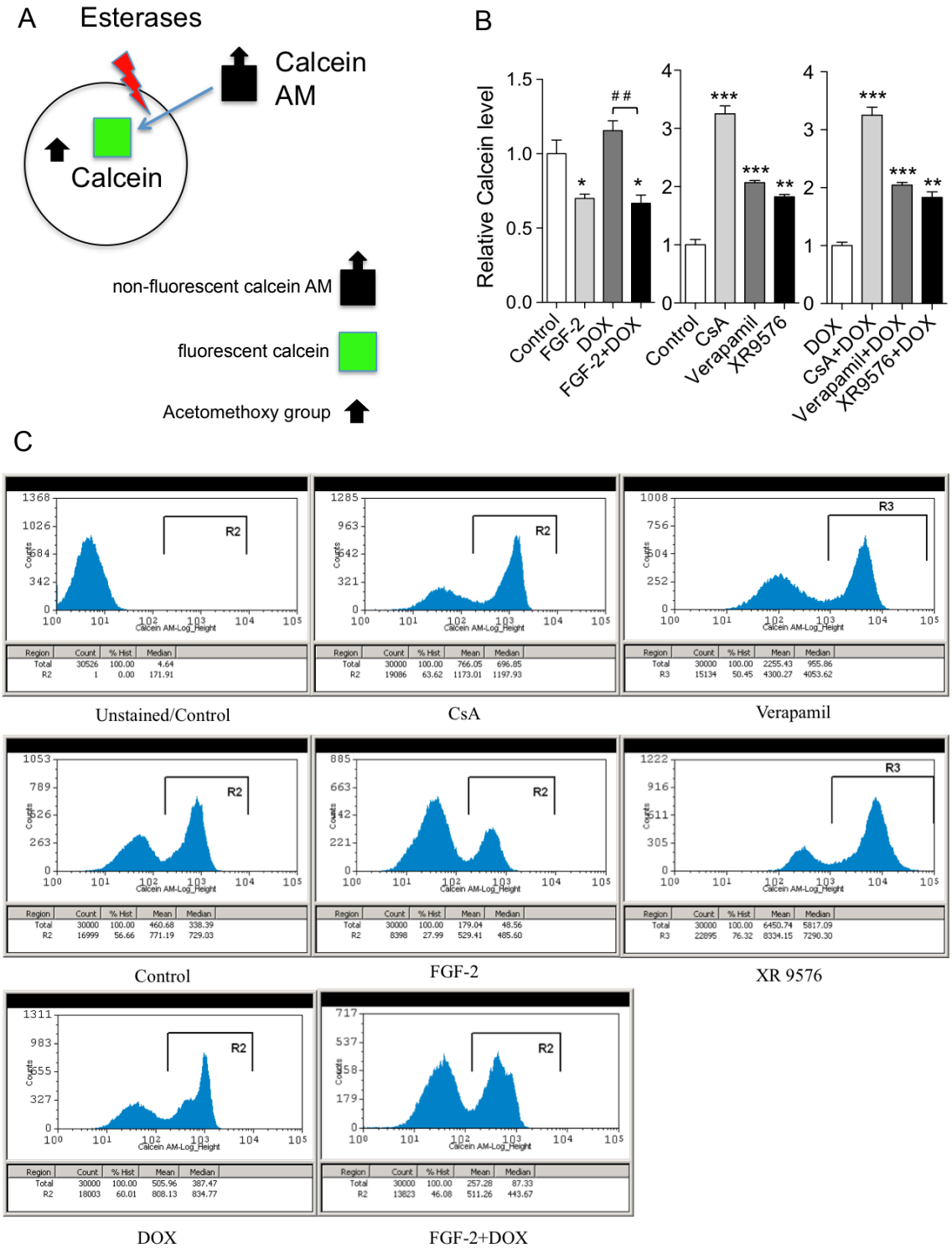

A
Esterases
Calcein AM
Calcein
non-fluorescent calcein AM
fluorescent calcein
Acetomethoxy group
B
Relative Calcein level
Control
FGF-2
DOX
FGF-2+DOX
CsA
Verapamil
XR9576
CsA+DOX
Verapamil+DOX
XR9576+DOX
C
Unstained/Control
CsA
Verapamil
Control
FGF-2
XR 9576
DOX
FGF-2+DOX

Figure 4. 7 - FGF-2 increases efflux transporter levels/function when measured by calcein retention/extrusion

Efflux of fluorescent calcein was assessed using the Multi-Drug Resistance Assay Kit (Calcein AM) according to the manufacturer's instructions (Cayman Chemical Company, Ann Arbor, USA). (A) Briefly, following intracellular esterase activity that removes the acetomethoxy (AM) group, calcein AM is converted to fluorescent calcein, which is retained in the cytoplasm. Cells were pelleted, resuspended in Assay Buffer and analyzed by FACS as described within 1 hour of staining. (B) Cultures were treated with doxorubicin (DOX) and/or FGF-2, or transporter inhibitors (e.g., 20 μM CsA, 2 μM verapamil or 1 μM XR9576) as a positive control for efflux inhibition. To load cells with calcein, cultures were treated with cell-permeable non-fluorescent substrate calcein AM solution 25 minutes prior to harvesting. Data were analyzed using the Summit v.5.2 program (Beckman). Gating for the analysis was based on determinations for untreated cells and calcein AM positive cells. (C) Examples of histograms of calcein levels are shown. Results are expressed as the mean plus or minus standard error of which is arbitrarily set to 1. Data were assessed by one-way ANOVA with the Tukey-Kramer post-test. A value of $p<0.05$ is considered statistically significant: $p<0.05$ */#; $p<0.01$, **/##; $p<0.001$, ***/###.

4.8 To test whether the positive effects of FGF-2 on efflux drug transport contributes to the increased resistance of neonatal rat cardiac myocytes to doxorubicin-induced plasma membrane damage

Rationale

The observations made are consistent with an increase in efflux drug transporter expression and function in response to FGF-2 pre-treatment in the presence of doxorubicin and, as a result, an expected increase in doxorubicin extrusion. A decrease in intracellular doxorubicin concentration in cardiac myocytes stimulated by FGF-2 may be a major reason for the increased resistance to doxorubicin-induced plasma membrane damage seen in the presence of FGF-2. As previously discussed (Chapter I.2.1) the major pathway for doxorubicin metabolism is not modification or breakdown to metabolites but rather extrusion or efflux of 'intact' doxorubicin.

Approaches

The possible relationship between the stimulation in efflux transport and an increase in resistance to doxorubicin-induced cell injury with FGF-2, as measured by LDH release, was examined by adding the transport inhibitors CsA, verapamil or XR9576 to neonatal rat cardiac myocyte cultures before treatment without or with FGF-2 and/or doxorubicin.

Results

There was no effect of CsA, verapamil or XR9576 alone on LDH release, but when each one was used in combination with doxorubicin, there was a significant increase in LDH release compared to doxorubicin treatment alone (Figure 4.8). This is consistent with retention of doxorubicin, and presumably increased neonatal rat cardiac myocyte

membrane damage and LDH release resulting from the higher intracellular doxorubicin concentrations. As expected, a significant decrease in LDH release, approaching ‘control’ levels was observed with combined FGF-2 and doxorubicin treatments (Figure 4.8A-C). By contrast, this beneficial effect of FGF-2 was not observed in the presence of CsA, verapamil or XR9576 pre-treatment (Figure 4.8A-C). The extent of doxorubicin-induced LDH release was however significantly reduced in cells treated with FGF-2 and CsA (or XR9576) compared to those treated with FGF-2 and verapamil, suggesting a partial 'rescue' by FGF-2. (Figure 4.8A-C). This partial rescue of the effect of CsA and XR9576 on increased retention and sensitivity to doxorubicin did not go beyond doxorubicin treatment alone and approach ‘control’ levels of LDH activity.

CsA is an effective immunosuppressant, and is also reported to be cardioprotective in the isolated rat heart in a dose-dependent manner [218]. While protection against ischemia-reperfusion-induced damage was seen at a low dose (0.2 μM), the protection was reversed when a high dose (1 μM) was used [218]. No effect on cell damage/LDH activity was seen when a higher dose (20 μM) of CsA was used alone on cardiac myocyte cultures, but LDH activity doubled when CsA was used in combination with doxorubicin [215, 219]. This is consistent with the reported role of CsA as an inhibitor and modulator of MDR1 and MRP2 efflux transporter function [215, 219], and suggests increased retention and sensitivity to the damaging effects of doxorubicin. Thus, the ability of pre-treatment with CsA to interfere with the cytoprotective effect of FGF-2 in our cultures is indicative of a mechanism involving efflux transport. This was supported when the beneficial effect of FGF-2, defined as a significant reduction in LDH release compared to doxorubicin treatment alone, was also suppressed with the MDR1/P-glycoprotein

inhibitors verapamil and XR9576.

Conclusions

The possible relationship between the stimulation in efflux transporter by FGF-2 and increased resistance to doxorubicin-induced cell injury by FGF-2 was addressed.

There was an apparent 'rescue' by FGF-2 of the negative effect of CsA and XR9576 in the presence of doxorubicin, on LDH release. FGF-2 was however found to be completely unable to reverse the verapamil induced potentiation of LDH release. No increase in neonatal cardiac myocyte damage was detected with CsA, XR9576 or verapamil, but an increase in LDH release was seen with CsA and XR9576 in the presence of doxorubicin. This presumably reflects the presence of a negative effect in addition to that on efflux transporter function, which is also limited by FGF-2. A partial rescue by FGF-2 of the negative effect of CsA and XR9576 on LDH release in the presence of doxorubicin was observed. This suggests that FGF-2 activity is not completely dependent on the signals targeted by CsA and XR9576, but it is fully dependent on signals inhibited by verapamil. There are at least four explanations for the lack of a similar effect with verapamil. These include:

(1) Specificity "theory": An absence or distinct property of the inhibitor(s), such as the specificity of individual drug transporters. CsA is a competitive inhibitor that can block the efflux drug transporter and compete with substrates of transporters preventing them from coming out of cells, while verapamil is a non-competitive inhibitor. In general, drug transporters work in a balance of both efflux and influx drug transporters. For this study, five efflux drug transporters were assessed. Although FGF-2 increased all five transporter

mRNA levels compared to doxorubicin alone, MDR2 mRNA level were still about 80% less compared to control (non-treated) levels, while the other four were all increased significantly. The abundance and significance for each efflux transporter in terms of efficiency or specificity for different drugs is not well understood. Thus, the blockage of transporters by different inhibitors as it relates to different drugs may vary from one to another.

(2) Concentration "theory": A difference in the effective concentration of each inhibitors contributing to the "full" or "half" inhibition, such that 2 μM verapamil was sufficient to negate 10 nM FGF-2 while 20 μM CsA and 1 μM XR9576 were not. In this case, increased concentration of inhibitors might increase the percentage of inhibition.

(3) L-type calcium channel "theory": Besides inhibition of efflux drug transporters, verapamil is also an L-type calcium channel blocker. Based on the literature, L-type calcium channel blockers that inhibit calcium influx also contribute to multidrug resistance independently of MDR protein overexpression [220]. L-type calcium channel blockers decrease cardiac myocyte contraction that can also lead to decreased release of drugs. This possibility also raises the idea that more than one mechanism might be involved in multidrug resistance [221]. Doxorubicin also induces mitochondrial dysfunction, possibly by loss of calcium homeostasis [222]. Thus, blocking the influx of calcium might interfere maintainance of calcium homeostasis. Further studies to evaluate the biological activities of verapamil, in addition to blocking L-type calcium channels, may provide further insight into the mechanism of action of cardiac myocyte protection by FGF-2.

(4) Contraction "theory": Endogenous FGF-2 is released upon cardiac myocyte contraction [154]. Verapamil would decrease heart contractility and energy expense [223], and thus in the context of neonatal cardiac myocytes might be expected to decrease endogenous FGF-2 release and, as a result, the frequency of influx and efflux doxorubicin.

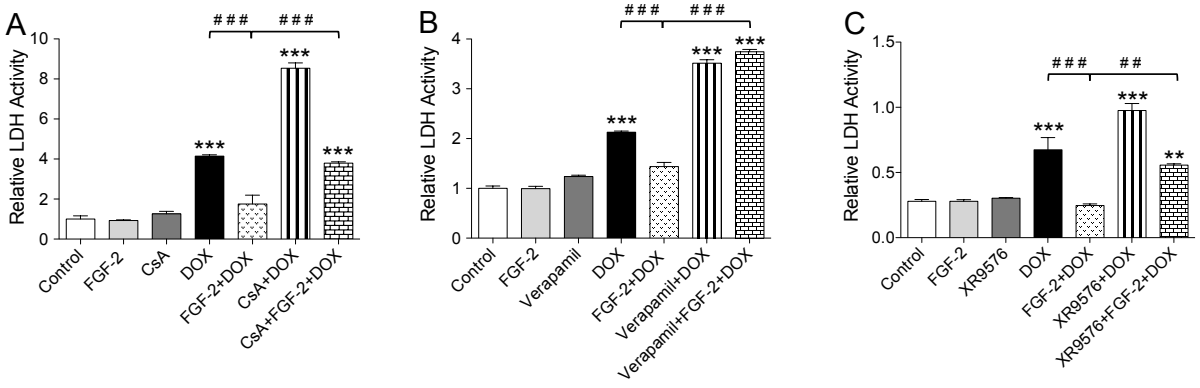


Figure 4. 8 - The FGF-2-induced upregulation of drug efflux transport contributes to the increased resistance of neonatal rat cardiac myocytes to doxorubicin-induced plasma membrane damage

Inhibition of efflux transport with (A) 20 μM cyclosporine A (CsA), (B) 2 μM verapamil and (C) 1 μM XR9576 interferes with the cytoprotective ability of FGF-2 against doxorubicin-induced neonatal rat cardiac myocyte damage. Cultures were treated with (i) CsA, verapamil, XR9576 or 0.5 μM doxorubicin alone, as well as (ii) CsA, verapamil or XR9576 with doxorubicin, and (iii) 10 nM FGF-2, FGF-2 and doxorubicin, and CsA, verapamil or XR9576, FGF-2 and doxorubicin. LDH activity in the medium was assessed at 24 hours. Results are expressed as the mean plus or minus standard error of the mean (+SEM), relative to the control (untreated) value, which is arbitrarily set to 1. Data were assessed by one-way ANOVA with the Tukey-Kramer post-test. A value of $p<0.05$ is considered statistically significant: $p<0.05$ */#; $p<0.01$, $^{**/\#\#}$; $p<0.001$, $^{***/\#\#\#}$.

CHAPTER V: DISCUSSION

The results of this study show for the first time that exogenous FGF-2 increases resistance to doxorubicin-induced damage in neonatal rat cardiac myocyte cultures. Pre-treatment with FGF-2 before doxorubicin resulted in a greater total cell number and a lower percentage of programmed cell death compared to doxorubicin treatment alone. Pre-treatment with FGF-2 was also able to decrease doxorubicin-induced LDH release indicating a significant effect on maintaining plasma membrane integrity. This increased resistance to damage was blunted by FGFR inhibition or PKC inhibition with chelerythrine or BIM-1, implicating FGFR and PKC activation in the beneficial effect of FGF-2. More specifically, pre-treatment with FGF-2 before treatment with doxorubicin was shown to increase efflux drug transporter, MDR1a, MDR1b, MDR2 and MRP1 mRNA levels, when compared to doxorubicin treatment alone. This increased mRNA level was sensitive to chelerythrine inhibition with the exception of MDR1a. Functionally, pre-treatment with FGF-2 before doxorubicin addition, was shown to increase efflux of substrates for drug transporters, specifically, doxorubicin and calcein. This indicates an increase in efflux drug transporter function or levels. Finally, the cytoprotective effect of FGF-2 was blunted when neonatal rat cardiac myocyte cultures were treated with the efflux transport inhibitors CsA, verapamil and XR9576, prior to FGF-2 and doxorubicin treatment. These observations implicate for the first time regulation of efflux drug transporter production and function as a component of the cytoprotective properties of FGF-2 for cardiac myocytes, and this is also linked to PKC activation.

Here, neonatal rat cardiac myocytes were used as a model, however, with respect to the *in vivo* myocardium, it is preferable to study adult myocytes [224]. During development, neonatal rat cardiac mycoytes change the expression of ion channels and contractile protein isoforms which make it problematic to extrapolate results to the fully differentiated adult myocardium [224]. That said, there are some characteristics that make neonatal cardiac myocytes a good choice for study. For example, primary neonatal rat cardiac myocyte cultures maintain a similar and stable contractile profile during hypoxia-reoxygenation when compared with *in vivo* hearts during ischemia-reperfusion injury [194]. This contrasts with adult cardiac myocyte cultures, where a distinct contractile profile is observed compared with hearts *in vivo* [194].

Doxorubicin and its metabolic products are cytotoxic to both cancer and heart cells and as a result multiple mechanisms are implicated [28, 29, 50-52]. In addition to side effects like cardiotoxicity, cancer cell resistance caused by effects on multidrug resistance transporters is also an obstacle that limits the use of chemotherapeutic agents like doxorubicin [66] (Figure 5). FGF-2 increases neonatal rat cardiac myocyte resistance to doxorubicin-induced injury by increasing efflux drug transporter levels, however, the effects of FGF-2 on cancer cell resistance mediated by efflux transporters is not well studied. If FGF-2 has similar effects on cancer cells to those on neonatal cardiac myocytes, further increased efflux drug transporter levels would be predicted to enhance the resistance of cancer to doxorubicin, which might compromise the protective effects on heart. If FGF-2 has a reduced or even opposite effect on cancer cells to that seen with neonatal cardiac myocytes, then decreased efflux drug transport levels might decrease the

resistance of cancer to the drug relative to the heart muscle cells, which would be benefical in terms of killing cancer while protecting heart.

An additional issue when considering FGF-2 as a cardioprotective agent, in the context of doxorubicin-based chemotherapy, is that FGF-2 alone has an effect on cancer cells, either by promoting cancer cell proliferation or inducing cancer cell death [225, 226]. In prostate cancer cells, exogenous administration of FGF-2 promoted proliferation and maintained cancer cell viability [225]. This similar beneficial effect on cancer cells would, perhaps then, offset any possible benefit of FGF-2 to decrease the vulnerability of cardiac myocytes to doxorubicin-induced injury *in vivo*. There is support, however, for differential effects on cardiac myocytes versus cancer cells that comes from data suggesting that FGF-2 can induce cancer cell death. In MCF-7 human breast cancer cells, overexpression of FGF-2 downregulates BCL-2 and promotes apoptotic cell death [226, 227]. In the Ewing's sarcoma family of tumors, FGF-2 induced cell death both *in vitro* and *in vivo* [227]. There is also evidence that FGF-2 can increase the sensitivity of tumor cells, including those of breast and ovarian origin, to anti-cancer platinum compounds like cisplatin as well as, but to a much lesser degree, doxorubicin [177, 228]. If true, the damaging effect on tumor cells may provide a second beneficial effect of FGF-2 to add to its ability to decrease the vulnerability of cardiac myocytes to doxorubicin-induced injury *in vivo*.

In this context, and although not explored, the number and type of FGFRs in the tumor cells might also be expected to affect the response of FGF-2 on cancer cell sensitivity. FGF-2 binds to FGFR and FGFR signaling has been linked to breast cancer cell growth

and death [229-232]. Human A431 skin epidermoid, and estrogen-independent breast cancer MDA-MB-231 tumor cells represent cancer cells responsive to doxorubicin treatment with low versus high FGFR1 levels and both express MDR1 and MRP1 [233-235]. Thus, besides cell survival, the differential expression level of FGF receptor in different cancer cells might influence the application of FGF-2 to maintain the protective effects on heart while provide no more benefit to cancer cells with anti-cancer drug treatment like doxorubicin.

In addition, FGF-2 and FGFR binding triggers multiple signaling pathways that can result in chemo-resistance and/or chemo-sensitization depending on dose and/or time of exposure [236]. The potential involvement of mutiple FGF-2 induced pathways to affect efflux drug transporters raises the possibility that conditions offering different or varying degrees of effects on cardiac myocytes versus cancer cells can be identified. As suggested by our data, MDR1a is not PKC dependent suggesting possible involvement of other signaling pathway(s), such as ERK1/2 and Akt. In addition, the distribution or expression level of efflux drug transporters is different in different tissue or cells in rats [214]. Thus, it might be important to increase our understanding of the FGF-2 signaling pathways regulating efflux drug transport and the major contributing transporters involved in drug resistance both in heart and cancer cells. Moreover, targeted inhibition of specific drug transporters like MDR1a or MDR1b might offer differential effects on ‘benefits’ versus ‘damage’ in different cell types. Thus, while complex, cell survival, the inherent variability in receptors and signaling pathways might provide the potential to identify conditions aimed at stimulating cardiac myocyte chemo-resistance while also increasing or maintaining the sensitivity of tumor cells to doxorubicin *in vivo*.

In addition to identifying and controlling differential expression of receptors and signaling pathways, it might also be possible to manipulate cancer resistance versus heart damage by considering efflux drug transporter substrate and inhibitor specificity. It has been reported that the breast cancer drug resistance protein (BCRP), which is a major drug transporter that contributes to breast cancer drug resistance, is not expressed in the heart [237]. Thus, specifically blocking the BCRP transporter activity may maintain the effectiveness in killing breast cancer cells while having less or no significant damaging effect on the heart.

Another aspect of manipulating FGF-2 to become less beneficial to cancer cell proliferation comes to manipulating its angiogenic properties. FGF-2 can be modified by substituting serine 117 with alanine (S117A) to diminish the ability of activating CK2 signaling pathway and stimulating cancer cell proliferation [150]. In addition, previous studies have shown that S117A-FGF-2 offers acute protective to the ischemic myocardium without or with reperfusion [150]. Short-term treatment with S117A-FGF-2 before or during ischemia occurs without or with perfusion provides protection to cardiac injuries [150]. Thus, modification of FGF-2 by diminishing the angiogenic property offers less benefit to cancer and still maintains the protection to heart.

Taken together, multiple aspects of applying FGF-2 as a benefical (endogenous or exogenous) growth factor to protect cardiac myocytes and the heart from doxorubicin-induced damage, while still permitting destruction of cancer cells, suggest this is a fruitful avenue worthwhile of further investigation even in a complex *in vivo* system (Figure 5.1).

In summary, FGF-2 increases resistance to injury in cardiac cells *in vitro* and in the myocardium *in vivo*, and positive effects on mitochondrial function, programmed cell death and cell-cell communications have been implicated in these events, via FGFR and PKC signaling [17, 154, 159]. Increased removal of cytotoxic agents can now be added as a potential mechanism of cardiac myocyte protection by FGF-2, and specifically increased resistance to doxorubicin-induced damage via an increase in efflux transporter production and function. This property would also be consistent with the concept of a role for FGF-2 in the 'self-protection' of the cardiac myocyte (and myocardium), which was raised previously in regard to autoregulation of FGF-2 in cardiac myocytes [131], and the observation that FGF-2 deficient mice are more susceptible to injury [141].

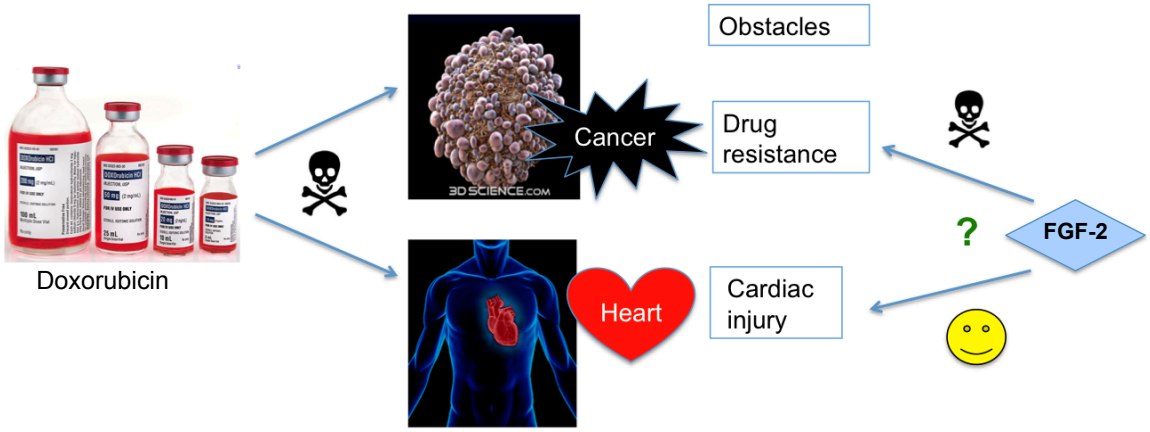


Figure 5. 1 - Doxorubicin: the heart versus cancer cell dilemma

Doxorubicin is among the most effect anti-cancer drugs, but there are two obstacles that limit its effectiveness: (i) increased cancer cell drug resistance and (ii) cardiac side effects. The observations made suggest FGF-2 may protect cardiac myocytes and the heart from doxorubicin damage via protein kinase C-dependent effects on efflux transporters. The effects of FGF-2 on cancer cell survival are controversial depending on different cancer cell types and may be influenced by FGFR levels/types.

REFERENCES

1. Heron, M., *Deaths: leading causes for 2008.* Natl Vital Stat Rep, 2012. **60**(6): p. 1-94.

2. Galbraith, E.M., P.K. Mehta, E. Veledar, V. Vaccarino, and N.K. Wenger, *Women and heart disease: knowledge, worry, and motivation.* J Womens Health (Larchmt), 2011. **20**(10): p. 1529-34.

3. Baptista, A., R.P. Costa, C. Ferreira, P. Mateus, A. Trigo Faria, and I. Moreira, *Pulmonary hypertension, heart failure and hyperthyroidism: A case report.* Rev Port Cardiol, 2013. pii: S0870-2551(12)00319-8.

4. Swirski, F.K. and M. Nahrendorf, *Leukocyte behavior in atherosclerosis, myocardial infarction, and heart failure.* Science, 2013. **339**(6116): p. 161-6.

5. Pfister, R., R. Cairns, E. Erdmann, and C.A. Schneider, *A clinical risk score for heart failure in patients with type 2 diabetes and macrovascular disease: an analysis of the PROactive study.* Int J Cardiol, 2013. **162**(2): p. 112-6.

6. Bunyamin, V., H. Spaderna, and G. Weidner, *Health behaviors contribute to quality of life in patients with advanced heart failure independent of psychological and medical patient characteristics.* Qual Life Res, 2012.

7. Haykowsky, M.J., D.M. Herrington, P.H. Brubaker, T.M. Morgan, W.G. Hundley, and D.W. Kitzman, *Relationship of flow-mediated arterial dilation and exercise capacity in older patients with heart failure and preserved ejection fraction.* J Gerontol A Biol Sci Med Sci, 2013. **68**(2): p. 161-7.

8. de Azambuja, E., P.L. Bedard, T. Suter, and M. Piccart-Gebhart, *Cardiac toxicity with anti-HER-2 therapies: what have we learned so far?* Target Oncol, 2009. **4**(2): p. 77-88.

9. Yoshida, M., I. Shiojima, H. Ikeda, and I. Komuro, *Chronic doxorubicin cardiotoxicity is mediated by oxidative DNA damage-ATM-p53-apoptosis*

pathway and attenuated by pitavastatin through the inhibition of Rac1 activity. J Mol Cell Cardiol, 2009. **47**(5): p. 698-705.

10. Costache, II and A. Petris, *[Cardiotoxicity of anthracyclines].* Rev Med Chir Soc Med Nat Iasi, 2011. **115**(4): p. 1200-7.

11. Jirkovsky, E., O. Popelova, P. Krivakova-Stankova, A. Vavrova, M. Hroch, P. Haskova, E. Brcakova-Dolezelova, S. Micuda, M. Adamcova, T. Simunek, Z. Cervinkova, V. Gersl, and M. Sterba, *Chronic anthracycline cardiotoxicity: molecular and functional analysis with focus on nuclear factor erythroid 2-related factor 2 and mitochondrial biogenesis pathways.* J Pharmacol Exp Ther, 2012. **343**(2): p. 468-78.

12. Wonders, K.Y., D.S. Hydock, C.M. Schneider, and R. Hayward, *Acute exercise protects against doxorubicin cardiotoxicity.* Integr Cancer Ther, 2008. **7**(3): p. 147-54.

13. Chelland Campbell, S., R.J. Moffatt, and B.A. Stamford, *Smoking and smoking cessation -- the relationship between cardiovascular disease and lipoprotein metabolism: a review.* Atherosclerosis, 2008. **201**(2): p. 225-35.

14. Frishman, W.H. and K. Pallerla, *Cardiovascular pharmacologic therapies under investigation: 2012.* Cardiol Rev, 2012. **20**(3): p. 102-10.

15. Mairesse, S., J. Blacher, and M.E. Safar, *[Focus on beta-blockers for vascular specialists in 2012].* J Mal Vasc, 2011. **36**(6): p. 339-47.

16. Frystyk, J., T. Ledet, N. Moller, A. Flyvbjerg, and H. Orskov, *Cardiovascular disease and insulin-like growth factor I.* Circulation, 2002. **106**(8): p. 893-5.

17. Kardami, E., K. Detillieux, X. Ma, Z. Jiang, J.J. Santiago, S.K. Jimenez, and P.A. Cattini, *Fibroblast growth factor-2 and cardioprotection.* Heart Fail Rev, 2007. **12**(3-4): p. 267-77.

18. Manning, J.R., G. Carpenter, D.R. Porter, S.L. House, D.A. Pietras, T. Doetschman, and J. Schultz Jel, *Fibroblast growth factor-2-induced cardioprotection against myocardial infarction occurs via the interplay between nitric oxide, protein kinase signaling, and ATP-sensitive potassium channels.* Growth Factors, 2012. **30**(2): p. 124-39.

19. Gobbi, P.G., A.J. Ferreri, M. Ponzoni, and A. Levis, *Hodgkin lymphoma.* Crit Rev Oncol Hematol, 2012.

20. Swystun, L.L., S. Mukherjee, and P.C. Liaw, *Breast cancer chemotherapy induces the release of cell-free DNA, a novel procoagulant stimulus.* J Thromb Haemost, 2011. **9**(11): p. 2313-21.

21. Zhao, X.Y., X.N. Hong, J.N. Cao, S.J. Leaw, Y. Guo, Z.T. Li, and J.H. Chang, *Clinical features and treatment outcomes of 14 cases of primary ovarian non-Hodgkin's lymphoma: a single-center experience.* Med Oncol, 2011. **28**(4): p. 1559-64.

22. San Miguel, J.F., M.V. Mateos, E. Ocio, and R. Garcia-Sanz, *Multiple myeloma: treatment evolution.* Hematology, 2012. **17 Suppl 1**: p. S3-6.

23. Wang, Y., M. Gonzalez, C. Cheng, A. Haouala, T. Krueger, S. Peters, L.A. Decosterd, H. van den Bergh, J.Y. Perentes, H.B. Ris, I. Letovanec, and E. Debefve, *Photodynamic induced uptake of liposomal doxorubicin to rat lung tumors parallels tumor vascular density.* Lasers Surg Med, 2012. **44**(4): p. 318-24.

24. Aguilo, J.I., M. Iturralde, I. Monleon, P. Inarrea, J. Pardo, M.J. Martinez-Lorenzo, A. Anel, and M.A. Alava, *Cytotoxicity of quinone drugs on highly proliferative human leukemia T cells: Reactive oxygen species generation and inactive shortened SOD1 isoform implications.* Chem Biol Interact, 2012. **198**(1-3): p. 18-28.

25. Arcamone, F., G. Cassinelli, G. Fantini, A. Grein, P. Orezzi, C. Pol, and C. Spalla, *Adriamycin, 14-hydroxydaunomycin, a new antitumor antibiotic from S. peucetius var. caesius. Reprinted from Biotechnology and Bioengineering, Vol. XI, Issue 6, Pages 1101-1110 (1969).* Biotechnol Bioeng, 2000. **67**(6): p. 704-13.

26. Ferrazzi, E., J.M. Woynarowski, A. Arakali, D.E. Brenner, and T.A. Beerman, *DNA damage and cytotoxicity induced by metabolites of anthracycline antibiotics, doxorubicin and idarubicin.* Cancer Commun, 1991. **3**(6): p. 173-80.

27. Licata, S., A. Saponiero, A. Mordente, and G. Minotti, *Doxorubicin metabolism and toxicity in human myocardium: role of cytoplasmic deglycosidation and carbonyl reduction.* Chem Res Toxicol, 2000. **13**(5): p. 414-20.

28. Mordente, A., E. Meucci, A. Silvestrini, G.E. Martorana, and B. Giardina, *New developments in anthracycline-induced cardiotoxicity.* Curr Med Chem, 2009. **16**(13): p. 1656-72.

29. Minotti, G., S. Recalcati, A. Mordente, G. Liberi, A.M. Calafiore, C. Mancuso, P. Preziosi, and G. Cairo, *The secondary alcohol metabolite of doxorubicin irreversibly inactivates aconitase/iron regulatory protein-1 in cytosolic fractions from human myocardium.* FASEB J, 1998. **12**(7): p. 541-52.

30. Minotti, G., *Reactions of adriamycin with microsomal iron and lipids.* Free Radic Res Commun, 1989. **7**(3-6): p. 143-8.

31. Pawlowska, J., J. Tarasiuk, C.R. Wolf, M.J. Paine, and E. Borowski, *Differential ability of cytostatics from anthraquinone group to generate free radicals in three enzymatic systems: NADH dehydrogenase, NADPH cytochrome P450 reductase, and xanthine oxidase.* Oncol Res, 2003. **13**(5): p. 245-52.

32. Vasquez-Vivar, J., P. Martasek, N. Hogg, B.S. Masters, K.A. Pritchard, Jr., and B. Kalyanaraman, *Endothelial nitric oxide synthase-dependent superoxide generation from adriamycin.* Biochemistry, 1997. **36**(38): p. 11293-7.

33. Fogli, S., P. Nieri, and M.C. Breschi, *The role of nitric oxide in anthracycline toxicity and prospects for pharmacologic prevention of cardiac damage.* FASEB J, 2004. **18**(6): p. 664-75.

34. Kassner, N., K. Huse, H.J. Martin, U. Godtel-Armbrust, A. Metzger, I. Meineke, J. Brockmoller, K. Klein, U.M. Zanger, E. Maser, and L. Wojnowski, *Carbonyl reductase 1 is a predominant doxorubicin reductase in the human liver.* Drug Metab Dispos, 2008. **36**(10): p. 2113-20.

35. Mordente, A., G. Minotti, G.E. Martorana, A. Silvestrini, B. Giardina, and E. Meucci, *Anthracycline secondary alcohol metabolite formation in human or rabbit heart: biochemical aspects and pharmacologic implications.* Biochem Pharmacol, 2003. **66**(6): p. 989-98.

36. Gutierrez, P.L., M.V. Gee, and N.R. Bachur, *Kinetics of anthracycline antibiotic free radical formation and reductive glycosidase activity.* Arch Biochem Biophys, 1983. **223**(1): p. 68-75.

37. Zelcer, N., T. Saeki, G. Reid, J.H. Beijnen, and P. Borst, *Characterization of drug transport by the human multidrug resistance protein 3 (ABCC3).* J Biol Chem, 2001. **276**(49): p. 46400-7.

38. Wohlfart, S., A.S. Khalansky, S. Gelperina, O. Maksimenko, C. Bernreuther, M. Glatzel, and J. Kreuter, *Efficient chemotherapy of rat glioblastoma using doxorubicin-loaded PLGA nanoparticles with different stabilizers.* PLoS One, 2011. **6**(5): p. e19121.

39. Chen, Y., Y. Wan, Y. Wang, H. Zhang, and Z. Jiao, *Anticancer efficacy enhancement and attenuation of side effects of doxorubicin with titanium dioxide nanoparticles.* Int J Nanomedicine, 2011. **6**: p. 2321-6.

40. Gewirtz, D.A., *A critical evaluation of the mechanisms of action proposed for the antitumor effects of the anthracycline antibiotics adriamycin and daunorubicin.* Biochem Pharmacol, 1999. **57**(7): p. 727-41.

41. Fry, D.W., T.J. Boritzki, J.A. Besserer, and R.C. Jackson, *In vitro DNA strand scission and inhibition of nucleic acid synthesis in L1210 leukemia cells by a new class of DNA complexers, the anthra[1,9-cd]pyrazol-6(2H)-ones (anthrapyrazoles).* Biochem Pharmacol, 1985. **34**(19): p. 3499-508.

42. Jensen, P.B., B.S. Sorensen, M. Sehested, E.J. Demant, E. Kjeldsen, E. Friche, and H.H. Hansen, *Different modes of anthracycline interaction with topoisomerase II. Separate structures critical for DNA-cleavage, and for overcoming topoisomerase II-related drug resistance.* Biochem Pharmacol, 1993. **45**(10): p. 2025-35.

43. Voest, E.E., E. Van Faassen, J.P. Neijt, J.J. Marx, and B.S. Van Asbeck, *Doxorubicin-mediated free radical generation in intact human tumor cells enhances nitroxide electron paramagnetic resonance absorption intensity decay.* Magn Reson Med, 1993. **30**(3): p. 283-8.

44. Zhang, Y.W., J. Shi, Y.J. Li, and L. Wei, *Cardiomyocyte death in doxorubicin-induced cardiotoxicity.* Arch Immunol Ther Exp (Warsz), 2009. **57**(6): p. 435-45.

45. Lu, W., Y.H. Li, X.F. He, J.B. Zhao, Y. Chen, and Q.L. Mei, *Necrosis and apoptosis in hepatocellular carcinoma following low-dose versus high-dose preoperative chemoembolization.* Cardiovasc Intervent Radiol, 2008. **31**(6): p. 1133-40.

46. Wang, Z., H. Kishimoto, P. Bhat-Nakshatri, C. Crean, and H. Nakshatri, *TNFalpha resistance in MCF-7 breast cancer cells is associated with altered subcellular localization of p21CIP1 and p27KIP1.* Cell Death Differ, 2005. **12**(1): p. 98-100.

47. Kotamraju, S., E.A. Konorev, J. Joseph, and B. Kalyanaraman, *Doxorubicin-induced apoptosis in endothelial cells and cardiomyocytes is ameliorated by nitrone spin traps and ebselen. Role of reactive oxygen and nitrogen species.* J Biol Chem, 2000. **275**(43): p. 33585-92.

48. Dartsch, D.C., A. Schaefer, S. Boldt, W. Kolch, and H. Marquardt, *Comparison of anthracycline-induced death of human leukemia cells: programmed cell death versus necrosis.* Apoptosis, 2002. **7**(6): p. 537-48.

49. Weiss, R.B., *The anthracyclines: will we ever find a better doxorubicin?* Semin Oncol, 1992. **19**(6): p. 670-86.

50. Pramanik, D., N.R. Campbell, S. Das, S. Gupta, V. Chenna, S. Bisht, P. Sysa-Shah, D. Bedja, C. Karikari, C. Steenbergen, K.L. Gabrielson, and A. Maitra, *A composite polymer nanoparticle overcomes multidrug resistance and ameliorates doxorubicin-associated cardiomyopathy.* Oncotarget, 2012. **3**(6): p. 640-50.

51. Octavia, Y., C.G. Tocchetti, K.L. Gabrielson, S. Janssens, H.J. Crijns, and A.L. Moens, *Doxorubicin-induced cardiomyopathy: from molecular mechanisms to therapeutic strategies.* J Mol Cell Cardiol, 2012. **52**(6): p. 1213-25.

52. Peng, X., B. Chen, C.C. Lim, and D.B. Sawyer, *The cardiotoxicology of anthracycline chemotherapeutics: translating molecular mechanism into preventative medicine.* Mol Interv, 2005. **5**(3): p. 163-71.

53. Witjes, J.A., M. Wullink, G.O. Oosterhof, and P. de Mulder, *Toxicity and results of MVAC (methotrexate, vinblastine, adriamycin and cisplatin) chemotherapy in advanced urothelial carcinoma.* Eur Urol, 1997. **31**(4): p. 414-9.

54. Love, R.R., H. Leventhal, D.V. Easterling, and D.R. Nerenz, *Side effects and emotional distress during cancer chemotherapy.* Cancer, 1989. **63**(3): p. 604-12.

55. Ferreira, A.L., L.S. Matsubara, and B.B. Matsubara, *Anthracycline-induced cardiotoxicity.* Cardiovasc Hematol Agents Med Chem, 2008. **6**(4): p. 278-81.

56. Lampidis, T.J., I.C. Henderson, M. Israel, and G.P. Canellos, *Structural and functional effects of adriamycin on cardiac cells in vitro.* Cancer Res, 1980. **40**(11): p. 3901-9.

57. Barnabe, N., R.A. Marusak, and B.B. Hasinoff, *Prevention of doxorubicin-induced damage to rat heart myocytes by arginine analog nitric oxide synthase inhibitors and their enantiomers.* Nitric Oxide, 2003. **9**(4): p. 211-6.

58. Howard, P.H., S. Payne, L. Wong, B. Gonzalez, and W. Lewis, *Lactate dehydrogenase activity in cultured neonatal rat heart cells exposed to doxorubicin.* Exp Mol Pathol, 1988. **48**(3): p. 311-6.

59. Ikegami, E., R. Fukazawa, M. Kanbe, M. Watanabe, M. Abe, M. Kamisago, M. Hajikano, Y. Katsube, and S. Ogawa, *Edaravone, a potent free radical scavenger, prevents anthracycline-induced myocardial cell death.* Circ J, 2007. **71**(11): p. 1815-20.

60. Lewis, W. and B. Gonzalez, *Anthracycline effects on actin and actin-containing thin filaments in cultured neonatal rat myocardial cells.* Lab Invest, 1986. **54**(4): p. 416-23.

61. Jones, R.L., *Utility of dexrazoxane for the reduction of anthracycline-induced cardiotoxicity.* Expert Rev Cardiovasc Ther, 2008. **6**(10): p. 1311-7.

62. Ludke, A., A.K. Sharma, A.K. Bagchi, and P.K. Singal, *Subcellular basis of vitamin C protection against doxorubicin-induced changes in rat cardiomyocytes.* Mol Cell Biochem, 2012. **360**(1-2): p. 215-24.

63. Kocak, G., K.M. Erbil, I. Ozdemir, S. Aydemir, B. Sunar, M. Tuncel, and S. Atalay, *The protective effect of melatonin on adriamycin-induced acute cardiac injury.* Can J Cardiol, 2003. **19**(5): p. 535-41.

64. Chae, H.J., H.R. Kim, J. Bae, S.U. Chae, K.C. Ha, and S.W. Chae, *Signal transduction of the protective effect of insulin like growth factor-1 on adriamycin-induced apoptosis in cardiac muscle cells.* Arch Pharm Res, 2004. **27**(3): p. 324-33.

65. Geisberg, C.A. and D.J. Lenihan, *Neuregulin in heart failure : reverse translation from cancer cardiotoxicity to new heart failure therapy.* Herz, 2011. **36**(4): p. 306-10.

66. Kaye, S. and S. Merry, *Tumour cell resistance to anthracyclines--a review.* Cancer Chemother Pharmacol, 1985. **14**(2): p. 96-103.

67. Melling, C.W., D.B. Thorp, K.J. Milne, and E.G. Noble, *Myocardial Hsp70 phosphorylation and PKC-mediated cardioprotection following exercise.* Cell Stress Chaperones, 2009. **14**(2): p. 141-50.

68. Vaziri, F., S.N. Peerayeh, Q.B. Nejad, and A. Farhadian, *The prevalence of aminoglycoside-modifying enzyme genes (aac (6')-I, aac (6')-II, ant (2")-I, aph (3')-VI) in Pseudomonas aeruginosa.* Clinics (Sao Paulo), 2011. **66**(9): p. 1519-22.

69. Jones, R.G. and C.B. Thompson, *Tumor suppressors and cell metabolism: a recipe for cancer growth.* Genes Dev, 2009. **23**(5): p. 537-48.

70. Martin, C., G. Berridge, C.F. Higgins, P. Mistry, P. Charlton, and R. Callaghan, *Communication between multiple drug binding sites on P-glycoprotein.* Mol Pharmacol, 2000. **58**(3): p. 624-32.

71. Broxterman, H.J., N. Feller, C.M. Kuiper, E. Boven, C.H. Versantvoort, T. Teerlink, H.M. Pinedo, and J. Lankelma, *Correlation between functional and molecular analysis of mdr1 P-glycoprotein in human solid-tumor xenografts.* Int J Cancer, 1995. **61**(6): p. 880-6.

72. Cui, T., Y. Liu, X. Men, Z. Xu, L. Wu, S. Liu, and A. Xing, *Bile acid transport correlative protein mRNA expression profile in human placenta with intrahepatic cholestasis of pregnancy.* Saudi Med J, 2009. **30**(11): p. 1406-10.

73. Flens, M.J., G.J. Zaman, P. van der Valk, M.A. Izquierdo, A.B. Schroeijers, G.L. Scheffer, P. van der Groep, M. de Haas, C.J. Meijer, and R.J. Scheper, *Tissue*

distribution of the multidrug resistance protein. Am J Pathol, 1996. **148**(4): p. 1237-47.

74. de Cerio, O.D., E. Bilbao, M.P. Cajaraville, and I. Cancio, *Regulation of xenobiotic transporter genes in liver and brain of juvenile thicklip grey mullets (Chelon labrosus) after exposure to Prestige-like fuel oil and to perfluorooctane sulfonate.* Gene, 2012. **498**(1): p. 50-8.

75. Gottesman, M.M., T. Fojo, and S.E. Bates, *Multidrug resistance in cancer: role of ATP-dependent transporters.* Nat Rev Cancer, 2002. **2**(1): p. 48-58.

76. Couture, L., J.A. Nash, and J. Turgeon, *The ATP-binding cassette transporters and their implication in drug disposition: a special look at the heart.* Pharmacol Rev, 2006. **58**(2): p. 244-58.

77. Hollenstein, K., R.J. Dawson, and K.P. Locher, *Structure and mechanism of ABC transporter proteins.* Curr Opin Struct Biol, 2007. **17**(4): p. 412-8.

78. Deeley, R.G., C. Westlake, and S.P. Cole, *Transmembrane transport of endo- and xenobiotics by mammalian ATP-binding cassette multidrug resistance proteins.* Physiol Rev, 2006. **86**(3): p. 849-99.

79. Sharom, F.J., *The P-glycoprotein multidrug transporter.* Essays Biochem, 2011. **50**(1): p. 161-78.

80. Schinkel, A.H., *P-Glycoprotein, a gatekeeper in the blood-brain barrier.* Adv Drug Deliv Rev, 1999. **36**(2-3): p. 179-194.

81. Cordon-Cardo, C., J.P. O'Brien, J. Boccia, D. Casals, J.R. Bertino, and M.R. Melamed, *Expression of the multidrug resistance gene product (P-glycoprotein) in human normal and tumor tissues.* J Histochem Cytochem, 1990. **38**(9): p. 1277-87.

82. Cui, Y.J., X. Cheng, Y.M. Weaver, and C.D. Klaassen, *Tissue distribution, gender-divergent expression, ontogeny, and chemical induction of multidrug*

resistance transporter genes (Mdr1a, Mdr1b, Mdr2) in mice. Drug Metab Dispos, 2009. **37**(1): p. 203-10.

83. Diestra, J.E., E. Condom, X.G. Del Muro, G.L. Scheffer, J. Perez, A.J. Zurita, J. Munoz-Segui, F. Vigues, R.J. Scheper, G. Capella, J.R. Germa-Lluch, and M.A. Izquierdo, *Expression of multidrug resistance proteins P-glycoprotein, multidrug resistance protein 1, breast cancer resistance protein and lung resistance related protein in locally advanced bladder cancer treated with neoadjuvant chemotherapy: biological and clinical implications.* J Urol, 2003. **170**(4 Pt 1): p. 1383-7.

84. Leonessa, F. and R. Clarke, *ATP binding cassette transporters and drug resistance in breast cancer.* Endocr Relat Cancer, 2003. **10**(1): p. 43-73.

85. Baghdasaryan, A., P. Fickert, A. Fuchsbichler, D. Silbert, J. Gumhold, G. Horl, C. Langner, T. Moustafa, E. Halilbasic, T. Claudel, and M. Trauner, *Role of hepatic phospholipids in development of liver injury in Mdr2 (Abcb4) knockout mice.* Liver Int, 2008. **28**(7): p. 948-58.

86. Buschman, E. and P. Gros, *The inability of the mouse mdr2 gene to confer multidrug resistance is linked to reduced drug binding to the protein.* Cancer Res, 1994. **54**(18): p. 4892-8.

87. Wang, R., J.A. Sheps, and V. Ling, *ABC transporters, bile acids, and inflammatory stress in liver cancer.* Curr Pharm Biotechnol, 2011. **12**(4): p. 636-46.

88. Klaassen, C.D. and L.M. Aleksunes, *Xenobiotic, bile acid, and cholesterol transporters: function and regulation.* Pharmacol Rev, 2010. **62**(1): p. 1-96.

89. Slot, A.J., S.V. Molinski, and S.P. Cole, *Mammalian multidrug-resistance proteins (MRPs).* Essays Biochem, 2011. **50**(1): p. 179-207.

90. Bellarosa, C., G. Bortolussi, and C. Tiribelli, *The role of ABC transporters in protecting cells from bilirubin toxicity.* Curr Pharm Des, 2009. **15**(25): p. 2884-92.

91. Young, L.C., B.G. Campling, S.P. Cole, R.G. Deeley, and J.H. Gerlach, *Multidrug resistance proteins MRP3, MRP1, and MRP2 in lung cancer: correlation of protein levels with drug response and messenger RNA levels.* Clin Cancer Res, 2001. **7**(6): p. 1798-804.

92. Zhou, S.F., L.L. Wang, Y.M. Di, C.C. Xue, W. Duan, C.G. Li, and Y. Li, *Substrates and inhibitors of human multidrug resistance associated proteins and the implications in drug development.* Curr Med Chem, 2008. **15**(20): p. 1981-2039.

93. Yu, X.Q., C.C. Xue, G. Wang, and S.F. Zhou, *Multidrug resistance associated proteins as determining factors of pharmacokinetics and pharmacodynamics of drugs.* Curr Drug Metab, 2007. **8**(8): p. 787-802.

94. Yamasaki, M., T. Makino, T. Masuzawa, Y. Kurokawa, H. Miyata, S. Takiguchi, K. Nakajima, Y. Fujiwara, N. Matsuura, M. Mori, and Y. Doki, *Role of multidrug resistance protein 2 (MRP2) in chemoresistance and clinical outcome in oesophageal squamous cell carcinoma.* Br J Cancer, 2011. **104**(4): p. 707-13.

95. Palmeira, A., E. Sousa, M.H. Vasconcelos, and M.M. Pinto, *Three decades of P-gp inhibitors: skimming through several generations and scaffolds.* Curr Med Chem, 2012. **19**(13): p. 1946-2025.

96. Tsuruo, T., H. Iida, S. Tsukagoshi, and Y. Sakurai, *Overcoming of vincristine resistance in P388 leukemia in vivo and in vitro through enhanced cytotoxicity of vincristine and vinblastine by verapamil.* Cancer Res, 1981. **41**(5): p. 1967-72.

97. Qadir, M., K.L. O'Loughlin, S.M. Fricke, N.A. Williamson, W.R. Greco, H. Minderman, and M.R. Baer, *Cyclosporin A is a broad-spectrum multidrug resistance modulator.* Clin Cancer Res, 2005. **11**(6): p. 2320-6.

98. Laupacis, A., P.A. Keown, R.A. Ulan, N. McKenzie, and C.R. Stiller, *Cyclosporin A: a powerful immunosuppressant.* Can Med Assoc J, 1982. **126**(9): p. 1041-6.

99. Cheung, B.M. and C.R. Kumana, *Calcium channel blockers revisited.* Hong Kong Med J, 2002. **8**(4): p. 300-1.

100. Denardo, S.J., F.H. Messerli, E. Gaxiola, J.M. Aranda, Jr., R.M. Cooper-Dehoff, E.M. Handberg, Y. Gong, A. Champion, Q. Zhou, and C.J. Pepine, *Characteristics and outcomes of revascularized patients with hypertension: an international verapamil SR-trandolapril substudy.* Hypertension, 2009. **53**(4): p. 624-30.

101. Chinushi, M., M. Tagawa, Y. Nakamura, and Y. Aizawa, *Shortening of the ventricular fibrillatory intervals after administration of verapamil in a patient with Brugada syndrome and vasospastic angina.* J Electrocardiol, 2006. **39**(3): p. 331-5.

102. Yamabe, H., Y. Tanaka, K. Morihisa, T. Uemura, K. Enomoto, H. Kawano, and H. Ogawa, *Analysis of the anatomical tachycardia circuit in verapamil-sensitive atrial tachycardia originating from the vicinity of the atrioventricular node.* Circ Arrhythm Electrophysiol, 2010. **3**(1): p. 54-62.

103. Ise, W., M. Heuser, K. Sanders, J. Beck, and V. Gekeler, *P-glycoprotein-associated resistance to taxol and taxotere and its reversal by dexniguldipine-HCl, dexverapamil-HCl, or cyclosporin A.* Int J Oncol, 1996. **8**(5): p. 951-6.

104. Malkhandi, J., D.R. Ferry, R. Boer, V. Gekeler, W. Ise, and D.J. Kerr, *Dexniguldipine-HCl is a potent allosteric inhibitor of [3H]vinblastine binding to P-glycoprotein of CCRF ADR 5000 cells.* Eur J Pharmacol, 1994. **288**(1): p. 105-14.

105. Fox, E. and S.E. Bates, *Tariquidar (XR9576): a P-glycoprotein drug efflux pump inhibitor.* Expert Rev Anticancer Ther, 2007. **7**(4): p. 447-59.

106. Pusztai, L., P. Wagner, N. Ibrahim, E. Rivera, R. Theriault, D. Booser, F.W. Symmans, F. Wong, G. Blumenschein, D.R. Fleming, R. Rouzier, G. Boniface, and G.N. Hortobagyi, *Phase II study of tariquidar, a selective P-glycoprotein inhibitor, in patients with chemotherapy-resistant, advanced breast carcinoma.* Cancer, 2005. **104**(4): p. 682-91.

107. Sharom, F.J., *ABC multidrug transporters: structure, function and role in chemoresistance.* Pharmacogenomics, 2008. **9**(1): p. 105-27.

108. Caceres, G., R.W. Robey, L. Sokol, K.L. McGraw, J. Clark, N.J. Lawrence, S.M. Sebti, M. Wiese, and A.F. List, *HG-829 is a potent noncompetitive inhibitor of the ATP-binding cassette multidrug resistance transporter ABCB1.* Cancer Res, 2012. **72**(16): p. 4204-13.

109. Chearwae, W., C.P. Wu, H.Y. Chu, T.R. Lee, S.V. Ambudkar, and P. Limtrakul, *Curcuminoids purified from turmeric powder modulate the function of human multidrug resistance protein 1 (ABCC1).* Cancer Chemother Pharmacol, 2006. **57**(3): p. 376-88.

110. Prevoo, B., D.S. Miller, F.M. van de Water, K.E. Wever, F.G. Russel, G. Flik, and R. Masereeuw, *Rapid, nongenomic stimulation of multidrug resistance protein 2 (Mrp2) activity by glucocorticoids in renal proximal tubule.* J Pharmacol Exp Ther, 2011. **338**(1): p. 362-71.

111. Dunne, G., L. Breen, D.M. Collins, S. Roche, M. Clynes, and R. O'Connor, *Modulation of P-gp expression by lapatinib.* Invest New Drugs, 2011. **29**(6): p. 1284-93.

112. Meissner, K., B. Sperker, C. Karsten, H. Meyer Zu Schwabedissen, U. Seeland, M. Bohm, S. Bien, P. Dazert, C. Kunert-Keil, S. Vogelgesang, R. Warzok, W. Siegmund, I. Cascorbi, M. Wendt, and H.K. Kroemer, *Expression and localization of P-glycoprotein in human heart: effects of cardiomyopathy.* J Histochem Cytochem, 2002. **50**(10): p. 1351-6.

113. Weiss, M. and W. Kang, *P-glycoprotein inhibitors enhance saturable uptake of idarubicin in rat heart: pharmacokinetic/pharmacodynamic modeling.* J Pharmacol Exp Ther, 2002. **300**(2): p. 688-94.

114. Jungsuwadee, P., R. Nithipongvanitch, Y. Chen, T.D. Oberley, D.A. Butterfield, D.K. St Clair, and M. Vore, *Mrp1 localization and function in cardiac mitochondria after doxorubicin.* Mol Pharmacol, 2009. **75**(5): p. 1117-26.

115. Kim, K.H., G.Y. Oudit, and P.H. Backx, *Erythropoietin protects against doxorubicin-induced cardiomyopathy via a phosphatidylinositol 3-kinase-dependent pathway.* J Pharmacol Exp Ther, 2008. **324**(1): p. 160-9.

116. Cai, C., L. Lothstein, R.R. Morrison, and P.A. Hofmann, *Protection from doxorubicin-induced cardiomyopathy using the modified anthracycline N-benzyladriamycin-14-valerate (AD 198).* J Pharmacol Exp Ther, 2010. **335**(1): p. 223-30.

117. Alshabanah, O.A., M.M. Hafez, M.M. Al-Harbi, Z.K. Hassan, S.S. Al Rejaie, Y.A. Asiri, and M.M. Sayed-Ahmed, *Doxorubicin toxicity can be ameliorated during antioxidant L-carnitine supplementation.* Oxid Med Cell Longev, 2010. **3**(6): p. 428-33.

118. Lee, C., F.M. Safdie, L. Raffaghello, M. Wei, F. Madia, E. Parrella, D. Hwang, P. Cohen, G. Bianchi, and V.D. Longo, *Reduced levels of IGF-I mediate differential protection of normal and cancer cells in response to fasting and improve chemotherapeutic index.* Cancer Res, 2010. **70**(4): p. 1564-72.

119. Ciftci, K., J. Su, and P.B. Trovitch, *Growth factors and chemotherapeutic modulation of breast cancer cells.* J Pharm Pharmacol, 2003. **55**(8): p. 1135-41.

120. Tarasenko, N., S.M. Cutts, D.R. Phillips, A. Inbal, A. Nudelman, G. Kessler-Icekson, and A. Rephaeli, *Disparate impact of butyroyloxymethyl diethylphosphate (AN-7), a histone deacetylase inhibitor, and doxorubicin in mice bearing a mammary tumor.* PLoS One, 2012. **7**(2): p. e31393.

121. Kottakis, F., C. Polytarchou, P. Foltopoulou, I. Sanidas, S.C. Kampranis, and P.N. Tsichlis, *FGF-2 regulates cell proliferation, migration, and angiogenesis through an NDY1/KDM2B-miR-101-EZH2 pathway.* Mol Cell, 2011. **43**(2): p. 285-98.

122. Lee, J.G. and E.P. Kay, *FGF-2-induced wound healing in corneal endothelial cells requires Cdc42 activation and Rho inactivation through the phosphatidylinositol 3-kinase pathway.* Invest Ophthalmol Vis Sci, 2006. **47**(4): p. 1376-86.

123. Comeau, W.L., E. Hastings, and B. Kolb, *Pre- and postnatal FGF-2 both facilitate recovery and alter cortical morphology following early medial prefrontal cortical injury.* Behav Brain Res, 2007. **180**(1): p. 18-27.

124. Fallon, J.F., A. Lopez, M.A. Ros, M.P. Savage, B.B. Olwin, and B.K. Simandl, *FGF-2: apical ectodermal ridge growth signal for chick limb development.* Science, 1994. **264**(5155): p. 104-7.

125. Powers, C.J., S.W. McLeskey, and A. Wellstein, *Fibroblast growth factors, their receptors and signaling.* Endocr Relat Cancer, 2000. **7**(3): p. 165-97.

126. Detillieux, K.A., F. Sheikh, E. Kardami, and P.A. Cattini, *Biological activities of fibroblast growth factor-2 in the adult myocardium.* Cardiovasc Res, 2003. **57**(1): p. 8-19.

127. Clarke, M.S., R.W. Caldwell, H. Chiao, K. Miyake, and P.L. McNeil, *Contraction-induced cell wounding and release of fibroblast growth factor in heart.* Circ Res, 1995. **76**(6): p. 927-34.

128. Kaye, D., D. Pimental, S. Prasad, T. Maki, H.J. Berger, P.L. McNeil, T.W. Smith, and R.A. Kelly, *Role of transiently altered sarcolemmal membrane permeability and basic fibroblast growth factor release in the hypertrophic response of adult rat ventricular myocytes to increased mechanical activity in vitro.* J Clin Invest, 1996. **97**(2): p. 281-91.

129. Santiago, J.J., X. Ma, L.J. McNaughton, B.E. Nickel, B.P. Bestvater, L. Yu, R.R. Fandrich, T. Netticadan, and E. Kardami, *Preferential accumulation and export of high molecular weight FGF-2 by rat cardiac non-myocytes.* Cardiovasc Res, 2011. **89**(1): p. 139-47.

130. Sun, G., B.W. Doble, J.M. Sun, R.R. Fandrich, R. Florkiewicz, L. Kirshenbaum, J.R. Davie, P.A. Cattini, and E. Kardami, *CUG-initiated FGF-2 induces chromatin compaction in cultured cardiac myocytes and in vitro.* J Cell Physiol, 2001. **186**(3): p. 457-67.

131. Jimenez, S.K., F. Sheikh, Y. Jin, K.A. Detillieux, J. Dhaliwal, E. Kardami, and P.A. Cattini, *Transcriptional regulation of FGF-2 gene expression in cardiac myocytes.* Cardiovasc Res, 2004. **62**(3): p. 548-57.

132. Sheikh, F., D.P. Sontag, R.R. Fandrich, E. Kardami, and P.A. Cattini, *Overexpression of FGF-2 increases cardiac myocyte viability after injury in isolated mouse hearts.* Am J Physiol Heart Circ Physiol, 2001. **280**(3): p. H1039-50.

133. Jiang, Z.S., M. Jeyaraman, G.B. Wen, R.R. Fandrich, I.M. Dixon, P.A. Cattini, and E. Kardami, *High- but not low-molecular weight FGF-2 causes cardiac hypertrophy in vivo; possible involvement of cardiotrophin-1.* J Mol Cell Cardiol, 2007. **42**(1): p. 222-33.

134. Ornitz, D.M., *FGFs, heparan sulfate and FGFRs: complex interactions essential for development.* Bioessays, 2000. **22**(2): p. 108-12.

135. Xu, C., E. Rosler, J. Jiang, J.S. Lebkowski, J.D. Gold, C. O'Sullivan, K. Delavan-Boorsma, M. Mok, A. Bronstein, and M.K. Carpenter, *Basic fibroblast growth factor supports undifferentiated human embryonic stem cell growth without conditioned medium.* Stem Cells, 2005. **23**(3): p. 315-23.

136. Amalric, F., G. Bouche, H. Bonnet, P. Brethenou, A.M. Roman, I. Truchet, and N. Quarto, *Fibroblast growth factor-2 (FGF-2) in the nucleus: translocation process and targets.* Biochem Pharmacol, 1994. **47**(1): p. 111-5.

137. Detillieux, K.A., P.A. Cattini, and E. Kardami, *Beyond angiogenesis: the cardioprotective potential of fibroblast growth factor-2.* Can J Physiol Pharmacol, 2004. **82**(12): p. 1044-52.

138. House, S.L., S.J. Melhorn, G. Newman, T. Doetschman, and J. Schultz Jel, *The protein kinase C pathway mediates cardioprotection induced by cardiac-specific overexpression of fibroblast growth factor-2.* Am J Physiol Heart Circ Physiol, 2007. **293**(1): p. H354-65.

139. Jimenez, S.K., D.S. Jassal, E. Kardami, and P.A. Cattini, *Protection by endogenous FGF-2 against isoproterenol-induced cardiac dysfunction is attenuated by cyclosporine A.* Mol Cell Biochem, 2011. **357**(1-2): p. 1-8.

140. Padua, R.R., R. Sethi, N.S. Dhalla, and E. Kardami, *Basic fibroblast growth factor is cardioprotective in ischemia-reperfusion injury.* Mol Cell Biochem, 1995. **143**(2): p. 129-35.

141. Virag, J.A., M.L. Rolle, J. Reece, S. Hardouin, E.O. Feigl, and C.E. Murry, *Fibroblast growth factor-2 regulates myocardial infarct repair: effects on cell proliferation, scar contraction, and ventricular function.* Am J Pathol, 2007. **171**(5): p. 1431-40.

142. Seghezzi, G., S. Patel, C.J. Ren, A. Gualandris, G. Pintucci, E.S. Robbins, R.L. Shapiro, A.C. Galloway, D.B. Rifkin, and P. Mignatti, *Fibroblast growth factor-2 (FGF-2) induces vascular endothelial growth factor (VEGF) expression in the endothelial cells of forming capillaries: an autocrine mechanism contributing to angiogenesis.* J Cell Biol, 1998. **141**(7): p. 1659-73.

143. Takeuchi, A., N. Yoshizawa, M. Yamamoto, Y. Sawasaki, T. Oda, A. Senoo, H. Niwa, and Y. Fuse, *Basic fibroblast growth factor promotes proliferation of rat glomerular visceral epithelial cells in vitro.* Am J Pathol, 1992. **141**(1): p. 107-16.

144. Goncalves, L.M., *Fibroblast growth factor-mediated angiogenesis for the treatment of ischemia. Lessons learned from experimental models and early human experience.* Rev Port Cardiol, 1998. **17 Suppl 2**: p. II11-20.

145. Sugimoto, Y., T. Koji, and S. Miyoshi, *Modification of expression of stem cell factor by various cytokines.* J Cell Physiol, 1999. **181**(2): p. 285-94.

146. House, S.L., C. Bolte, M. Zhou, T. Doetschman, R. Klevitsky, G. Newman, and J. Schultz Jel, *Cardiac-specific overexpression of fibroblast growth factor-2 protects against myocardial dysfunction and infarction in a murine model of low-flow ischemia.* Circulation, 2003. **108**(25): p. 3140-8.

147. Detillieux, K.A., J.T. Meij, E. Kardami, and P.A. Cattini, *alpha1-Adrenergic stimulation of FGF-2 promoter in cardiac myocytes and in adult transgenic mouse hearts.* Am J Physiol, 1999. **276**(3 Pt 2): p. H826-33.

148. Bertrand, S., I. Somorjai, J. Garcia-Fernandez, T. Lamonerie, and H. Escriva, *FGFRL1 is a neglected putative actor of the FGF signalling pathway present in all major metazoan phyla.* BMC Evol Biol, 2009. **9**: p. 226.

149. Cornish, E.E., R.C. Natoli, A. Hendrickson, and J.M. Provis, *Differential distribution of fibroblast growth factor receptors (FGFRs) on foveal cones: FGFR-4 is an early marker of cone photoreceptors.* Mol Vis, 2004. **10**: p. 1-14.

150. Jiang, Z.S., W. Srisakuldee, F. Soulet, G. Bouche, and E. Kardami, *Non-angiogenic FGF-2 protects the ischemic heart from injury, in the presence or absence of reperfusion.* Cardiovasc Res, 2004. **62**(1): p. 154-66.

151. Jiang, Z.S., G.B. Wen, Z.H. Tang, W. Srisakuldee, R.R. Fandrich, and E. Kardami, *High molecular weight FGF-2 promotes postconditioning-like*

cardioprotection linked to activation of the protein kinase C isoforms Akt and p70 S6 kinase. Can J Physiol Pharmacol, 2009. **87**(10): p. 798-804.

152. Schlessinger, J., *Cell signaling by receptor tyrosine kinases.* Cell, 2000. **103**(2): p. 211-25.

153. Bailly, K., F. Soulet, D. Leroy, F. Amalric, and G. Bouche, *Uncoupling of cell proliferation and differentiation activities of basic fibroblast growth factor.* FASEB J, 2000. **14**(2): p. 333-44.

154. Jiang, Z.S., R.R. Padua, H. Ju, B.W. Doble, Y. Jin, J. Hao, P.A. Cattini, I.M. Dixon, and E. Kardami, *Acute protection of ischemic heart by FGF-2: involvement of FGF-2 receptors and protein kinase C.* Am J Physiol Heart Circ Physiol, 2002. **282**(3): p. H1071-80.

155. Padua, R.R., P.L. Merle, B.W. Doble, C.H. Yu, P. Zahradka, G.N. Pierce, V. Panagia, and E. Kardami, *FGF-2-induced negative inotropism and cardioprotection are inhibited by chelerythrine: involvement of sarcolemmal calcium-independent protein kinase C.* J Mol Cell Cardiol, 1998. **30**(12): p. 2695-709.

156. Park, S.J., S.H. Kim, H.S. Choi, Y. Rhee, and S.K. Lim, *Fibroblast growth factor 2-induced cytoplasmic asparaginyl-tRNA synthetase promotes survival of osteoblasts by regulating anti-apoptotic PI3K/Akt signaling.* Bone, 2009. **45**(5): p. 994-1003.

157. Cui, X., H. Wang, H. Guo, C. Wang, H. Ao, X. Liu, and Y.Z. Tan, *Transplantation of mesenchymal stem cells preconditioned with diazoxide, a mitochondrial ATP-sensitive potassium channel opener, promotes repair of myocardial infarction in rats.* Tohoku J Exp Med, 2010. **220**(2): p. 139-47.

158. Okyar, A., C. Dressler, A. Hanafy, G. Baktir, B. Lemmer, and H. Spahn-Langguth, *Circadian variations in exsorptive transport: in situ intestinal perfusion data and in vivo relevance.* Chronobiol Int, 2012. **29**(4): p. 443-53.

159. Jiang, Z.S., G.B. Wen, Z.H. Tang, W. Srisakuldee, R.R. Fandrich, and E. Kardami, *High molecular weight FGF-2 promotes postconditioning-like cardioprotection linked to activation of protein kinase C isoforms, as well as Akt and p70 S6 kinases. [corrected]*. Can J Physiol Pharmacol, 2009. **87**(10): p. 798-804.

160. Hausenloy, D.J. and D.M. Yellon, *New directions for protecting the heart against ischaemia-reperfusion injury: targeting the Reperfusion Injury Salvage Kinase (RISK)-pathway.* Cardiovasc Res, 2004. **61**(3): p. 448-60.

161. Langford, D., R. Hurford, M. Hashimoto, M. Digicaylioglu, and E. Masliah, *Signalling crosstalk in FGF2-mediated protection of endothelial cells from HIV-gp120.* BMC Neurosci, 2005. **6**: p. 8.

162. Jensen, L., P. Schjerling, and Y. Hellsten, *Regulation of VEGF and bFGF mRNA expression and other proliferative compounds in skeletal muscle cells.* Angiogenesis, 2004. **7**(3): p. 255-67.

163. Steinberg, S.F., *Cardiac actions of protein kinase C isoforms.* Physiology (Bethesda), 2012. **27**(3): p. 130-9.

164. Doble, B.W., P. Ping, and E. Kardami, *The epsilon subtype of protein kinase C is required for cardiomyocyte connexin-43 phosphorylation.* Circ Res, 2000. **86**(3): p. 293-301.

165. Bogoyevitch, M.A., P.J. Parker, and P.H. Sugden, *Characterization of protein kinase C isotype expression in adult rat heart. Protein kinase C-epsilon is a major isotype present, and it is activated by phorbol esters, epinephrine, and endothelin.* Circ Res, 1993. **72**(4): p. 757-67.

166. Duquesnes, N., F. Lezoualc'h, and B. Crozatier, *PKC-delta and PKC-epsilon: Foes of the same family or strangers?* J Mol Cell Cardiol, 2011. **51**(5): p.665-73.

167. Srisakuldee, W., B.E. Nickel, R.R. Fandrich, Z.S. Jiang, and E. Kardami, *Administration of FGF-2 to the heart stimulates connexin-43 phosphorylation at protein kinase C target sites.* Cell Commun Adhes, 2006. **13**(1-2): p. 13-9.

168. Krejci, P., B. Masri, L. Salazar, C. Farrington-Rock, H. Prats, L.M. Thompson, and W.R. Wilcox, *Bisindolylmaleimide I suppresses fibroblast growth factor-mediated activation of Erk MAP kinase in chondrocytes by preventing Shp2 association with the Frs2 and Gab1 adaptor proteins.* J Biol Chem, 2007. **282**(5): p. 2929-36.

169. Iwai-Kanai, E., K. Hasegawa, M. Fujita, M. Araki, T. Yanazume, S. Adachi, and S. Sasayama, *Basic fibroblast growth factor protects cardiac myocytes from iNOS-mediated apoptosis.* J Cell Physiol, 2002. **190**(1): p. 54-62.

170. Izevbigie, E.B., J.S. Gutkind, and P.E. Ray, *Isoproterenol inhibits fibroblast growth factor-2-induced growth of renal epithelial cells.* Pediatr Nephrol, 2000. **14**(8-9): p. 726-34.

171. House, S.L., K. Branch, G. Newman, T. Doetschman, and J. Schultz Jel, *Cardioprotection induced by cardiac-specific overexpression of fibroblast growth factor-2 is mediated by the MAPK cascade.* Am J Physiol Heart Circ Physiol, 2005. **289**(5): p. H2167-75.

172. Juarez, J.C., M. Manuia, M.E. Burnett, O. Betancourt, B. Boivin, D.E. Shaw, N.K. Tonks, A.P. Mazar, and F. Donate, *Superoxide dismutase 1 (SOD1) is essential for H2O2-mediated oxidation and inactivation of phosphatases in growth factor signaling.* Proc Natl Acad Sci U S A, 2008. **105**(20): p. 7147-52.

173. Evseenko, D.A., J.W. Paxton, and J.A. Keelan, *Independent regulation of apical and basolateral drug transporter expression and function in placental trophoblasts by cytokines, steroids, and growth factors.* Drug Metab Dispos, 2007. **35**(4): p. 595-601.

174. Welti, J.C., M. Gourlaouen, T. Powles, S.C. Kudahetti, P. Wilson, D.M. Berney, and A.R. Reynolds, *Fibroblast growth factor 2 regulates endothelial cell sensitivity to sunitinib.* Oncogene, 2011. **30**(10): p. 1183-93.

175. Katayama, K., S. Yoshioka, S. Tsukahara, J. Mitsuhashi, and Y. Sugimoto, *Inhibition of the mitogen-activated protein kinase pathway results in the down-regulation of P-glycoprotein.* Mol Cancer Ther, 2007. **6**(7): p. 2092-102.

176. Song, S., M.G. Wientjes, Y. Gan, and J.L. Au, *Fibroblast growth factors: an epigenetic mechanism of broad spectrum resistance to anticancer drugs.* Proc Natl Acad Sci U S A, 2000. **97**(15): p. 8658-63.

177. Coleman, A.B., *Positive and negative regulation of cellular sensitivity to anti-cancer drugs by FGF-2.* Drug Resist Updat, 2003. **6**(2): p. 85-94.

178. Cole, S.P., G. Bhardwaj, J.H. Gerlach, J.E. Mackie, C.E. Grant, K.C. Almquist, A.J. Stewart, E.U. Kurz, A.M. Duncan, and R.G. Deeley, *Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line.* Science, 1992. **258**(5088): p. 1650-4.

179. Lin, S.T., H.C. Chou, Y.W. Chen, and H.L. Chan, *Redox-proteomic analysis of doxorubicin-induced altered thiol activity in cardiomyocytes.* Mol Biosyst, 2013. **9**(3): p. 447-56.

180. Krishnamurthy, K., K. Vedam, R. Kanagasabai, L.J. Druhan, and G. Ilangovan, *Heat shock factor-1 knockout induces multidrug resistance gene, MDR1b, and enhances P-glycoprotein (ABCB1)-based drug extrusion in the heart.* Proc Natl Acad Sci U S A, 2012. **109**(23): p. 9023-8.

181. Savill, J., V. Fadok, P. Henson, and C. Haslett, *Phagocyte recognition of cells undergoing apoptosis.* Immunol Today, 1993. **14**(3): p. 131-6.

182. Koopman, G., C.P. Reutelingsperger, G.A. Kuijten, R.M. Keehnen, S.T. Pals, and M.H. van Oers, *Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis.* Blood, 1994. **84**(5): p. 1415-20.

183. Ura, H., K. Hirata, and T. Katsuramaki, *[Mechanisms of cell death in hypoxic stress]*. Nihon Geka Gakkai Zasshi, 1999. **100**(10): p. 656-62.

184. Nakajima, T., T.R. Shearer, and M. Azuma, *Involvement of calpain 2 in ionomycin-induced cell death in cultured mouse lens epithelial cells.* Curr Eye Res, 2011. **36**(10): p. 930-6.

185. Karukstis, K.K., E.H. Thompson, J.A. Whiles, and R.J. Rosenfeld, *Deciphering the fluorescence signature of daunomycin and doxorubicin.* Biophys Chem, 1998. **73**(3): p. 249-63.

186. Orchard, A., G.A. Schamerhorn, B.D. Calitree, G.A. Sawada, T.W. Loo, M. Claire Bartlett, D.M. Clarke, and M.R. Detty, *Thiorhodamines containing amide and thioamide functionality as inhibitors of the ATP-binding cassette drug transporter P-glycoprotein (ABCB1).* Bioorg Med Chem, 2012. **20**(14): p. 4290-302.

187. Chlopcikova, S., J. Psotova, and P. Miketova, *Neonatal rat cardiomyocytes--a model for the study of morphological, biochemical and electrophysiological characteristics of the heart.* Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub, 2001. **145**(2): p. 49-55.

188. Estevez, M.D., A. Wolf, and U. Schramm, *Effect of PSC 833, verapamil and amiodarone on adriamycin toxicity in cultured rat cardiomyocytes.* Toxicol In Vitro, 2000. **14**(1): p. 17-23.

189. Limaye, D.A. and Z.A. Shaikh, *Cytotoxicity of cadmium and characteristics of its transport in cardiomyocytes.* Toxicol Appl Pharmacol, 1999. **154**(1): p. 59-66.

190. Acosta, D. and K. Ramos, *Cardiotoxicity of tricyclic antidepressants in primary cultures of rat myocardial cells.* J Toxicol Environ Health, 1984. **14**(2-3): p. 137-43.

191. Orita, H., M. Fukasawa, S. Hirooka, H. Uchino, K. Fukui, M. Kohi, and M. Washio, *Cytoprotective effects of nicorandil on hypothermic injury to immature*

cardiac myocytes--comparison with nitroglycerin, diltiazem and prostaglandin E1. Jpn Circ J, 1994. **58**(8): p. 653-61.

192. Lu, S.Y., D.P. Sontag, K.A. Detillieux, and P.A. Cattini, *FGF-16 is released from neonatal cardiac myocytes and alters growth-related signaling: a possible role in postnatal development.* Am J Physiol Cell Physiol, 2008. **294**(5): p. C1242-9.

193. Wang, J., M.W. Nachtigal, E. Kardami, and P.A. Cattini, *FGF-2 protects cardiomyocytes from doxorubicin damage via protein kinase C-dependent effects on efflux transporters*. Cardiovasc Res, 2013. **98**(1): p.56-63

194. Yamashita, N., M. Nishida, S. Hoshida, T. Kuzuya, M. Hori, N. Taniguchi, T. Kamada, and M. Tada, *Induction of manganese superoxide dismutase in rat cardiac myocytes increases tolerance to hypoxia 24 hours after preconditioning.* J Clin Invest, 1994. **94**(6): p. 2193-9.

195. Ito, H., S.C. Miller, M.E. Billingham, H. Akimoto, S.V. Torti, R. Wade, R. Gahlmann, G. Lyons, L. Kedes, and F.M. Torti, *Doxorubicin selectively inhibits muscle gene expression in cardiac muscle cells in vivo and in vitro.* Proc Natl Acad Sci U S A, 1990. **87**(11): p. 4275-9.

196. Andrieu-Abadie, N., J.P. Jaffrezou, S. Hatem, G. Laurent, T. Levade, and J.J. Mercadier, *L-carnitine prevents doxorubicin-induced apoptosis of cardiac myocytes: role of inhibition of ceramide generation.* FASEB J, 1999. **13**(12): p. 1501-10.

197. Zang, M.F., Y.M. Zhang, Y.H. Zhi, Z. Zhai, M. Zhang, F. Gu, and X.C. Zhi, *[Comparative study of chemosensitivity and efficacy between pirarubicin and epirubicin in breast cancer]*. Zhonghua Yi Xue Za Zhi, 2011. **91**(20): p. 1388-92.

198. Zhang, Y., T.S. Li, S.T. Lee, K.A. Wawrowsky, K. Cheng, G. Galang, K. Malliaras, M.R. Abraham, C. Wang, and E. Marban, *Dedifferentiation and proliferation of mammalian cardiomyocytes.* PLoS One, 2010. **5**(9): p. e12559.

199. Cook, S.A. and P.A. Poole-Wilson, *Cardiac myocyte apoptosis.* Eur Heart J, 1999. **20**(22): p. 1619-29.

200. Lockshin, R.A. and J. Beaulaton, *Programmed cell death. Cytochemical appearance of lysosomes when death of the intersegmental muscles is prevented.* J Ultrastruct Res, 1974. **46**(1): p. 63-78.

201. Guicciardi, M.E., M. Leist, and G.J. Gores, *Lysosomes in cell death.* Oncogene, 2004. **23**(16): p. 2881-90.

202. Ono, K., S.O. Kim, and J. Han, *Susceptibility of lysosomes to rupture is a determinant for plasma membrane disruption in tumor necrosis factor alpha-induced cell death.* Mol Cell Biol, 2003. **23**(2): p. 665-76.

203. Cuevas, P., F. Carceller, R.M. Lozano, A. Crespo, M. Zazo, and G. Gimenez-Gallego, *Protection of rat myocardium by mitogenic and non-mitogenic fibroblast growth factor during post-ischemic reperfusion.* Growth Factors, 1997. **15**(1): p. 29-40.

204. Tarasenko, N., G. Kessler-Icekson, P. Boer, A. Inbal, H. Schlesinger, D.R. Phillips, S.M. Cutts, A. Nudelman, and A. Rephaeli, *The histone deacetylase inhibitor butyroyloxymethyl diethylphosphate (AN-7) protects normal cells against toxicity of anticancer agents while augmenting their anticancer activity.* Invest New Drugs, 2012. **30**(1): p. 130-43.

205. Alavi, A.S., L. Acevedo, W. Min, and D.A. Cheresh, *Chemoresistance of endothelial cells induced by basic fibroblast growth factor depends on Raf-1-mediated inhibition of the proapoptotic kinase, ASK1.* Cancer Res, 2007. **67**(6): p. 2766-72.

206. Coleman, A.B., J. Momand, and S.E. Kane, *Basic fibroblast growth factor sensitizes NIH 3T3 cells to apoptosis induced by cisplatin.* Mol Pharmacol, 2000. **57**(2): p. 324-33.

207. Kiyonari, H., M. Kaneko, S. Abe, and S. Aizawa, *Three inhibitors of FGF receptor, ERK, and GSK3 establishes germline-competent embryonic stem cells of C57BL/6N mouse strain with high efficiency and stability.* Genesis, 2010. **48**(5): p. 317-27.

208. Ahmad, S., J.B. Trepel, S. Ohno, K. Suzuki, T. Tsuruo, and R.I. Glazer, *Role of protein kinase C in the modulation of multidrug resistance: expression of the atypical gamma isoform of protein kinase C does not confer increased resistance to doxorubicin.* Mol Pharmacol, 1992. **42**(6): p. 1004-9.

209. Ahmad, S. and R.I. Glazer, *Expression of the antisense cDNA for protein kinase C alpha attenuates resistance in doxorubicin-resistant MCF-7 breast carcinoma cells.* Mol Pharmacol, 1993. **43**(6): p. 858-62.

210. Orr, G.A., E.K. Han, P.C. Browne, E. Nieves, B.M. O'Connor, C.P. Yang, and S.B. Horwitz, *Identification of the major phosphorylation domain of murine mdr1b P-glycoprotein. Analysis of the protein kinase A and protein kinase C phosphorylation sites.* J Biol Chem, 1993. **268**(33): p. 25054-62.

211. Pantos, C., C. Xinaris, I. Mourouzis, V. Malliopoulou, E. Kardami, and D.V. Cokkinos, *Thyroid hormone changes cardiomyocyte shape and geometry via ERK signaling pathway: potential therapeutic implications in reversing cardiac remodeling?* Mol Cell Biochem, 2007. **297**(1-2): p. 65-72.

212. McCubrey, J.A., L.S. Steelman, S.L. Abrams, J.T. Lee, F. Chang, F.E. Bertrand, P.M. Navolanic, D.M. Terrian, R.A. Franklin, A.B. D'Assoro, J.L. Salisbury, M.C. Mazzarino, F. Stivala, and M. Libra, *Roles of the RAF/MEK/ERK and PI3K/PTEN/AKT pathways in malignant transformation and drug resistance.* Adv Enzyme Regul, 2006. **46**: p. 249-79.

213. Lee, J.T., Jr., L.S. Steelman, and J.A. McCubrey, *Phosphatidylinositol 3'-kinase activation leads to multidrug resistance protein-1 expression and subsequent chemoresistance in advanced prostate cancer cells.* Cancer Res, 2004. **64**(22): p. 8397-404.

214. Brady, J.M., N.J. Cherrington, D.P. Hartley, S.C. Buist, N. Li, and C.D. Klaassen, *Tissue distribution and chemical induction of multiple drug resistance genes in rats.* Drug Metab Dispos, 2002. **30**(7): p. 838-44.

215. Tang, F. and R.T. Borchardt, *Characterization of the efflux transporter(s) responsible for restricting intestinal mucosa permeation of the coumarinic acid-based cyclic prodrug of the opioid peptide DADLE.* Pharm Res, 2002. **19**(6): p. 787-93.

216. Glavinas, H., P. Krajcsi, J. Cserepes, and B. Sarkadi, *The role of ABC transporters in drug resistance, metabolism and toxicity.* Curr Drug Deliv, 2004. **1**(1): p. 27-42.

217. Karaszi, E., K. Jakab, L. Homolya, G. Szakacs, Z. Hollo, B. Telek, A. Kiss, L. Rejto, S. Nahajevszky, B. Sarkadi, and J. Kappelmayer, *Calcein assay for multidrug resistance reliably predicts therapy response and survival rate in acute myeloid leukaemia.* Br J Haematol, 2001. **112**(2): p. 308-14.

218. Griffiths, E.J. and A.P. Halestrap, *Protection by Cyclosporin A of ischemia/reperfusion-induced damage in isolated rat hearts.* J Mol Cell Cardiol, 1993. **25**(12): p. 1461-9.

219. Shen, F., S. Chu, A.K. Bence, B. Bailey, X. Xue, P.A. Erickson, M.H. Montrose, W.T. Beck, and L.C. Erickson, *Quantitation of doxorubicin uptake, efflux, and modulation of multidrug resistance (MDR) in MDR human cancer cells.* J Pharmacol Exp Ther, 2008. **324**(1): p. 95-102.

220. Chiu, L.Y., J.L. Ko, Y.J. Lee, T.Y. Yang, Y.T. Tee, and G.T. Sheu, *L-type calcium channel blockers reverse docetaxel and vincristine-induced multidrug resistance independent of ABCB1 expression in human lung cancer cell lines.* Toxicol Lett, 2010. **192**(3): p. 408-18.

221. Tsuruo, T., *Acquired vs innate multidrug resistance and the effect of calcium channel blockers.* Prog Clin Biol Res, 1986. **223**: p. 203-16.

222. Wallace, K.B., *Adriamycin-induced interference with cardiac mitochondrial calcium homeostasis.* Cardiovasc Toxicol, 2007. **7**(2): p. 101-7.

223. Maulik, S.K., S.D. Seth, S.C. Manchanda, K.S. Reddy, Y.K. Gupta, and M.G. Maulik, *Effect of verapamil post-treatment in myocardial reperfusion injury.* Indian J Exp Biol, 1993. **31**(2): p. 120-4.

224. Mitcheson, J.S., J.C. Hancox, and A.J. Levi, *Cultured adult cardiac myocytes: future applications, culture methods, morphological and electrophysiological properties.* Cardiovasc Res, 1998. **39**(2): p. 280-300.

225. Shain, S.A., *Exogenous fibroblast growth factors maintain viability, promote proliferation, and suppress GADD45alpha and GAS6 transcript content of prostate cancer cells genetically modified to lack endogenous FGF-2.* Mol Cancer Res, 2004. **2**(11): p. 653-61.

226. Maloof, P., Q. Wang, H. Wang, D. Stein, T.N. Denny, J. Yahalom, E. Fenig, and R. Wieder, *Overexpression of basic fibroblast growth factor (FGF-2) downregulates Bcl-2 and promotes apoptosis in MCF-7 human breast cancer cells.* Breast Cancer Res Treat, 1999. **56**(2): p. 153-67.

227. Williamson, A.J., B.C. Dibling, J.R. Boyne, P. Selby, and S.A. Burchill, *Basic fibroblast growth factor-induced cell death is effected through sustained activation of p38MAPK and up-regulation of the death receptor p75NTR.* J Biol Chem, 2004. **279**(46): p. 47912-28.

228. Coleman, A.B., M.Z. Metz, C.A. Donohue, R.E. Schwarz, and S.E. Kane, *Chemosensitization by fibroblast growth factor-2 is not dependent upon proliferation, S-phase accumulation, or p53 status.* Biochem Pharmacol, 2002. **64**(7): p. 1111-23.

229. Penault-Llorca, F., F. Bertucci, J. Adelaide, P. Parc, F. Coulier, J. Jacquemier, D. Birnbaum, and O. deLapeyriere, *Expression of FGF and FGF receptor genes in human breast cancer.* Int J Cancer, 1995. **61**(2): p. 170-6.

230. Cailliau, K., D. Perdereau, A. Lescuyer, H. Chen, C. Garbay, J.P. Vilain, A.F. Burnol, and E. Browaeys-Poly, *FGF receptor phosphotyrosine 766 is a target for Grb14 to inhibit MDA-MB-231 human breast cancer cell signaling.* Anticancer Res, 2005. **25**(6B): p. 3877-82.

231. Wang, Q., P. Maloof, H. Wang, E. Fenig, D. Stein, G. Nichols, T.N. Denny, J. Yahalom, and R. Wieder, *Basic fibroblast growth factor downregulates Bcl-2 and promotes apoptosis in MCF-7 human breast cancer cells.* Exp Cell Res, 1998. **238**(1): p. 177-87.

232. Fenig, E., T. Livnat, S. Sharkon-Polak, L. Wasserman, E. Beery, G. Lilling, J. Yahalom, R. Wieder, and J. Nordenberg, *Basic fibroblast growth factor potentiates cisplatinum-induced cytotoxicity in MCF-7 human breast cancer cells.* J Cancer Res Clin Oncol, 1999. **125**(10): p. 556-62.

233. Kunjachan, S., A. Blauz, D. Mockel, B. Theek, F. Kiessling, T. Etrych, K. Ulbrich, L. van Bloois, G. Storm, G. Bartosz, B. Rychlik, and T. Lammers, *Overcoming cellular multidrug resistance using classical nanomedicine formulations.* Eur J Pharm Sci, 2012. **45**(4): p. 421-8.

234. Chen, Y.J., C.D. Kuo, S.H. Chen, W.J. Chen, W.C. Huang, K.S. Chao, and H.F. Liao, *Small-molecule synthetic compound norcantharidin reverses multi-drug resistance by regulating Sonic hedgehog signaling in human breast cancer cells.* PLoS One, 2012. **7**(5): p. e37006.

235. Rasmussen, N., J.H. Andersen, H. Jespersen, O.G. Mouritsen, and H.J. Ditzel, *Effect of free fatty acids and lysolipids on cellular uptake of doxorubicin in human breast cancer cell lines.* Anticancer Drugs, 2010. **21**(7): p. 674-7.

236. Pardo, O.E., J. Latigo, R.E. Jeffery, E. Nye, R. Poulsom, B. Spencer-Dene, N.R. Lemoine, G.W. Stamp, E.O. Aboagye, and M.J. Seckl, *The fibroblast growth factor receptor inhibitor PD173074 blocks small cell lung cancer growth in vitro and in vivo.* Cancer Res, 2009. **69**(22): p. 8645-51.

237. Hegedus, C., G. Szakacs, L. Homolya, T.I. Orban, A. Telbisz, M. Jani, and B. Sarkadi, *Ins and outs of the ABCG2 multidrug transporter: an update on in vitro functional assays.* Adv Drug Deliv Rev, 2009. **61**(1): p. 47-56.