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Cytotoxic effects of electron carriers are inhibited by Hsp27 in breast cancer cell lines

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CYTOTOXIC EFFECTS OF ELECTRON CARRIERS ARE INHIBITED BY HSP27 IN
BREAST CANCER CELL LINES

by

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Bachelor of Science
University of Nevada, Las Vegas
2000

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A thesis submitted in partial fulfillment
of the requirements for the

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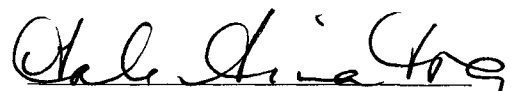
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ABSTRACT

Cytotoxic Effects of Electron Carriers Are Inhibited by HSP27 in Breast Cancer Cell Lines

by

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Human breast cancer cell lines DC4, DB46, MCF-7, MDA-MB-435 were incubated with cytochrome c at 5×10^{-7} M, 5×10^{-6} M and 5×10^{-5} M. Human colon cancer cell line, RKO, and normal human lung fibroblast cell line, HFL1, were incubated with cytochrome c at 5.6×10^{-8} M, 5.6×10^{-7} M and 5.6×10^{-6} M. Cytochrome c exposure showed a dose response including cell death in breast cancer cell lines. RKO showed no sensitivity to cytochrome c exposure at any of the concentrations and HFL1 showed sensitivity only at the highest concentration. FAD, NADH, Q_{10} and ATP were incubated with cells at 1×10^{-9} M, 1×10^{-8} M and 1×10^{-7} M. Incubation of FAD with DC4 and DB46 was also done at 5×10^{-7} M, 5×10^{-6} M and 5×10^{-5} M. FAD, ATP and NADH were more effective in cell death induction at much lower concentrations than cytochrome c. Q_{10} was least effective in cell death induction.

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CHAPTER 1

INTRODUCTION

The following subchapters will discuss the concepts of heat shock proteins, heat shock protein 27, apoptosis, caspases, mitochondria, electron carriers and cancer. The subsequent hypotheses derived from these concepts are stated on page 22.

Heat Shock Proteins

The name heat shock proteins (HSPs) was coined in 1962 as Ritossa discovered that a certain group of proteins became expressed in *Drosophila* larvae following exposure to temperatures that were above those allowing optimal growth (1). Through further research by Tissières it was soon realized that high temperatures were not the only method of induction (2,3). Tissières et al. showed that metals, amino acid analogs and anoxia were able to elicit similar responses; hence, HSPs are also referred to as stress proteins. HSPs are divided into five major families according to their molecular weight: HSP100, 90, 70, 60 and the small HSPs (sHSPs)/ α -crystallins (3). sHSPs are evolutionary related to α -crystallin which is known to be a major structural protein of the vertebrate eye lens. A common characteristic of the α -crystallin/sHSP family is a conserved homologous sequence of 90-100 residues (4). sHSPs/ α -crystallins form a diverse family of proteins, are between 15-30 kDa in size and can be induced by mild

heat shock or other forms of stress. They have a tendency to aggregate and are involved in protein-protein interactions (4).

HSPs are found in all organisms from bacteria to humans and are among the most conserved proteins. The synthesis of HSPs allows an organism to gain tolerance to insult. An insult constitutes elevated temperature or stress. It was found that HSPs are synthesized in stressed cells as well as in unstressed cells, leading to the understanding that HSPs fulfilled another function. The major role for large HSPs, which are HSP100, 90, 70 and 60, appears to be molecular chaperones (3,4). Molecular chaperones have been defined as proteins that bind to and stabilize an otherwise unstable conformer of another protein. The controlled binding and release of the client protein determines its correct fate *in vivo*, which may be folding, oligomeric assembly, transport to a particular subcellular compartment or controlled switching between active versus inactive conformations (2). HSP100/Clp proteins protect cells under stress conditions. They function as chaperones, solubilize protein aggregates in an ATP-dependent manner and lead either to renaturation or proteolytic degradation of individual proteins (3). HSP90 under normal conditions is an abundant, essential, cytosolic protein that in a complex with other proteins engages steroid receptors to possibly maintain them in an inert, folded state allowing for the binding with and reaction to the correct steroid signal (3). Stressed cells show a marked increase of HSP90 (3). HSP70 is a constitutive and stress-inducible protein. It binds to nascent polypeptide chains on ribosomes, maintains the membrane translocation-competent state of the precursor proteins, assists protein transport into mitochondria and the endoplasmic reticulum and also protects proteins under stress (3).

HSP60 belongs to the chaperonin family, which includes GroEL, from bacteria among

others, which is a group of proteins with a distinct ring-shaped, or toroid (double donut) quaternary structure. The chaperonins appear to mediate native folding of proteins in an ATP-dependent manner, which seems to proceed through cooperation of HSP70 (3). It is suggested that in this way large HSPs contribute to the maintenance and restoration of cellular homeostasis during and after stress (3). The focus of this discussion will now turn to the small heat shock protein 27, HSP27, and its role in programmed cell death (apoptosis).

Heat Shock Protein 27

HSP27 belongs to the family of sHSP/ α -crystallin. This small heat shock protein is constitutively expressed in many cell types and tissues at specific stages of development and differentiation (5). A variety of stimuli including elevated temperature, oxidative stress, staurosporine, Fas ligand and cytotoxic drugs induce HSP27 protection against apoptosis (5). Intracellular localization, level of oligomerization and phosphorylation status seem to determine the biological reactivity of HSP27 (2). Nonphosphorylated HSP27 exists in large complexes made up of about 24 subunits (6 tetramers) while phosphorylated HSP27 forms small rod-like oligomers, which may be tetrameric. Dissociation of large oligomers to tetramers as a result of phosphorylation or of mutation of all three serine residues to aspartate has been shown to lead to a significant decrease in chaperone activity (6). HSP27 forms large oligomers, which can act as molecular chaperones *in vitro* and protect cells from heat shock and oxidative stress when overexpressed (6). The function of a molecular chaperone is to prevent unfolded proteins from irreversible aggregation (6). In cooperation with other factors, *e.g.* HSP70 and ATP,

HSP27 helps to facilitate productive refolding of unfolded proteins (6). HSP27 shows stress-induced increase in expression as well as phosphorylation (11). The phosphorylation and stress-induced expression of HSP27 shows different kinetics leading to the assumption that the phosphorylation of the pre-existing constitutively expressed sHSPs is a first phase stress response and the elevated expression at a time when their phosphorylation is already down-regulated comprises the second phase (6).

A commonality found between α -crystallin and HSP27 is their phosphorylation at specific serine residues. The mammalian HSP27 has three major phosphorylation sites, which are at Ser-15, Ser-78 and Ser-82 (4,7). The monomeric, non-phosphorylated HSP27 has been shown to be the only form that inhibits actin polymerization *in vitro* (2). Elevated levels of HSP27, particularly phosphorylated isoforms, have been associated with increased resistance of human breast cancer cells to doxorubicin (4). Doxorubicin is an anticancer drug that inhibits both DNA and RNA synthesis and has preferential toxicity for cells in the S phase of the cell cycle (8).

It has been suggested that the phosphorylation of HSP27 is a possible signal for development of thermotolerance (4). A shift from the unphosphorylated to the phosphorylated isoforms of HSP27 within minutes after heat shock associated with the rapid translocation of the small HSPs from cytosol to nucleus has been observed in HeLa cells (4). Heat-induced phosphorylation of HSP27 is reduced in thermotolerant cells, as is the redistribution from cytoplasm to nucleus (4). Rapid stress-induced phosphorylation is induced by a p38 MAP (mitogen-activated protein) kinase cascade with subsequent activation of MAPKAP (mitogen-activated protein kinase-activated protein) kinases 2 and 3, which directly phosphorylate mammalian sHSPs at several distinct sites (6).

Increased phosphorylation of mammalian sHSPs occurs in response to a number of mitogenic stimuli including serum, growth factors and tumor promoters. Calcium ionophores and chelators, cadmium, arsenite, cycloheximide and phorbol esters are known to induce phosphorylation at normal growing temperature (4).

HSP27 is an estrogen responsive protein (4,9,10). The estrogen receptor, in absence of hormone, is not bound to its respective hormone response element (HRE). The hormone receptors are present within the nucleus bound to a complex consisting of two molecules of HSP90, one molecule of HSP70 and other proteins (11). Upon hormone binding, the receptors are released from the HSP complex (11). This homodimer has a greatly increased affinity for binding to HREs in DNA. The HREs are usually found several hundred base pairs upstream of target genes (11). The estrogen homodimer can bind to the HRE in the promoter region of the HSP27 gene and transcription of the HSP27 gene is initiated. Thus, when estrogen is present the HSP27 protein can become expressed in elevated levels (12).

Elevated levels of HSP27 may be in response to environmental stresses or exposure to estrogen (10). HSP27 is overexpressed in several human breast carcinomas and correlates well with their levels of estrogen receptors (9). It has been postulated that there is a link with hormone response (9). Treatment of human breast cancer cell lines with 17β -estradiol was able to elicit HSP27 expression (10). Breast cancer cell lines overexpressing HSP27 showed an increase in cell survival when treated with doxorubicin or heat (10). HSP27 has been shown to correlate with the oncogenic status of cells and plays a role in their tumorigenicity (9). Well differentiated tumors have been shown to have the highest levels of HSP27 (9). It has been found that estrogen⁺ HSP27⁺ tumors

recur more rapidly than estrogen⁺ HSP27⁺ tumors (9). High levels of HSP27 have been found to correlate with a shorter disease-free survival period in lymph node-negative patients but showed prolonged survival from first occurrence (9). Elevated levels of HSP27 in advanced cancers have been shown to be indicative of long survival. It is not understood why the overall survival of the patient is not affected with a high recurrence of the cancer (9).

HSP27 has been shown to block apoptosis induced by Fas (death cell surface receptor belonging to the tumor necrosis factor (TNF) superfamily of surface receptors) as it binds to Daxx (13). Daxx is an adapter protein that is normally associated with the nuclear substructures named ND-10 or PML oncogenic domain (POD) (13,14). This adapter protein can bind to the Fas receptor, mediate Fas-induced apoptosis and activate apoptosis signal-regulated kinase 1 (Ask 1) (13). Activation of this kinase can then activate the stress-activated protein kinase 1-Jun N-terminal kinase (SAPK1/JNK) (13). Thus, the binding of HSP27 to Daxx prevents the interaction of Daxx with Fas (13).

In neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis and progressive supranuclear palsy the production of reactive oxygen species (ROS) has been implicated in the pathogenesis. Unphosphorylated, large oligomers of HSP27 protect the cell against ROS and play a role in confluent-dependent resistance (15,16).

Cells in a tissue culture flask become confluent as a result of space limitation resulting in the cells making contact with neighboring cells via their cell walls. It is believed that confluent cultures are better models than actively growing cells for growth conditions in a solid tumor (16). It has been reported that cancer cell sensitivity to most anticancer

drugs continuously decreased as cells reached confluence (16). Some of the mechanisms proposed to explain confluence-dependent resistance include decreased intracellular drug accumulation, modifications of the drug target in confluent cells and decreased ability of confluent tumor cells to undergo cell death in response to specific damage induced by the drug (16).

It is suggested that the protection that HSP27 confers against oxidative stress is independent of its ability to bind cytochrome c, which is an electron carrier in the mitochondrial electron transport chain (15). HSP27 has been shown to play a significant role in inhibiting apoptosis by binding to cytochrome c (5,17,18). It appears that both, *in vitro* chaperone properties and the ability to protect against oxidative stress depend on the existence of large oligomers of HSP27 meaning that *in vivo* chaperoning by HSP27 is the basis for its protective activity at the cellular level (6).

Apoptosis

Apoptosis plays a pivotal role in maintaining the balance between cell proliferation, regulation of healthy development, and protection against disease (14). It is involved in fundamental processes such as the regulation of immune cell maturation, target cell death induction by cytotoxic T lymphocytes and natural killer (NK) cells as well as the response of hormone-growth-factor-dependent tissues to the addition or withdrawal of ligands (14). Apoptosis is a morphologically distinct form of cell death characterized by rapid removal of unwanted and potentially dangerous cells. The inappropriate regulation of apoptosis is associated with a variety of diseases, including cancer, AIDS, neurodegenerative diseases and ischemic stroke (19).

Cells undergoing apoptosis are characterized by distinct morphological alterations including cell shrinkage, nuclear condensation, and the blebbing of apoptotic vesicles containing intracellular components. Those vesicles are specifically recognized and engulfed by phagocytic cells; hence, an inflammatory response is not elicited because intracellular contents are not released into the extracellular environment (14). Apoptosis is fundamentally different from necrosis. Necrosis is the result of acute cellular disfunction due to extreme trauma or injury to the cell. This passive and disruptive process results in rapid loss of ion flux, leading to the uptake of water, which gives rise to cell and organelle swelling ending in cytolysis. The release of the cell contents into surrounding tissue solicits a local inflammatory response (2).

Characteristic biochemical alterations that occur during apoptosis include DNA cleavage between nucleosomes to form fragments that are multiples of approximately 180 basepairs and/or into 50-to 300-kb fragments, the reorientation of phosphatidylserine from the cytoplasmic to the extracellular face of the plasma membrane and the loss of the mitochondrial membrane potential (14).

Cells are exceptionally sensitive to small changes in their environment and have to be able to adapt quickly to new conditions. Many times a rapid response is facilitated by the constitutive expression of key components of signal transduction or effector pathways (14). Many cells are able to undergo apoptosis in the presence of inhibitors of macromolecular synthesis, leading to the indication that the distal components of the apoptotic pathway are constitutively expressed. It has been shown that such inhibitors are capable of inducing apoptosis in some cell lines independent of additional stimulus. Thus, it appears that the constitutively expressed effectors of apoptosis are normally

inhibited by suppressive molecules (14). The apoptotic pathways were elucidated from studies performed in the nematode *Caenorhabditis elegans* (14,20-27). It was discovered that the gene *ced-3* shares homology with a family of cysteine proteases known as caspases in higher animals (14). The term caspase refers to the enzymatic specificity: Cys protease cleaving after Asp residues (14,23,24,27). The human *ced-4* homologue is *apaf-1* (apoptotic protease-activating factor-1).

Caspases

More than 14 caspases have been identified adding to the complexity of the pathways controlling apoptosis in higher animals (14,20,21). All caspases have similar amino acid sequences and substrate specificities, all cleave after an aspartic acid residue and all recognize a motif of at least four amino acids proximal to the cleavage site; yet, other structural elements must be required for substrate recognition since not all target proteins with a particular motif are cleaved (14).

Caspases are synthesized as proenzymes (zymogens), consisting of three domains (N-terminal prodomain together with large (~p20) and small (~p10) subunits), remain inactive in most healthy cells and require proteolytic cleavage to be active (16,14,18-24). Activation occurs as the single-chain procaspases are cleaved at specific aspartic acid residues to remove an inhibitory N-terminal pro-domain as well as cleavage at a specific recognition site between the large and small subunits to generate two distinct subunits. These differ from most non-caspase proteases, which are activated by removal of the inhibitory domain only (19,20). The resulting two subunits associate to form a

homodimer (14,19). Mature (activated) caspases form a tetramer, which consist of two homodimers bound together as two large and two small subunits (22).

Activated caspases exhibit considerable substrate specificity, particularly in relation to effector caspases (20). Caspases that are involved in apoptosis are divided into two groups: group III, which is involved in the initiation of the apoptotic signal (initiator or apical caspases) and group II, which is involved in the disassembly of the cell (effector or down-stream executioner caspases) (17,14,20). Initiator caspases preferentially cleave at recognition sites that consist of the following four amino acid sequences YEXD (Y→I, L, V and X→any amino acid) whereas effector caspases prefer DEXD sequences (each capital letter represents an amino acid, listed in Table 1, p 23) (19). As mentioned earlier, all caspases exist in cells as inactive zymogens that have to be activated in order to be able to induce apoptosis. Effector caspases (e.g. caspase-3 or -7) are activated by initiator caspases (such as caspase-9) via cleavage at specific internal aspartate residues separating the large and small subunits (14,23,24,27).

There are two pathways that can induce the activation of initiator caspases. One pathway leading to caspase activation is initiated by engaging cell surface death receptors with their specific ligands and the other is known as mitochondrion-dependent apoptosis (14,27-29). An example of death receptor apoptosis will be briefly discussed and the focus will then be turned to mitochondrion-dependent apoptosis. In the apoptotic pathway of cell surface death receptors (Fig. 1, p. 26), a number of cytokines that belong to the tumor necrosis factor receptor (TNFR) superfamily (a family of transmembrane proteins) induce apoptosis (2,14,28,29). Mammalian death receptors include FAS/APO-1/CD95, TNFR1, DR-3/APO-3/WSL-1/TRAMP, and the TRAIL receptors

DR4/TRAIL-R1, DR5/TRAIL R2/TRICK2/ KILLER (14,28,29). These death receptors consist of characteristic cysteine-rich extracellular repeats but lack substantial homology in their cytoplasmic regions except in the death domain (14,28,29). The death domain spans approximately 80 amino acids and is required for the death signal transduction (13,14). Fas and TNFR share a region of homology at the death domain at the cytoplasmic face (68 amino acids), which is required for apoptotic signaling by both Fas and TNFR (14). The activation ligands for these death receptors are structurally related molecules that belong to the TNF gene superfamily; hence, Fas/CD95 ligand (FasL) binds to Fas, TNF and lymphotoxin α bind to TNFR1, Apo 3 ligand (Apo3L) binds to DR3 and Apo2 ligand (Apo2L or TRAIL) binds to DR4 and DR5 (14,28,29). The Fas receptor is the only member that appears to act primarily in the apoptotic pathway. It is expressed in diverse tissues but is mainly involved in the modulation of the immune response (14). Binding of FasL to Fas receptor induces trimerization of Fas (19,28). A death domain-containing (DD- containing) adaptor molecule designated FADD/Mort1 (Fas-associating protein with death domain/Mort1), which contains a DD at its C-terminus, is then recruited to the cytoplasmic region of Fas where it binds to the DD of Fas, which is then called the ligand bound-death receptor complex (death-inducing signaling complex or DISC) (14,19,28). The N-terminus of FADD, termed the death effector domain (DED) is essential for recruiting the upstream initiator procaspase-8 and/or procaspase-10 (14,28,29). Procaspase-8 immediately undergoes autoactivation upon the recruitment to DISC and then activates procaspase-3. Procaspase-3 is the procaspase that mitochondrial-dependent apoptosis and death receptor apoptosis pathways converge on (14).

Another protein that normally localizes to sub-nuclear structures known as ND-10 or PODs (PML-oncogenic domains) can become associated with the Fas receptor (13,14). This protein is known as Daxx and its association with the Fas receptor activates the JNK (1-Jun N-terminal kinase) apoptotic pathway (14).

Death receptor induced apoptosis is regulated by three distinct mechanisms (28,29). In the first mechanism the recruitment and/or activation of procaspase-8 at the DISC is prevented by a group of inhibitors belonging to a family of viral proteins, FADD-like ICE inhibitory proteins (vFLIPS) that contain two DEDs (28,29). The two DEDs compete with procaspases for binding to the DED of FADD; thus, preventing the recruitment of procaspases to the DISC (28,29). The mammalian homologue of vFLIP was identified and is known as Casper, I-FLICE, FLAME, CASH or MERIT (28). Two alternatively spliced forms of FLIP, FLIP-long and FLIP-short, exist (28). FLIP-long has a C-terminal that resembles caspase-8 but lacks protease activity because several conserved residues at the caspase active site are absent (28). Both isoforms of cellular FLIP bind to FADD, procaspase-8 and procaspase-10 by way of DED interactions (28).

A second mechanism is through the expression of decoy receptors for TRAIL (19,28). Decoy receptors are closely related to the TRAIL receptors DR4 and DR5. These receptors lack the cytoplasmic domain (DcR1) or contain a cytoplasmic region with a truncated death domain (DcR2), thereby specifically inhibiting TRAIL-induced apoptosis by sequestering the TRAIL ligand away from the death receptors DR4 and DR5 (28).

The third mechanism involves viral protein crmA, a member of the serpin family that is a potent inhibitor of procaspase-8 (28). CrmA can inhibit the autoproteolytic activation of procaspase-8 as well as the ability of caspase-8 to cleave Bid, which leads to the

release of cytochrome c from mitochondria resulting in the activation of downstream caspases (28).

Mitochondria

In mitochondrion-dependent apoptosis (Fig. 2, p. 27), mitochondria play a critical role in the induction of apoptosis by responding to a myriad of stimuli (30). In response to apoptotic stimuli these organelles release proteins into the cytosol that trigger caspase activation or other functions relevant to apoptosis, including cytochrome c, caspases, AIF (apoptosis-inducing factor), and SMAC/Diablo (second mitochondria-derived activator of caspases and murine homologue) (30,31).

A mitochondrion is a dynamic organelle comprised of an inner and outer membrane (32-34). Both membranes have differing levels of permeability and selectivity for cytoplasmic molecules and the organelle employs a diverse set of channels, pumps and transporters to move molecules across these membranes (32). Cytochrome c, whose gene resides in the nucleus as opposed to the mitochondria, is transported to the mitochondrial intermembrane space (IMS) upon synthesis (32). The addition of a heme group to cytochrome c serves to lock it in the IMS preventing its access to cytoplasmic *apaf-1* (32). In the IMS, cytochrome c is a one-electron transfer agent involved in the mitochondrial electron transport chain (30,33,34). Apo-cytochrome c (lacking the heme) does not activate *apaf-1*, thus protecting the cell from cytochrome c while it is being synthesized, transported and contained within the mitochondria (32). It is a possibility that a minimum level of cytochrome c is required to activate the apoptosome (32). In healthy cells, mitochondria maintain an electrochemical gradient ($\Delta\psi$) across their

inner membranes that is created by pumping protons from the mitochondrial matrix to the mitochondrial IMS in conjunction with electron transport through the respiratory chain (30,33,34). The electrochemical gradient is made up of ΔpH (pH gradient) and ΔV_m (electrical gradient) components and is essentially caused by protons that are driven out of the matrix to the outer surface of the inner membrane (30,33,34). This proton pumping therefore creates a pH gradient and an electrical gradient across the inner membrane (33,34). The influx of protons back into the matrix is believed to be mediated mainly by the F_0F_1 -ATPase/ H^+ pump (30,33).

Depolarization of mitochondria and loss of the electrochemical gradient ($\Delta\psi$) is universally associated with apoptosis and cell death (30). A wide variety of apoptotic stimuli induce the release of cytochrome c from mitochondria prior to membrane depolarization (30,33,34). These stimuli include growth factor or neurotrophin withdrawal, DNA-damaging drugs, radiation, protein kinase inhibitors such as staurosporine, elevations of pro-apoptotic Bcl-2 family proteins, a variety of agonists or antagonists of the retinoid/steroid hormone family of nuclear receptors and cell surface death receptors (28,30). In response to these stimuli, mitochondrial depolarization typically occurs after caspase activation as a late event in the pathway (30).

There are four distinct protein complexes that make up the mitochondrial respiratory chain (Fig. 3, p. 28) (33,34). They are: (I) NADH-coenzyme Q reductase, (II) succinate-coenzyme Q reductase, (III) coenzyme Q-cytochrome c reductase, and (IV) cytochrome c oxidase. Briefly, NADH donates electrons to Complex I, serving as a link between glycolysis, the TCA cycle, fatty acid oxidation and the electron transport chain (33,34). Complex I and II produce a common product, reduced coenzyme Q (UQH_2), which

becomes the substrate for Complex III (33,34). Complex III oxidizes UQH₂ and reduces cytochrome c. Reduced cytochrome c then is the substrate for Complex IV and this is where molecular oxygen is reduced (33,34).

The release of cytochrome c prior to membrane depolarization and its role in the formation of the apoptosome has been of interest in cancer research. As already mentioned, cytochrome c is part of the electron transport chain. It also depends on other electron carriers of this chain for the transfer of electrons so that it can carry one electron at a time to Complex IV. These electron carriers will be discussed following this subchapter. What role these other electron carriers play in apoptosis regarding cancer treatment was investigated for this thesis.

The apoptotic induction of cytochrome c from mitochondria into the cytosol marks the beginning of caspase activation. Cytochrome c, which is present in the concentration of between 0.5 and 5 mM in the mitochondrial intermembrane space, is released from the mitochondria within 5 minutes (32,35,36). Once in the cytosol, cytochrome c binds its cytosolic partner *apaf-1* and induces the oligomerization of *apaf-1*•cytochrome c complex in a dATP/ATP-dependent manner (34,35,39). *Apaf-1* is a 130-kDa protein that consists of the following three domains: the N-terminal contains 85 amino acids that show homology with the prodomain of several caspases including caspase-1, -2 and -9 (20,21,24,25,28,39). This domain appears to function as the caspase recruitment domain (CARD) to bind caspases with a similar CARD (20,21,28). It is of interest that caspase-9 is the only procaspase of all the CARD-carrying caspases to be activated by *apaf-1* (28). After the CARD domain follows a stretch consisting of 310 amino acids that have 50% homology to the *C. elegans* death promoting domain *ced-4* (20,21,28,39). The most

obvious conserved regions of this domain are the Walker's A and B boxes, which are believed to be necessary for nucleotide binding since mutations in this nucleotide-binding site abolish both *apaf-1* and *ced-4* function (20,21,28,39). The ATP synthase complex contains alpha- and beta-subunits (A and B subunits), which binds ATP and ADP (33,34,38). The beta subunit contributes to the catalytic sites whereas the alpha subunit appears to be involved in the regulation of the ATP synthase activity (40). Amino acid sequences of alpha- and beta-subunits are weakly homologous to each other and may have common functions in catalysis (40). Related sequences in both, alpha- and beta-subunits as well as other enzymes that bind ATP and ADP, have helped to identify regions contributing to an adenine nucleotide binding fold in both ATP synthase subunits (40). The C-terminal half of *apaf-1* contains 12-13 WD-40 repeats that mediate protein-protein interactions (20,21,22,28,39). WD-40 repeats are highly conserved repeating units found in all eukaryotes usually ending with residues WD (41). A truncated *apaf-1*, in which the WD-40 repeats have been removed, renders *apaf-1* constitutively active *in vitro*, independent of dATP/ATP and cytochrome c (28,38,39). Conversely, activated caspase-9 cannot be released from *apaf-1* when the WD-40 repeats are truncated, signifying that this domain normally fulfills dual functions to inhibit *apaf-1* activity and to help release the activated caspase-9 (20,28,37). The critical role of *apaf-1* was confirmed by the analysis of *apaf-1*-deficient mice displaying abnormalities in several tissues, particularly the brain, as well as lack of developmental cell death (20,24,42). On the transcriptional level, the regulation of *apaf-1* has been implicated in several important biological processes particularly in tumorigenesis and during the development of the mammalian central nervous system (43). *Apaf-1* has been described to be the core of the apoptosome

and cytochrome c, which is essential for mitochondrial respiration and energy production and functions as a cofactor in the activation of caspases (42). All four components, cytochrome c, *apaf-1*, dATP/ATP and procaspase-9, form the apoptosome complex (18,21,22,28,37,39,42,43).

Cytochrome c is pivotal to the apoptosome assembly. Cell lines lacking cytochrome c and grown under conditions that mitigate the loss of this electron carrier, failed to form an apoptosome and failed to activate procaspase-9 in response to proapoptotic stimuli (39). Mitochondrion-dependent apoptosis requires activation of procaspase-9 to initiate the caspase cascade (37,45). This activation is dependent on the protein *apaf-1*, which has to be activated by cofactors cytochrome c and dATP (45). Cytochrome c binds *apaf-1* strongly in the absence of dATP; however, addition of dATP to the cytochrome c•*apaf-1* complex results in further assembly producing a larger complex made up of 8-10 *apaf-1* molecules along with associated cytochrome c molecules (23,45). Fluorescence polarization, ECL (enhanced chemiluminescence) and acrylamide fluorescence quenching results show the stoichiometry of this complex to be 2 cyt c•1 *apaf-1* and has been shown not to change with the addition of dATP (45). The role of dATP is to assist formation of the apoptosome complex (23,45). *Apaf-1* alone binds dATP poorly and in the presence of cytochrome c, nucleotide binding of *apaf-1* increases about 10-fold, confirming the temporal sequence of binding events that take place (23,37). Furthermore, procaspase-9 increases dATP binding to *apaf-1* indicating that the formation of the apoptosome is a synergistic event that stabilizes nucleotide binding (37). The stoichiometry of *apaf-1* and procaspase-9 binding is believed to be 1:1 due to the observed CARD:CARD interactions (20,28). The assumption is that procaspase-9

autodigests at *apaf-1* and cleaves a neighboring procaspase-9 to form a tetramer; thus, the activated caspase is formed (20). Lastly, activated caspase-9 is released from the apoptosome and goes on to cleave and activate downstream caspases such as procaspases-3, -6, and -7 (28). Specific target substrates are then cleaved leading to apoptosis (18).

The apoptosome complex has been isolated and found to exist in two different sizes. There is one ~ 1.4 MDa complex that appears to be inactive and may be due to inappropriate oligomerization of the *apaf-1* (20,21,27). Then there is an ~ 700 kDa complex that is capable of processing and activating effector caspases. It is this complex that has been found to be primarily assembled during apoptosis (20,21). The structure of the apoptosome has been hypothesized to be a wheel-like particle with seven spokes that radiate from a concave, central hub (39). The hub and bent spokes combine to give the apoptosome a puckered shape (39). Acehan et al. suggest in this model that cytochrome c interacts with WD-40 repeats that are contained in the spokes, place *apaf-1* in the upper region of the hub and have dATP binding to the *ced-4* homology region of the *apaf-1* with procaspase-9 being tethered to the apoptosome via CARD:CARD interactions (39).

Electron Carriers

Several electron carriers participate in the mitochondrial electron transport chain (Fig. 3, p. 28) (33,34,44).

Complex I contains the NADH-coenzyme Q reductase (33,34,44). Nicotinamide adenine dinucleotide (NAD^+) is a major electron acceptor in oxidative (catabolic) pathways (33,34). It is involved in hydride anion transfer either to NAD^+ or from NADH

(the reduced form) and because the hydride anion contains two electrons, acts solely as a two-electron carrier (33). NADH binds to the enzyme on the matrix side of the inner mitochondrial membrane and transfers its electrons to tightly bound flavin mononucleotide (FMN) (33). FMN belongs to the flavin coenzymes, can participate in one- and two-electron transfers and is a stronger oxidizing agent than NAD^+ (33). FMNH_2 then transfers electrons to a series of Fe-S proteins, which results in the two-electron transfers to coenzyme Q (33,34,44). Coenzyme Q is also known as ubiquinone, CoQ or UQ (33). It owes its hydrophobicity to its isoprenoid tail, which allows it to freely diffuse in the inner mitochondrial membrane (33). This allows the shuttling of electrons between Complex I, II and III (33).

In Complex II, succinate dehydrogenase contains two Fe-S proteins, flavoprotein 2 that has an FAD covalently bound to a histidine residue and three Fe-S centers. FAD is reduced to FADH_2 .

Complex III, CoQ- cytochrome c reductase, has three different cytochromes and one Fe-S protein. The cytochromes are: *b* cytochrome (b_L and b_H) and *c* cytochrome. They are one- electron carriers. It is only cytochrome *c* that transfers one electron at a time from Complex III to Complex IV (33). Electrons traveling through Complex III are passed through cytochrome c_1 to cytochrome *c* (33,34). Cytochrome *c* is the only watersoluble one of the cytochromes and like UQ a mobile electron carrier. It associates loosely with the inner mitochondrial membrane, in the IMS on the cytosolic side of the inner membrane, to acquire an electron from the Fe-S-cyt c_1 aggregate of Complex III (33,34). It migrates from Complex III along the membrane surface in the reduced state to Complex IV (33,34).

Complex IV, Cytochrome c Oxidase, consists of cytochrome c a and a₃ as well as two copper atoms. The reduction of molecular oxygen to water is accomplished here.

In summary, the major electron carriers of the respiratory chain are: NADH, coenzyme Q, cytochrome c and FAD. Table 2, page 25, shows their standard reduction potentials.

Cancer

Cancer has two characteristics. One is uncontrolled cellular proliferation and the other is the failure of cells to undergo apoptosis (43). Normally, cells develop a specific morphology and function, which is known as differentiation (8). The process of differentiation causes normal cells to lose their ability to proliferate (8). This process is kept in balance through the replacement of less-mature precursor cells also known as stem cells in renewal tissue (44). Precursor cells then go on to differentiate and it is from these precursor cells that cancer most likely originates (8). Evidence points to the transformation of a single precursor cell that proliferates to form a clone since all cells in a tumor appear to share some particular characteristic(s) of the original precursor cell (8).

Cancer cells are defined by two properties. The first property is that they and their progeny reproduce without regard of normal constraints; the second property is that they invade and colonize areas that are needed by other cells (44).

Out of control proliferation gives rise to a tumor, or neoplasm, which can either stay clustered or can invade surrounding tissue. The ability to invade other tissue depends on the ability to break loose, enter the bloodstream or lymphatic vessels, and form secondary tumors, or metastases (44,8). There are several mechanisms that ensure that cell

proliferation does not exceed the needs of a normal tissue. To name a few, there is promotion of cell differentiation by tumor suppressor genes, limitation of the total number of cell generations before senescence and apoptosis (46). Numerous normal developing tissues eliminate improperly developing cells by initiating apoptosis (46).

The p53 gene, which is a tumor suppressor gene, appears to play a regulatory role in apoptosis (46). Activated p53 functions as transcriptional transactivator, transrepressor and participates in numerous protein-protein interactions (46). The ability of p53 to transcriptionally activate *apaf-1* has been shown in mitochondria-induced apoptosis (46). Furthermore, activated p53 regulates the expression of genes that are in control of the mitochondrial membrane permeability, hence, the release of cytochrome c (46). Two sites in the *apaf-1* promoter relative to the transcriptional start site were found to correspond to a p53-responsive element and a radiolabeled *apaf-1* wild-type oligonucleotide containing the p53-responsive element was shown to bind p53 (46). A mutation in this gene, resulting in loss of function, prevents the physiological crucial elimination of unneeded cells (46). The p53 gene has been found inactivated in several neoplasms (47).

As has been described thus far, the molecular pathways that lead to the activation of caspases are tightly regulated. This regulation can be interfered with at the level of HSPs. The role of HSP27 in the inhibition of apoptosis has been investigated. It has been found that cytosolic HSP27, in human leukemic cells, interacts with cytochrome c released from the mitochondrial IMS, therefore inhibiting apoptosome formation as well as caspase activation (5). It was shown by Garrido et al. that in a cell free system, HSP27 co-immunoprecipitated with cytochrome c and not with procaspase-9, *apaf-1* or

procaspase-3 (5). The protection that HSP27 gives to cells against apoptosis aids the survival of cancerous cells.

Hypothesis

The purpose of this study is to investigate if the electron carriers, cytochrome c, FAD, NADH, Q₁₀ and one component, ATP, of the mitochondrial electron transport chain have any cytotoxic effects on various cell lines. Treatments with these electron carriers and ATP will be applied in increasing concentrations. The main goal is to see if HSP27 can provide protection against these agents. The first hypothesis of this study is that mitochondrial electron transport carriers and ATP can reduce cell proliferation in human cell lines. The second hypothesis is that HSP27 can protect human breast cancer cells against the toxic effects of these electron carriers and ATP.

The techniques used to achieve these goals are clonogenic survival assays and Western blotting analysis.

Table I
Alphabetical list of amino acid abbreviations

One-letter Symbol	Three-letter Abbreviation	Amino Acid
A	Ala	Alanine
B	Asx	Asparagine or Aspartic acid
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine

Table I (continued)
Alphabetical list of amino acid abbreviations

One-letter Symbol	Three-letter Abbreviation	Amino Acid
W	Trp	Tryptophan
Y	Tyr	Tyrosine
Z	Glx	Glutamine or Glutamic acid

Table II
Standard reduction potentials of the four major electron carriers of the respiratory chain

Oxidant	Reductant	Transferred e ⁻	E'°(V)
Cytochrome c ⁽⁺³⁾	Cytochrome c ⁽⁺²⁾	1	+ 0.254
CoQ (oxidized)	CoQ (reduced)	2	+ 0.060
FAD ⁺	FADH ₂	2	- 0.219
NAD ⁺	NADH + H ⁺	2	- 0.320

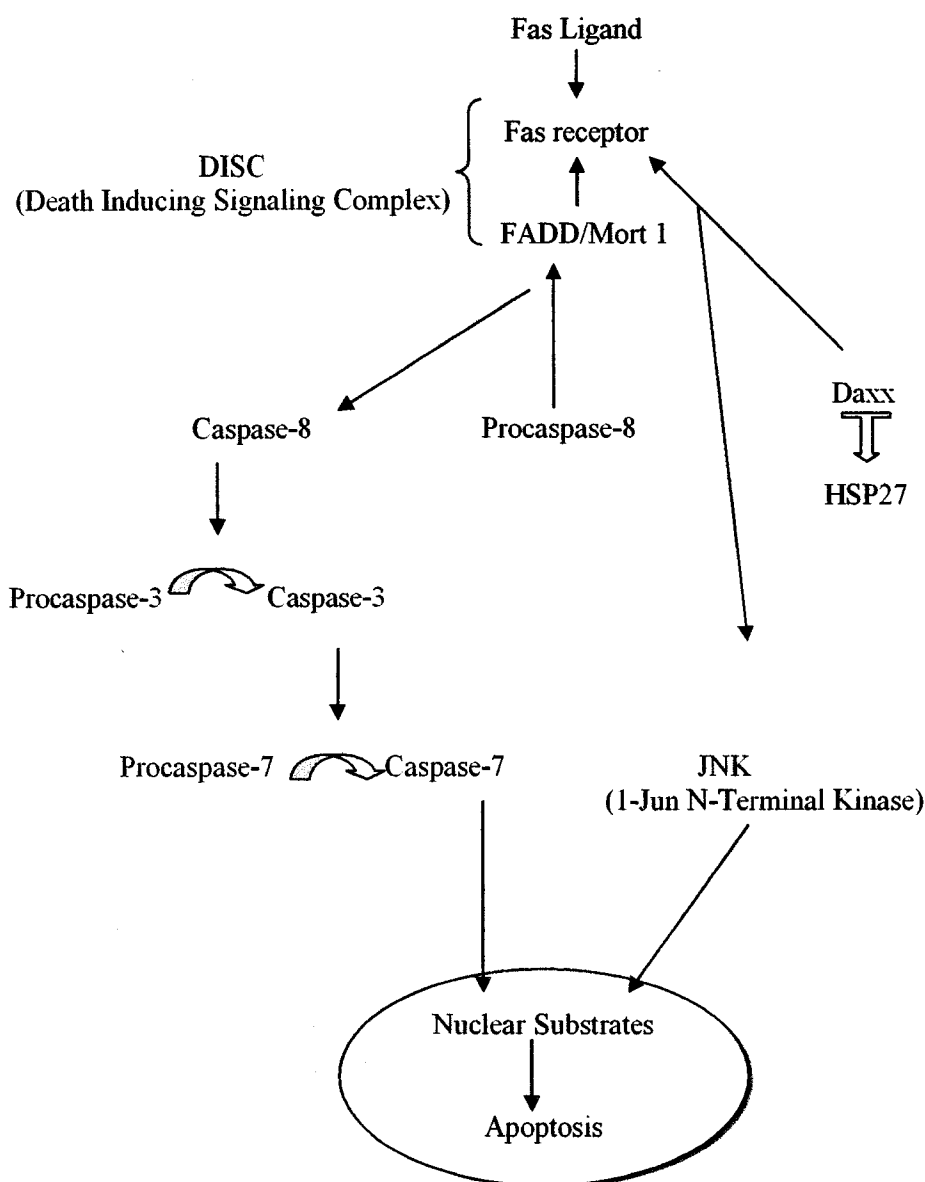


FIG. 1 Death receptor apoptosis. Binding of FasL (Fas/CD95 ligand) to Fas receptor induces trimerization of Fas. The death domain-containing (DD-containing) adaptor molecule, FADD/Mort1, contains a DD at its C-terminus, is recruited to the cytoplasmic region of Fas and binds to the DD of Fas forming death-inducing signaling complex (DISC). The N-terminus of FADD is needed for recruiting initiator procaspase-8 and/or procaspase-10. Procaspase-8 has two death effector domains (DEDs) at its N-terminal region where it binds FADD. Procaspase-8 autoactivates at the DISC, then activates procaspase-3, which is the procaspase that death receptor and mitochondrion-dependent apoptosis pathways converge on. Daxx can associate with the Fas receptor and activate the JNK apoptotic pathway.

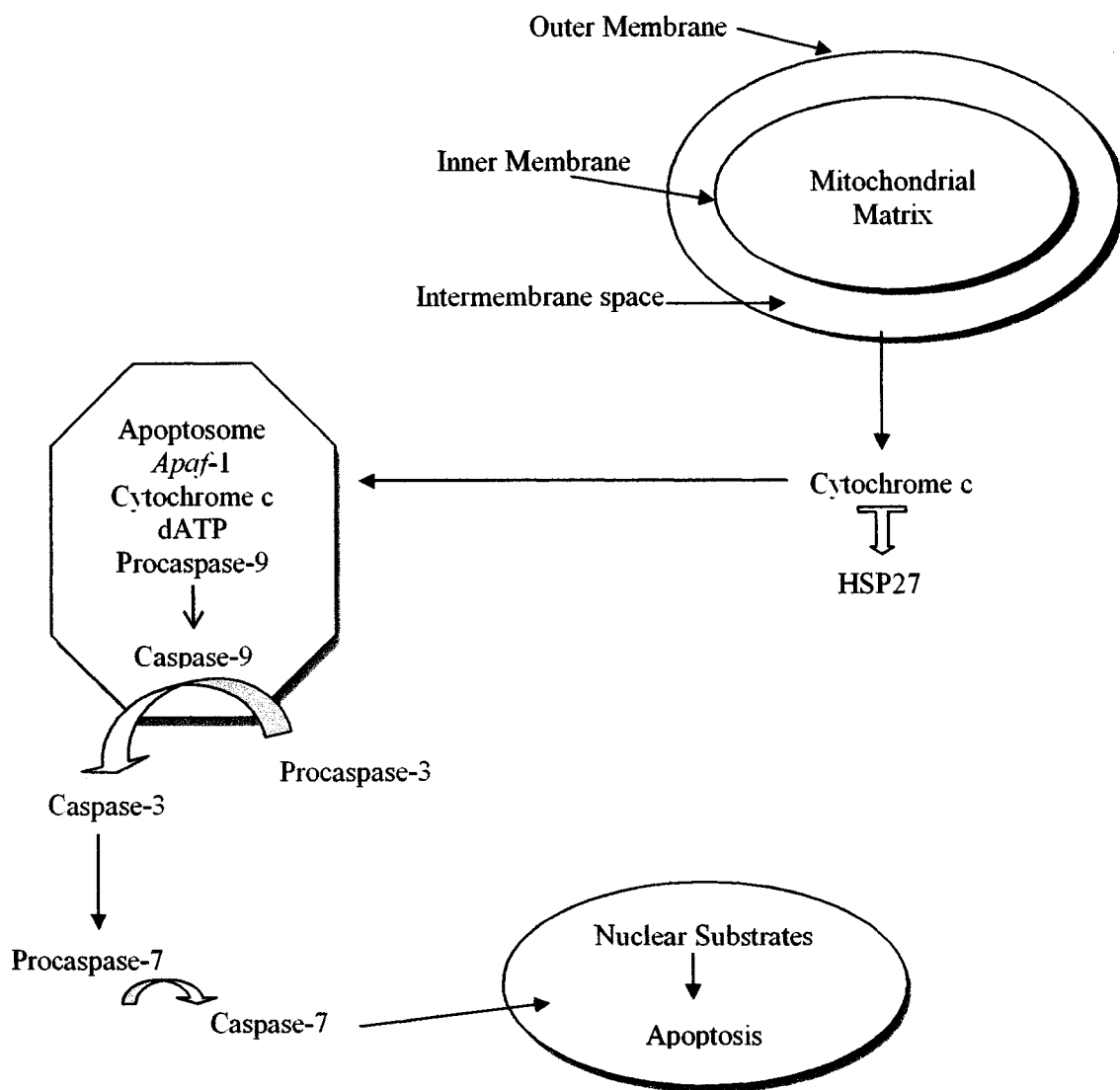
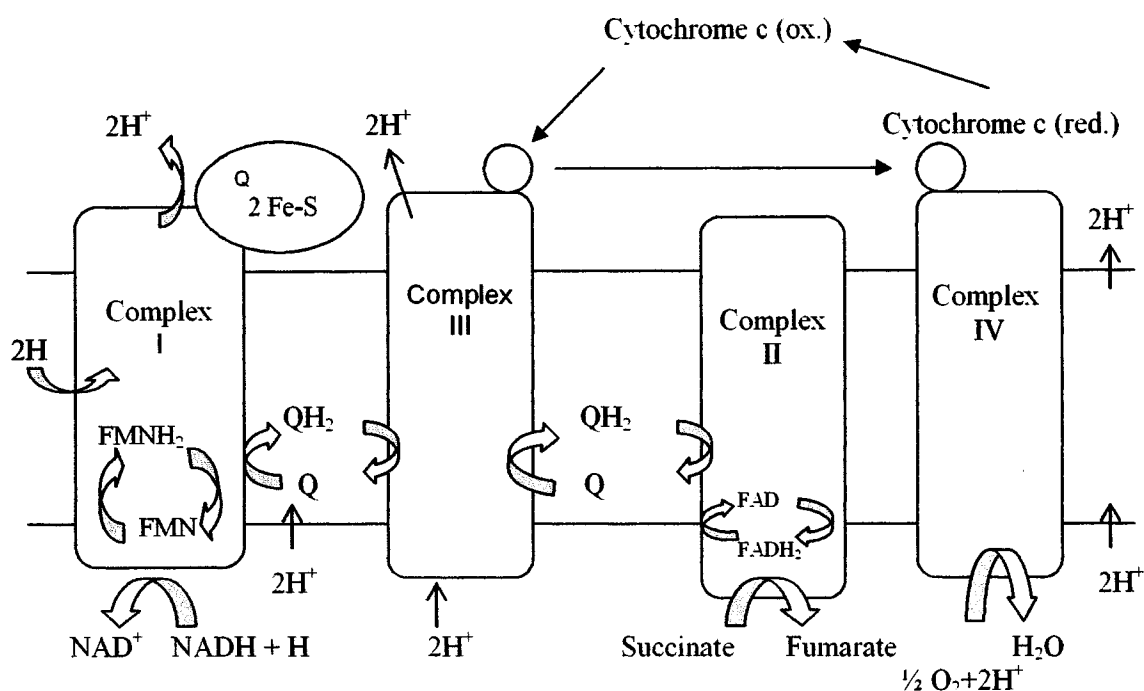


FIG. 2 Mitochondrion-dependent apoptosis. In response to apoptotic stimuli the mitochondrion releases cytochrome c, caspases, AIF (apoptosis-inducing factor), and SMAC/Diablo (second mitochondria-derived activator of caspases and murine homologue) into the cytosol to trigger caspase activation. In the cytosol cytochrome c is essential to the apoptosome, which consists of *apaf-1*, dATP and procaspase-9. Procaspase-9 autoactivates at the apoptosome, then cleaves procaspase-3, which cleaves procaspase-7, which cleaves specific target substrates leading to apoptosis. The binding of HSP27 to cytochrome c prevents the formation of the apoptosome; therefore inhibiting apoptosis.

Intermembrane Space



Matrix space

FIG. 3 The mitochondrial electron transport chain (respiratory chain). Four distinct protein complexes make up the mitochondrial respiratory chain: (I) NADH-coenzyme Q reductase, (II) succinate-coenzyme Q reductase, (III) coenzyme Q-cytochrome c reductase, and (IV) cytochrome c oxidase. NADH donates electrons to Complex I, serving as a link between glycolysis, the TCA cycle, fatty acid oxidation and the electron transport chain. Complex I and II produce a common product, reduced coenzyme Q (UQH₂), which becomes the substrate for Complex III. Complex III oxidizes UQH₂ and reduces cytochrome c, which is the substrate for Complex IV and this is where molecular oxygen is reduced to water.

Table III
Glossary

Terms	Definitions
AIF	Apoptosis inducing factor
Apaf-1	Apoptotic protease activating factor-1
Apoptosis	Programmed cell death
Apoptosome	A complex whose assembly is central to the activation of apoptosis. It consists of Apaf-1, holo-cytochrome c (containing the heme group), dATP/ATP and procaspase-9.
Ask 1	Apoptosis signal-regulated kinase 1
CARD	Caspase recruitment domain
Caspase	CysteinyI aspartic acid - protease (recognizes aspartic acid residues and cuts at those sites).
Caspase - 3	A cysteinyI aspartic acid – protease that is cleaved by caspase - 9 and is part of the mitochondrion dependent apoptotic pathway.
Caspase - 7	A cysteinyI aspartic acid - protease that cleaves essential nuclear substrates, completing apoptosis.
Caspase - 8	A cysteinyI aspartic acid - protease involved in receptor - mediated apoptosis.
Caspase - 9	A cysteinyI aspartic acid - protease that self-activates at the apoptosome.
Daxx	Daxx is a Fas receptor binding protein and can activate the JNK apoptotic pathway.

Table III (continued)
Glossary

Terms	Definitions
DD	Death domain
DED	Death effector domain
DISC	Death inducing signaling pathway
ECL	Enhanced chemiluminescence
FADD	Fas associated death domain
Fas	Fas is a member of the TNF (Tumor necrosis factor) receptor family. It is involved in receptor mediated apoptosis.
FasL	Fas ligand binds to Fas
HRE	Hormone response element
HSP	Heat shock protein
IMS	Intermembrane space
MAPKAP	Mitogen-activated protein kinase-activated protein
NK	Natural killer cells
POD	PML-oncogenic domain
p38 MAP	Mitogen-activated protein
ROS	Reactive oxygen species
SAPK1/JNK	Stress-activated protein kinase1-Jun N-terminal kinase
sHSP	Small heat shock protein

Table III (continued)
Glossary

Terms	Definitions
SMAC/Diablo	Second mitochondria-derived activator of caspases and murine homologue

CHAPTER 2

MATERIALS AND METHODS

Cell Lines

Four human breast cancer cell lines (DC4, DB46, MCF-7 & MDA-MB-435), one human colon cancer cell line (RKO) and one “normal”, human lung fibroblast cell line (HFL1) were used. RKO and HFL1 were a kind gift from Dr. R. Gary (UNLV). DC4 & DB46 were derived from an estrogen-receptor negative human breast cancer cell line MDA-MB-231 (7). DB46, which has the constitutive expression vector p β 27, expresses elevated levels of heat shock protein 27. The vector was constructed by cloning an *EcoRI* cDNA fragment of HSP27 into the *SalI* site of pH β Apr-1-neo by a blunt end ligation after filling in the fragments (48). DC4, which is the control, contains only the vector pH β Apr-1-neo; therefore, it expresses basal levels of heat shock protein 27 (7). MCF-7 has cytoplasmic estrogen receptors and MDA-MB-435 does not (9). All cell lines were grown in Minimal Essential Media (MEM), supplemented with 10% fetal bovine serum, 25 mM HEPES buffer (pH 7.4), 100 IU penicillin/ml, 100 μ g/ml streptomycin, 2 mM L-glutamine, and for DC4, DB46 cell lines, 400 μ g/ml G418 sulfate. RKO was supplemented with 1.0 mM sodium pyruvate. All reagents used in cell culture were purchased from Gibco BRL, Grand Island, NY. All cell lines were maintained in humidified incubators at 37°C with 5% CO₂:95% air. Forty-eight hours before an

experiment the cell lines, DC4 & DB46, were washed and resuspended in supplemented MEM lacking G418.

Electron Carriers and Reagents

The following electron carriers and reagents were purchased from Sigma: cytochrome c, Q₁₀, DMSO, FAD, β-NADH and ATP. MgCl₂•2H₂O was obtained from Fisher Scientific.

Cytochrome c was suspended in 5 ml of PBS (phosphate buffered saline, pH 7.2, (1X), no Mg⁺², no Ca⁺²) from which serial dilutions were made to yield experimental concentrations (PBS was purchased from Gibco BRL). FAD, β-NADH, ATP and MgCl₂ were suspended in 10 ml of PBS from which dilutions were made. MgCl₂ was used as a control in the ATP experiment. It was used at 1x10⁻⁷ M, which was the highest experimental concentration of ATP utilized. Q₁₀ was suspended in heated DMSO. A 500 ml beaker was filled with 200 ml of water so that a 15 ml centrifuge tube containing DMSO and Q₁₀ can be submerged halfway. First the water was heated for one minute in a microwave on high power. After removing the beaker from the microwave the 15 ml centrifuge tube was submerged halfway for 15 seconds at a time (3x). After each submersion the 15ml centrifuge tube was vortexed. After the third vortex Q₁₀ was dissolved. DMSO was used as a control in the following molarities: 276 mM, 138 mM, 27.6 mM and 2.76 mM. All reagents and electron carriers were made fresh and filter sterilized prior to dilutions.

Heat Shock

Twenty-four hours before the experiment, a water bath was filled with deionized water and heated to $45^{\circ}\text{C} \pm 0.01^{\circ}\text{C}$. A DC4 flask (T-25) was taken under the hood and sprayed with a spray bottle containing 70% ethanol and 30% water. Its vented cap was replaced with a solid cap and the entire flask was wrapped with parafilm so that no water could enter the flask once it was submerged in the water bath. The flask was then submerged in the heated water for 10 minutes after which it was removed. Parafilm was then removed and paper towels were used to collect any water that might have gotten under the cap. The flask was sprayed with again with a 70% ethanol/ 30% water dilution and taken under the hood. There the cap was exchanged and secured. The flask was then transferred back to the incubator.

Clonogenic Survival

The cell lines were exposed to the above mentioned electron carriers and reagents in various concentrations. Each exposure was for one hour at 37°C after which the cells were washed once in 5 ml of PBS, trypsinized, resuspended in supplemented MEM and counted using a Coulter Counter (Model ZF). Cells were plated in 60 mm tissue culture dishes. Each experiment was set up in triplicates. Cells were incubated at 37°C in a humidified incubator with 5% CO_2 :95% air for eleven days. After incubation, cells were removed from incubator, stained with crystal violet (0.5% Crystal violet:95% ethanol), allowed to dry and counted. Colonies consisting of > 50 cells were counted (cells survived). Results are graphed as percent survival.

Western Blotting

For Western blotting, cells from each cell line were trypsinized in T-25 flasks. When cells of a cell line were freely floating in trypsin, media was added and the contents of the flasks were emptied into a 15 ml centrifuge tube. The cells were centrifuged at 2000 rpm for 5 minutes in a Beckman Model TJ-6 Centrifuge at 4°C and the media was drained. The pellets were resuspended in 1 ml of PBS buffer. A 50 ml beaker was halfway filled with ice, the 15 ml centrifuge tube was put into the ice and held under a sonifier micro tip for 10 sec (3x) at power 3 (Branson Sonifier 450). The contents were transferred into a microfuge tube and spun for 2 min in a Fisher Scientific Micro Centrifuge (Model 235C). The supernatant was then transferred into a microfuge tube. 9 µl of the supernatant was added to 3 µl of 4x cracking buffer (4x cb), which was then subjected to SDS-PAGE (15%). 4x cb consisted of 60% sucrose, 100mM Tris, 8% SDS, 0.8% bromophenol blue, 8% β-mercapto ethanol, which was adjusted to pH 6.8 with HCL. The SDS-PAGE was electrophoresed at 40 mAmps for 40-45 min. The resolved proteins were then electroblotted onto a PDVF membrane in Western transfer buffer 1x at a constant 25 mV for 1 hr. 1x Western transfer buffer consisted of 12 mM trizma, 96 mM glycine in 800 ml water and 200 ml ethanol. The membrane was blocked with 10% non-fat dry milk (Carnation®) in 1x Western wash (40 ml total volume) and rocked for one hour at room temperature (R. T.) 1x Western wash was obtained from 10x Western wash. 10x Western wash consisted of 10x TBS and 0.1% Triton x-100. 10x TBS consisted of 0.5 M trizma, 1.5 M NaCl which was adjusted to a pH of 7.4 with HCL. Water was added until $V_{\text{tot}} = 0.5\text{L}$. Following the blocking, a 1° mouse monoclonal antibody (Ab) against HSP27 (Stressgen # SPA-800) was used. 8µl of Ab were dissolved in 800µl of 10%

milk, which was put in 7.2 ml of 1x Western wash thus bringing $V_{\text{tot}} = 8$ ml. After removing the blocking agent, the 8 ml Ab solution was added and then rocked in cold room (4°C) overnight. After 24 hrs the membrane was rinsed 4x in Western wash (volume 20 ml each time) at R. T. The procedure for washing: 5 min rocked, flipped, 5 min rocked, after which solution was changed. Total time elapsed was 40 min after which the 2° Ab Alexa Fluor 532 goat anti-mouse (Molecular Probes # A-11002) was added. The volume again was 8µl of Ab in 800µl of 10% milk put in 7.2 ml of 1x Western wash. This was rocked for 1.5 hrs at R.T. after which it was subjected to the same washing procedures as the 1° Ab and was stored in Western wash. The sheets were then scanned by Typhoon™ 9410. Emission filter was set at 555 BP 20 Alexafluor 532 and the following settings were used: PMT – 400, laser – green (532), sensitivity – normal, pixel size – 200 microns, focal plane – platen.

Statistical Analysis

The data is presented as the mean \pm S.D.; statistical analysis of variance (ANOVA) was performed with the JMP program. In this program, the LS Means Differences Student's t with regard to significance of treatment, cell type and treatment to cell type categories were utilized.

Each bar graph in a figure is representative of up to six replicates of 60 mm dishes. Error bars are representative of standard deviation.

CHAPTER 3

RESULTS

Incubation with Cytochrome c Induces Cell Death

The mechanism of cytochrome c induced apoptosis has been documented and is described in Chapter 1 (Figure 2, p. 27) (5). With this mind, the effects of exogenous addition of various cytochrome c concentrations to several cell lines were investigated. Breast cancer cell lines DC4, DB46, MCF-7 and MDA-MB-435 were treated with the following concentrations of cytochrome c: T0 = 0, T1 = 5×10^{-7} M, T2 = 5×10^{-6} M and T3 = 5×10^{-5} M (Fig. 4, 5, pp. 51, 52). DC4 was heat shocked for this experiment to see if more protection from cytochrome c toxicity could be observed. RKO and HFL1 were treated with the following cytochrome c concentrations: T0 = 0, T1 = 5.6×10^{-8} M, T2 = 5.6×10^{-7} M and T3 = 5.6×10^{-6} M (Fig. 6, p. 53).

DC4, HS DC4 and DB46 cells showed a dose response to T2 and T3 (Figure 4, p. 51). Cell survival was most affected in response to T3. HS DC4 survival rate at 44% was similar to DB46 at 41%. DC4 survival rate was 18%. Western blotting (Fig. 18 A, p. 65) shows the HSP27 expression in DC4 con. (con. = control = not heat shocked), HS DC4 (HS = heat shocked, as described in Chapter 2, p. 34), DB46 con. and DB46 HS. Relative quantification of the results to 0.1 μ g of HSP27 in Western blotting (Fig. 18 A, p. 65) resulted in the following values: DC4 con. - 0.053, HS DC4 - 1.16, DB46

con. - 1.16, HS DB46 - 5.38. From these values it can be seen that HSP27 expression in HS DC4 is the same as in DB46 control. This increased level of HSP27 expression in HS DC4 gives the cells about the same protection against cytochrome c toxicity as is observed in DB46. Control DC4, which shows a very small quantification number of HSP27 expression, does not have this protection due to the very low basal levels of HSP27 and therefore has a much lower survival rate as mentioned above. HSP27 expression increases about five-fold in DB46 following heat shock.

MCF-7 and MDA-MB-435 also responded in a dose dependent manner to cytochrome c treatments with the highest percent of cells killed at T3 (Fig. 5, p. 52). At T3, MCF-7 had the highest survival rate of all the breast cancer cell lines with 64.5%, which can be explained by constitutive expression of HSP27 in the cell line and MDA-MB-435, which does not constitutively express HSP27, had a survival rate of 36%. The treatment response of the cells can also be explained by looking at Western blotting (Fig. 18 B, p. 65). Quantification values are reported relative to 0.1 μ g of HSP27 expression in Western blotting (Fig. 18 A & B, p. 65) and are as follows: MCF-7 con. - 14.56, MCF-7 HS 15.07, RKO con. - 0.63, RKO HS - 0.44, MDA-MB-435 con. - 0.23, MDA-MB-435 HS - 0.78. These values show that, following heat shock, HSP27 expression does not change much in the MCF-7 cell line. A reduction of HSP27 expression is observed in the RKO cell line following heat shock and about a three-fold increase is seen in the MDA-MB-435 cell line following heat shock treatment. The difference in HSP27 expression in the two breast cancer cell lines and the resulting survival rates are analogous to the survival rates of DC4 and DB46 in the previous experiment. HSP27 protects the MCF-7 cells against the cytotoxic effects of cytochrome c and the low levels of HSP27 present in MDA-MB-

435 are not able to give that protection against cytochrome c toxicity in that cell line.

RKO was not sensitive to any of the cytochrome c concentrations and HFL1 was sensitive only at the highest cytochrome c concentration T3 with about a 45% cell survival (Fig. 6, p. 53). Western blotting (Fig. 18 B, p. 65) shows low HSP27 expression in the RKO cell line. Unfortunately, Western blotting could not be done with the HFL1 cell line because of loss to mold and therefore no data is available with regard to HSP27 levels in that cell line. It is not clear what mechanism was induced to protect the two cell lines against cell death. It is clear that the RKO cell line was much more resistant to cytochrome c toxicity than the breast cancer cell lines.

In summary, the survival rates at the highest cytochrome c concentrations are as follows: RKO - 100%, HFL1 - 55.0%, MCF-7 - 64.0%, HS DC4 - 44%, DB46 - 41%, MDA-MB-435 - 36% and DC4 - 18%.

Statistical analyses were performed on the three experiments above to see if there were significant differences between treatments, between cell lines and treatment effect between cell lines.

A significant effect of cytochrome c treatments (ANOVA, $p < 0.0001$) on mean cell survival in each cell line DC4, DB46 and HS DC4 exists (Fig. 4, p. 51). With regard to treatment there are no significant differences (LS Means Differences Student's t , $p > 0.05$) between control and T1. T2 is significantly different (LS Means Differences Students t , $p < 0.05$) from control, T1 and T3. T3 is significantly different from control, T1 and T2. Cell type (LS Means Differences Student's t , $p < 0.05$) differences were significant between DC4 and HS DC4 as well as DC4 and DB46. Treatment to cell type (LS Means Differences Student's t , $p < 0.05$) confirms that the DC4 cell line is significantly different

from HS DC4 and the cell line DB46 and vice versa in response to treatment.

Furthermore, HS DC4 and DB46 are not significantly different in their response to treatment.

A significant effect of cytochrome c treatments (ANOVA, $p < 0.0001$) was seen on mean cell survivals across the cell lines MCF-7 and MDA-MB-435 (Fig. 5, p. 52). Again, with regard to treatment there are no significant differences (LS Means Differences Student's t , $p > 0.05$) between control and T1. T2 is significantly different from control, T1 and T3. Conversely, T3 is significantly different from control, T1 and T2. A significant difference across means between cell types was not found (LS Means Differences Student's t , $p > 0.05$). Treatment effects between cell lines showed that only T3 on MCF-7 and MDA-MB-435 was significantly different from all other treatments. Cytochrome c treatments in RKO and HFL1 (ANOVA, $p > 0.0001$) showed no significant differences on mean cell survival across these two cell lines (Fig. 6, p. 53). Also no significant differences were found between cell types (LS Means Differences Student's t , $p > 0.05$). Finally, the only significant difference was found to exist between T3 in HFL1 and all other treatments including all treatments with the RKO cell line. The results for this experiment show that the cell type and response to treatment are only significantly different in the HFL1 cell line with regard to T3.

FAD Can Induce Cell Death

This lab has shown that treatment of breast cancer cell lines with exogenous addition of cytochrome c has varying cytotoxic effects among those cell lines. Given that cytochrome c is an electron carrier involved in the mitochondrial electron transport chain,

the decision was made to look at FAD, NADH, Q_{10} as well as ATP, which are the other electron carriers and one component that play a role in the mitochondrial electron transport chain. The purpose was to use these agents as controls to help show that only exogenous cytochrome c treatment can induce cell death. The same cell lines as in the previous experiments were used (Fig. 7, 9, 10, pp. 54, 56, 57). DC4 was not heat shocked for these experiments.

FAD was applied in the following concentrations: $T_0 = 0$, $T_1 = 1 \times 10^{-9}$ M, $T_2 = 1 \times 10^{-8}$ M and $T_3 = 1 \times 10^{-7}$ M. These concentrations are much smaller than the cytochrome c concentrations in the previous experiments; yet, FAD was found to be more cytotoxic in the DC4, DB46, MCF-7, MDA-MB-435, RKO and HFL-1 cell lines.

The DC4 and DB46 graph (Fig. 7, p. 54) shows a step function in response to FAD treatment. DC4 had a survival rate of 30.0% and DB46 had a survival rate of 57.0% at T_3 . Higher cell survival in DB46 is attributed to the elevated levels of HSP27 in that cell line.

It was of interest to see what effects FAD would have at the same concentrations as cytochrome c and therefore the following concentrations: $T_0 = 0$, $T_1 = 5 \times 10^{-7}$ M, $T_2 = 5 \times 10^{-6}$ M and $T_3 = 5 \times 10^{-5}$ M were applied in another experiment involving the cell lines DC4 and DB46 (Fig. 8, p. 55). A dose response was observed at these concentrations. At T_2 , DB46 had about 16% and DC4 about 13% cell survival. At T_3 , the cell survival of DB46 was almost negligible (0.33%) and DC4 had no survival.

MCF-7 and MDA-MB-435 (Fig. 9, p. 56) were given FAD treatments at the following concentrations: $T_0 = 0$, $T_1 = 1 \times 10^{-9}$ M, $T_2 = 1 \times 10^{-8}$ M and $T_3 = 1 \times 10^{-7}$ M. MCF-7 and MDA-MB-435 showed similar patterns to FAD treatments when compared

to DC4 and DB46 responses at these concentrations. MCF-7 has more protection against FAD toxicity just like DB46 and MDA-MB-435 this electron carrier in DB46 and MCF-7 since these two cell lines express high levels of this protein and both behave similarly.

The next experiment regarding FAD toxicity involved the cell lines RKO and HFL1. Both cell lines responded to FAD treatments (Fig. 10, p. 57). Survival rates for RKO and HFL1 at T3 were 75.0% and 61.0%, respectively. It not clear what gave RKO protection against FAD toxicity since the Western blotting results did not show high levels of HSP27 in that cell line.

A summary of dose responses observed in all cell lines and the survival rates at the highest FAD concentration (T3) are as follows: RKO - 75.0%, MCF-7 - 64.0%, HFL1- 61.0%, DB46 - 57.0%, MDA-MB-435 - 41.0% and DC4 - 30.0%.

Again, statistical analyses were performed on the four experiments above to see if there were significant differences between treatments, between cell lines and treatment effect between cell lines.

A significant effect of FAD treatments ($T_0 = 0$, $T_1 = 1 \times 10^{-9}$ M, $T_2 = 1 \times 10^{-8}$ M and $T_3 = 1 \times 10^{-7}$ M) (ANOVA, $p < 0.0001$) on mean cell survival across the cell lines DC4 and DB46 exists (Fig. 7, p. 54). Results of treatment analysis (LS Mean Differences Student's t, $p < 0.05$) show T_1 , T_2 and T_3 being significantly different from T_0 . Furthermore, T_1 and T_2 are not significantly different from one another but T_3 is significantly different from T_2 . Significant differences were found between cell lines DC4 and DB46 (LS Mean Differences Student's t, $p < 0.05$). Results from treatment to cell type analysis (LS Mean Differences Student's t, $p < 0.05$) revealed that the significant

differences exist between the cell lines and treatment and not within the cell lines and treatment, thus the two cell lines respond differently to FAD treatment.

Highly significant effects of FAD treatments ($T_0 = 0$, $T_1 = 5 \times 10^{-7} \text{ M}$, $T_2 = 5 \times 10^{-6} \text{ M}$ and $T_3 = 5 \times 10^{-5} \text{ M}$) (ANOVA, $p < 0.0001$) on mean cell survival across the cell lines DC4 and DB46 exist (Fig. 8, p. 55). Results of treatment analysis (LS Mean Differences Student's t , $p < 0.05$) show T_1 , T_2 and T_3 to be significantly different from one another as well as from T_0 . Also significant differences exist between the cell lines (LS Mean Differences Student's t , $p < 0.05$) and finally treatment to cell type analysis (LS Mean Differences Student's t , $p < 0.05$) shows that both cell lines respond with a similar pattern to treatment, thus significance lies in the treatment.

In the MCF-7/MDA-MB-435 experiment significant effects of FAD treatments ($T_0 = 0$, $T_2 = 1 \times 10^{-9} \text{ M}$, $T_3 = 1 \times 10^{-8} \text{ M}$ and $T_4 = 1 \times 10^{-7} \text{ M}$) (ANOVA, $p < 0.0001$) on mean cell survival across the cell lines exist (Fig. 9, p. 56). Treatment analysis (LS Mean Differences Student's t , $p < 0.05$) shows T_1 , T_2 and T_3 to be significantly different from T_0 . T_1 and T_2 are not significantly different from one another but T_3 is significantly different from T_1 . The cell type ($p < 0.05$) is significantly different and analysis of treatment to cell type (LS Mean Differences Student's t , $p < 0.05$) shows significant differences between the cell lines with respect to treatment.

Significant effects regarding FAD treatments ($T_0 = 0$, $T_1 = 1 \times 10^{-9} \text{ M}$, $T_2 = 1 \times 10^{-8} \text{ M}$ and $T_3 = 1 \times 10^{-7} \text{ M}$) (ANOVA, $p < 0.0001$) on mean cell survival across the cell lines RKO and HFL1 exist (Fig. 10, p. 57). Treatment analysis (LS Mean Differences Student's t , $p < 0.05$) shows T_1 , T_2 and T_3 to be significantly different from T_0 , but T_1 , T_2 and T_3 are not significantly different from one another. An analysis of cell type (LS Mean

Differences Student's *t*, $p < 0.05$) shows that the cell types are significantly different.

Finally, treatment to cell type (LS Mean Differences Student's *t*, $p < 0.05$) shows that the significance of treatment is between not within the cell lines.

ATP Can Affect Cell Survival

FAD cytotoxicity was not an anticipated outcome of the above experiment. ATP consists of an AMP moiety and two attached phosphates. FAD and ATP both have an AMP moiety. Upon this observation the decision was made to use ATP in this experiment. The same experimental concentrations as in the FAD experiment were applied. $MgCl_2$ was used as a control to make certain it did not induce cell death. The concentration for $MgCl_2$ was 1×10^{-7} M and results showed that it did not have any effect on the cells (Fig. 11, p. 58).

DC4 and DB46 were the only cell lines used in this experiment given the trend of similar cell survivals among the breast cancer cell lines and keeping in mind that the research is pertaining to the effects of electron carriers with regard to breast cancer research (Fig. 12, p. 59). Cell survivals for DC4 at increasing concentrations from $T_0 = 0$, $T_1 = 1 \times 10^{-9}$ M, $T_2 = 1 \times 10^{-8}$ M to $T_3 = 1 \times 10^{-7}$ M were 100%, 50.6%, 55.0% and 52%, respectively. For DB46 the survival rates at the same increasing concentrations were 100%, 52.0%, 61.0% and 63.0%, respectively. These results showed a response to ATP treatments.

Statistical analysis was performed on the experiment above to see if there were significant differences between treatments, between cell lines and treatment effect between cell lines.

Significant effects regarding ATP treatments ($T_0 = 0$, $T_1 = 1 \times 10^{-9}$ M, $T_2 = 1 \times 10^{-8}$ M and $T_3 = 1 \times 10^{-7}$ M) (ANOVA, $p < 0.0001$) on mean cell survival across the cell lines DC4 and DB46 exist (Figure 12, p. 59). Once again treatment analysis (LS Mean Differences Student's t , $p < 0.05$) shows T_1 , T_2 and T_3 to be significantly different from T_0 but no significant differences exist between T_1 , T_2 and T_3 . The cell types are not significantly different with respect to their means in this experiment (LS Mean Differences Student's t , $p > 0.05$) and finally analysis of treatment to cell type (LS Mean Differences Student's t , $p > 0.05$) shows the cell types are not significantly different and the treatments do not elicit significantly different responses of the cell lines.

NADH Can Induce Cell Death

Since FAD and cytochrome c are able to induce cell death and are both electron carriers, we were interested in the toxicity of another electron carrier. NADH, like FAD has an AMP moiety attached to its structure. The concentrations applied were again $T_0 = 0$, $T_1 = 1 \times 10^{-9}$ M, $T_2 = 1 \times 10^{-8}$ M and $T_3 = 1 \times 10^{-7}$ M.

NADH did have an effect on the cell lines DC4 and DB46 (Fig. 13, p. 60). At T_3 cell survival for DC4 and DB46 were 38.0% and 40.0% respectively. Interestingly T_1 cell survival for DC4 was 27% followed by T_2 with 52% cell survival.

The RKO cell line responded to NADH treatment with the following percent survivals: T_0 - 100%, T_1 - 117%, T_2 - 124% and T_3 - 103%, thus cell proliferation not cell death was the outcome of treatment with this electron carrier. HFL1 showed a non-significant dose response as concentrations were increased with a 71.0% cell survival at T_3 (Fig. 14, p. 61).

Statistical analyses were performed on the two experiments above to see if there were significant differences between treatments, between cell lines and treatment effects between cell lines.

Significant effects regarding NADH treatments ($T_0 = 0$, $T_1 = 1 \times 10^{-9}$ M, $T_2 = 1 \times 10^{-8}$ M and $T_3 = 1 \times 10^{-7}$ M) (ANOVA, $p < 0.0001$) on mean cell survival across the cell lines DC4 and DB46 exist (Fig. 13, p. 60). Significances of treatments (LS Mean Differences Student's t , $p < 0.05$) are not between T_1 , T_2 and T_3 but rather between the three treatments with respect to T_0 . The cell lines are not significantly different in their means (LS Mean Differences Student's t , $p > 0.05$) and treatment to cell type analysis (LS Mean Differences Student's t , $p < 0.05$) shows to have significance within the cell line DC4 (T_2 with respect to T_0 and T_1) but no significant effects of treatments between or within the cell lines DC4 and DB46.

Significant effects regarding NADH treatments ($T_0 = 0$, $T_1 = 1 \times 10^{-9}$ M, $T_2 = 1 \times 10^{-8}$ M and $T_3 = 1 \times 10^{-7}$ M) (ANOVA, $p > 0.0001$) on mean cell survival across the cell lines RKO and HFL1 do not exist (Fig. 14, p. 61). The cell lines are significantly different (LS Mean Differences Student's t , $p < 0.05$). An analysis of treatment to cell type (LS Mean Differences Student's t , $p < 0.05$) shows that there is a significant difference between the cell lines RKO and HFL1 in T_2 and T_3 but no significant differences within the RKO cell line. Significant differences within a cell line are only found in HFL1 between T_1 and T_3 . In summary this shows that the cell lines are significantly different and treatment significance within the cell line is between T_3 and T_1 (HFL1) whereas between the cell lines the significance is between T_2 in RKO and T_2 , T_3 in HFL1 and T_1 in RKO and T_3 in HFL1.

Q₁₀ May Induce Cell Death

The last electron carrier of the electron transport chain left to investigate was Q₁₀. DMSO was used as a solvent for Q₁₀ as described in Chapter 2 (Materials and Methods, p. 33) and was found not to affect cell survival (Fig. 15, p. 62).

Following this observation, Q₁₀, which was dissolved in DMSO, was applied in the same concentrations as in the above experiments; T₀ = 0, T₁ = 1x10⁻⁹ M, T₂ = 1x10⁻⁸ M and T₃ = 1x10⁻⁷ M. DC4 and DB46 displayed cell death only at T₃ with cell survival rates of 53.0% and 74.0% respectively (Fig. 16, p. 63). RKO and HFL1 were both resistant to all of the concentrations applied (Fig. 17, p. 64).

Statistical analyses were performed on the two experiments above to see if there were significant differences between treatments, between cell lines and treatment effect between cell lines.

Significant effects regarding Q₁₀ treatments (T₀ = 0, T₁ = 1x10⁻⁹ M, T₂ = 1x10⁻⁸ M and T₃ = 1x10⁻⁷ M) (ANOVA, p>0.0001) on mean cell survival across the cell lines DC4 and DB46 exist only between T₃ relative to T₀, T₁, T₂. No significant differences were found between T₀ relative to T₁ or T₂, T₁ relative to T₀ or T₂ and T₂ relative to T₀ or T₁. The means of cell lines are not significantly different (LS Mean Differences Student's t, p>0.05). An analysis of treatment to cell type (LS Mean Differences Student's t, p>0.05) shows that there are no significant differences within or between the cell lines DC4 and DB46 with respect to different concentrations except for the following two observations. T₂ to T₃ within the DB46 cell line, T₃ between cell lines DC4 and DB46 as well as within the cell line DC4 with respect to T₀, T₁ and T₂. In summary, this shows that the cell lines and treatment are not significantly different except for T₃.

Significant effects regarding Q_{10} treatments ($T_0 = 0$, $T_1 = 1 \times 10^{-9}$ M, $T_2 = 1 \times 10^{-8}$ M and $T_3 = 1 \times 10^{-7}$ M) (ANOVA, $p > 0.0001$) on mean cell survival across the cell lines RKO and HFL1 do not exist. The means of cell lines are not significantly different (LS Mean Differences Student's t , $p > 0.05$). An analysis of treatment to cell type (LS Mean Differences Student's t , $p > 0.05$) shows that there are no significant differences within or between the cell lines RKO and HFL1. In summary, the results of the analyses show that the cell lines and treatment do not respond significantly different.

Table IV (p. 49) is a summary containing the responses of the cell lines after treatments with the various agents that were used in the above experiments. The results of cytochrome c toxicity were taken from this table and compiled (Fig. 19, p. 66). A trend line shows that the higher the level of HSP27 expression is in a breast cancer cell line the more protection is provided against electron carrier cytotoxicity. It is not clear what mechanism of protection is present in the RKO cell line since the level of HSP27 expression is not enough to provide the observed protection. The results of FAD toxicity (Fig. 20, p. 67) show the same trend in these cell lines. Again, it is not clear what mechanism of protection is present in the RKO cell line. The breast cancer cell lines behave consistently in that the higher the levels of HSP27, the more protection is provided against a toxic agent. It is important to mention here that the concentrations of FAD were much lower than those of cytochrome c (Table IV, p. 49) and therefore showing FAD to be more toxic than cytochrome c.

Table IV

Toxicity Summary

Agent	Cell Line	Concentration (M)	Cell Survival (%)	Level of Toxicity
Cytochrome c	DC4	5×10^{-5}	18.0	****
	HS DC4	5×10^{-5}	44.0	***
	DB46	5×10^{-5}	41.0	***
	MCF-7	5×10^{-5}	64.0	**
	MDA-MB-435	5×10^{-5}	36.0	***
	RKO	5.6×10^{-6}	100.0	-
	HFL1	5.6×10^{-6}	55.3	**
FAD	DC4	5×10^{-5}	0	****
	DB46	5×10^{-5}	.33	****
	DC4	1×10^{-7}	34.0	***
	DB46	1×10^{-7}	57.0	**
	MCF-7	1×10^{-7}	64.0	***
	MDA-MB-435	1×10^{-7}	41.0	***
	RKO	1×10^{-7}	75.0	*
	HFL1	1×10^{-7}	61.0	***
ATP	DC4	1×10^{-7}	52.0	***
	DB46	1×10^{-7}	63.0	***
Q ₁₀	DC4	1×10^{-7}	53.0	***
	DB46	1×10^{-7}	74.	*
	RKO	1×10^{-7}	98.0	-
	HFL1	1×10^{-7}	102.0	-

- = no cell death

* = 100% - 70% cell survival

*** = 49% - 30% cell survival

** = 69% - 50% cell survival

**** = 29% - 0% cell survival

Table IV (continued)

Toxicity Summary

Agent	Cell Line	Concentration (M)	Cell Survival (%)	Level of Toxicity
DMSO	DC4	.276	99.0	-
	DB46	.276	99.0	-

- = no cell death

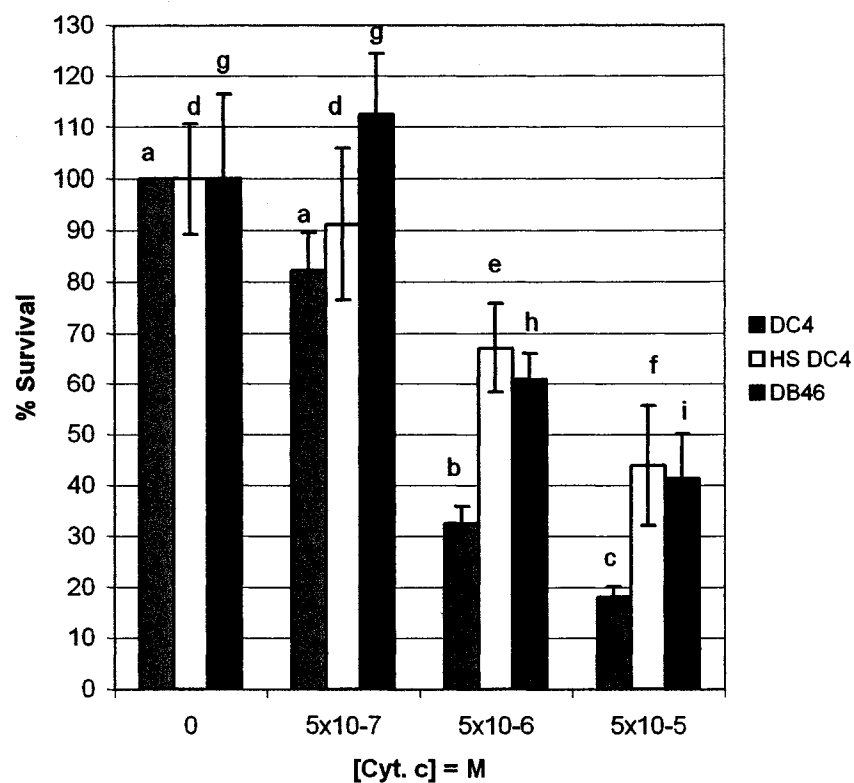


FIG. 4. Cytochrome c toxicity observed in DC4, HS DC4 and DB46 cell lines. Increasing concentrations of cytochrome c elicit a dose response (lowest percent survival at highest concentration) in DC4, HS DC4 and DB46. (Different letters indicate significantly different means $p < 0.05$). In this experiment no significant differences were found between treatments T0 (Control) and T1 (5×10^{-7} M) in all cell lines, hence letters a,d,g are assigned to both treatments. In DC4, T2 (5×10^{-6} M) and T3 (5×10^{-5} M) are significantly different from T0 as well as each other; hence letters b and c are assigned. In HS DC4, T2 and T3 are significantly different from T0 as well as each other, therefore letters e and f are assigned. In DB46, T2 and T3 are significantly different from T0 and as well as each other, which are indicated with the letters h and i. The assignments of letters also show that treatments in relation to cell type have significant differences. DC4 was more sensitive to cytochrome c to treatments. HS DC4 and DB46 showed similar responses to T2 and T3, thus the cell lines DC4 and DB46 respond differently to treatments in this experiment. (No error bars are in DC4 T0, because all but one 60 mm dish was lost to mold contamination. Experiment was performed twice with similar results.)

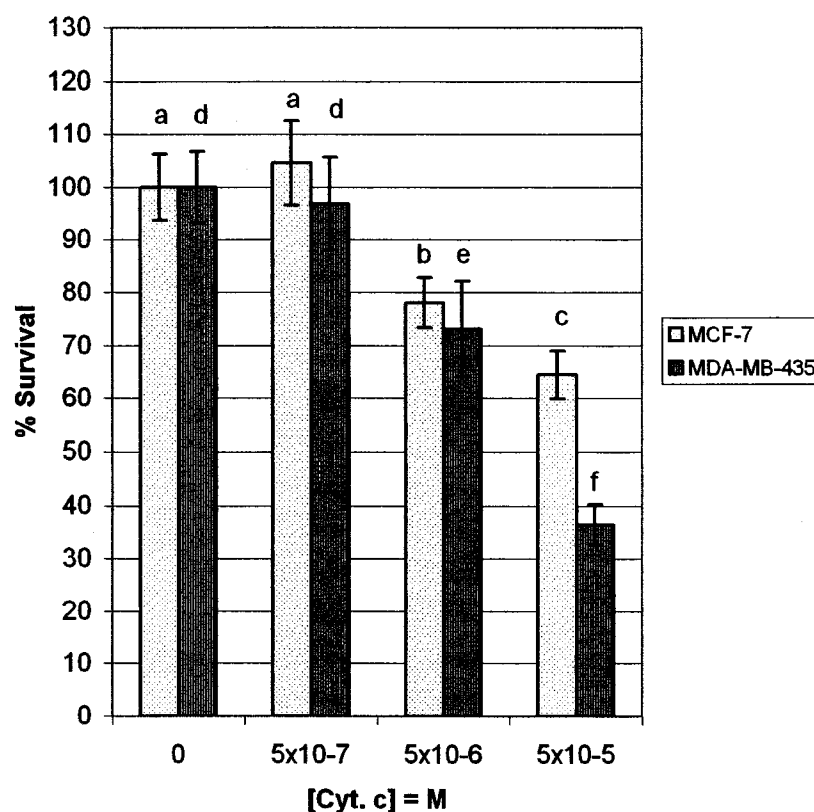


FIG. 5. Cytochrome c toxicity observed in cell lines MCF-7 and MDA-MB-435. A dose response is observed in both cell lines with the most cell kill at the highest concentration. MCF-7 was less sensitive to cytochrome c treatments than MDA-MB-435. (Different letters indicate significantly different means, $p < 0.05$). No significant differences were found between T0 (Control) and T1 (5×10^{-7} M) in both cell lines as letters a and d indicate. Significant differences in MCF-7 are between T0, T2 (5×10^{-6} M) and T3 (5×10^{-5} M) as shown by letters b and c. Significant differences in MDA-MB-435 are between T0, T2, T3 indicated by letters e and f. Cell type to treatment response (LS Means Differences Student's T, $p < 0.05$) revealed that the cell lines MCF-7 and MDA-MB-435 responded differently only to T3 otherwise no significant differences to treatment responses were found between cell types.

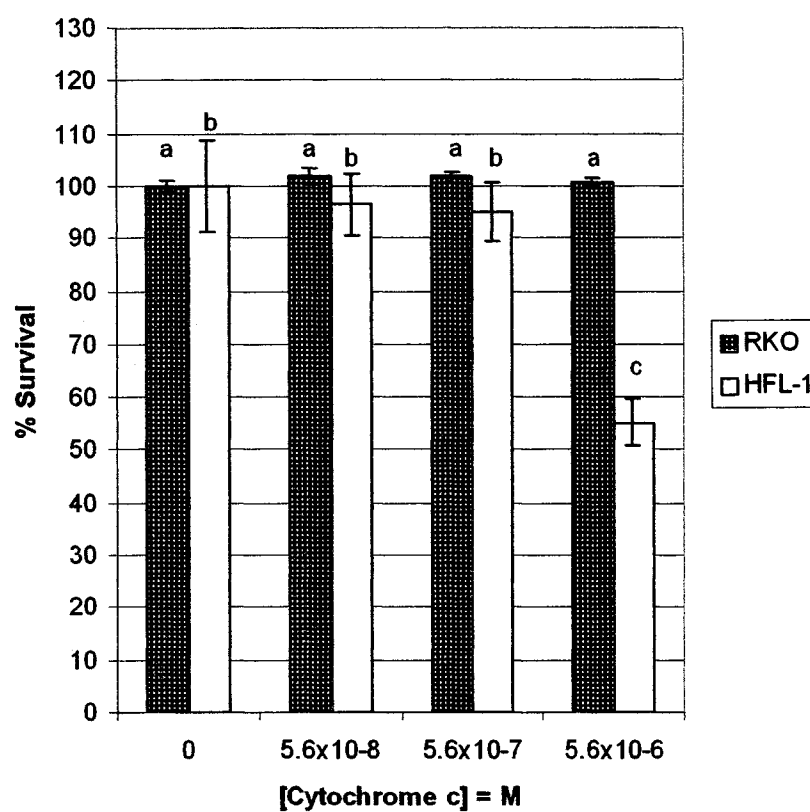


FIG. 6. Cytochrome c effects observed in cell lines RKO and HFL1. No significant differences on mean cell survival were found across the cell lines ($p > 0.05$) as indicated for RKO with letter a and HFL1 with letter b. RKO is resistant to all concentrations of cytochrome c. HFL1 is sensitive to cytochrome c only at the highest concentration. The only significant difference was found in HFL1 with regard to T3 and the other treatments in that cell line. With the exception of T3 (HFL1) no significant differences were found to exist between cell types and treatment response.

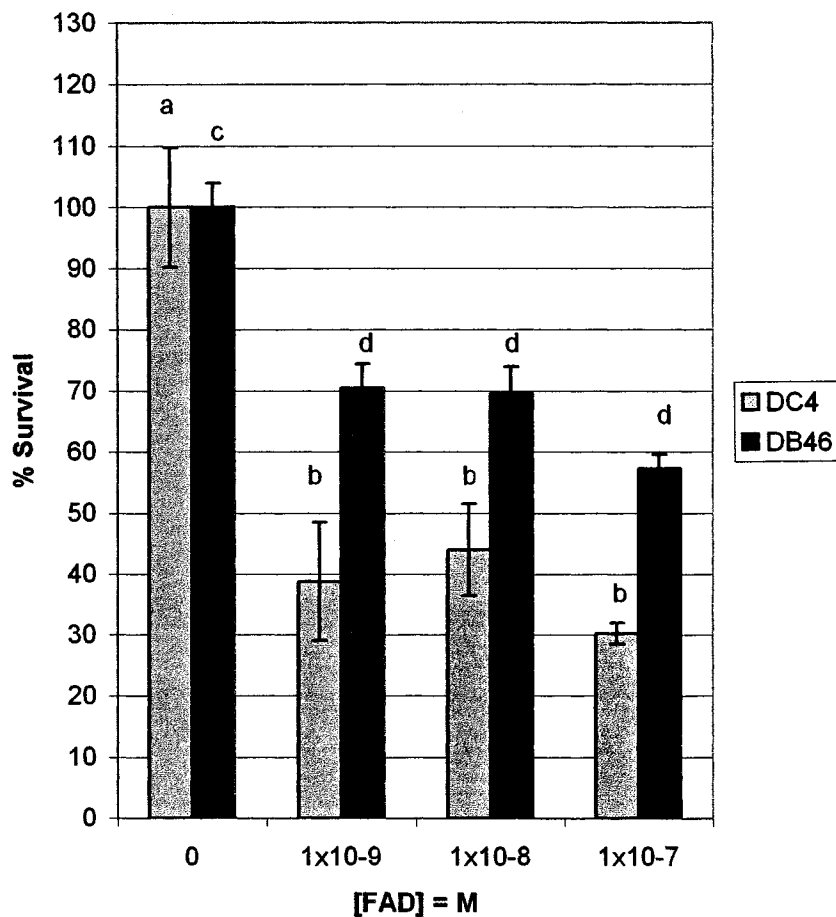


FIG. 7. FAD toxicity observed in DC4 and DB46 cell lines. All concentrations have an effect on both cell lines. This experiment shows a step function as response to cytochrome c treatments in both cell lines. DC4 is more sensitive to FAD treatments than DB46. The highest concentration of FAD yields 70% cell death in DC4 and 48% cell death in DB46. (Different letters indicate significantly different means, $p > 0.05$) Significant differences were found between T0 (control) and all three treatments but no significant differences were found within ($p > 0.05$) the cell lines between treatments T1 ($1 \times 10^{-9} \text{M}$), T2 ($1 \times 10^{-8} \text{M}$) and T3 ($1 \times 10^{-7} \text{M}$). A significant effect of cell type to FAD treatments response ($p < 0.05$) between the cell lines exists, thus no treatment effects exist within the cell lines to the various FAD treatments but the cell types respond differently to various FAD treatments.

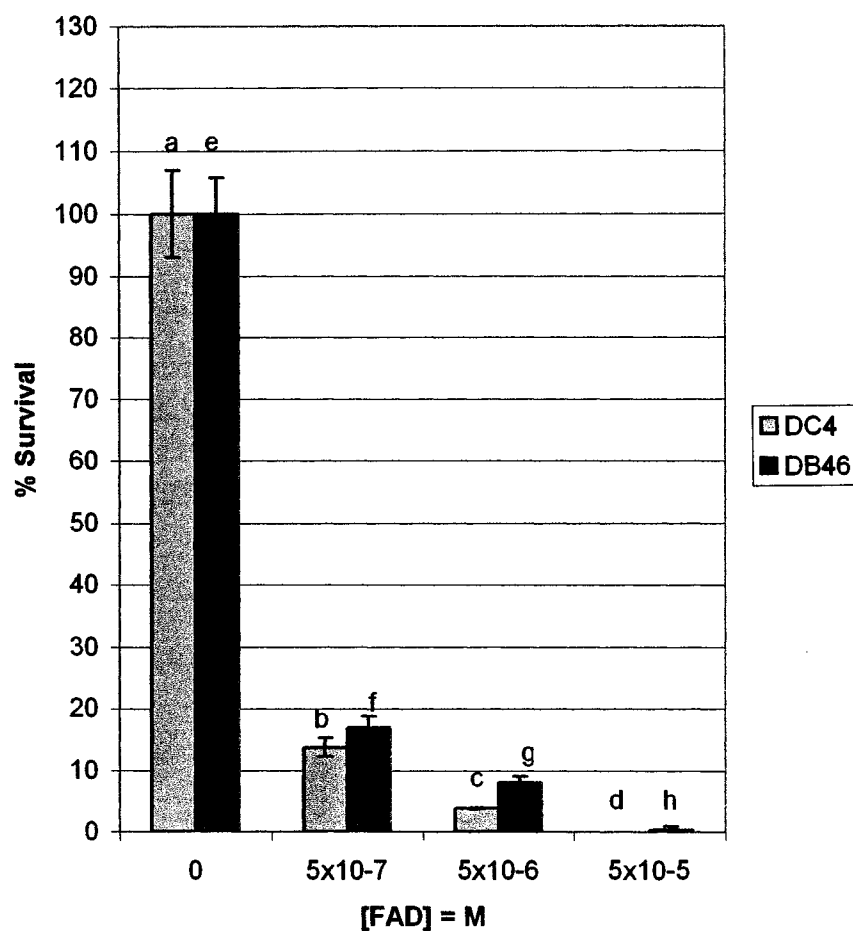


FIG. 8. FAD toxicity observed in DC4 and DB46 cell lines. A dose response is observed in this experiment. FAD applied in the same concentrations as cytochrome c overwhelms both cell lines at T3 (5×10^{-5} M). DC4 has no cell survival and DB46 has 0.33% cell survival at T3. (Different letters indicate significantly different means, $p < 0.05$). All three concentrations are significantly different from T0 (Control) as well as from one another in both cell lines. Treatment response with respect to cell type ($p < 0.05$) shows that the cell types respond the same to treatment and that the significance of difference is in the treatments.

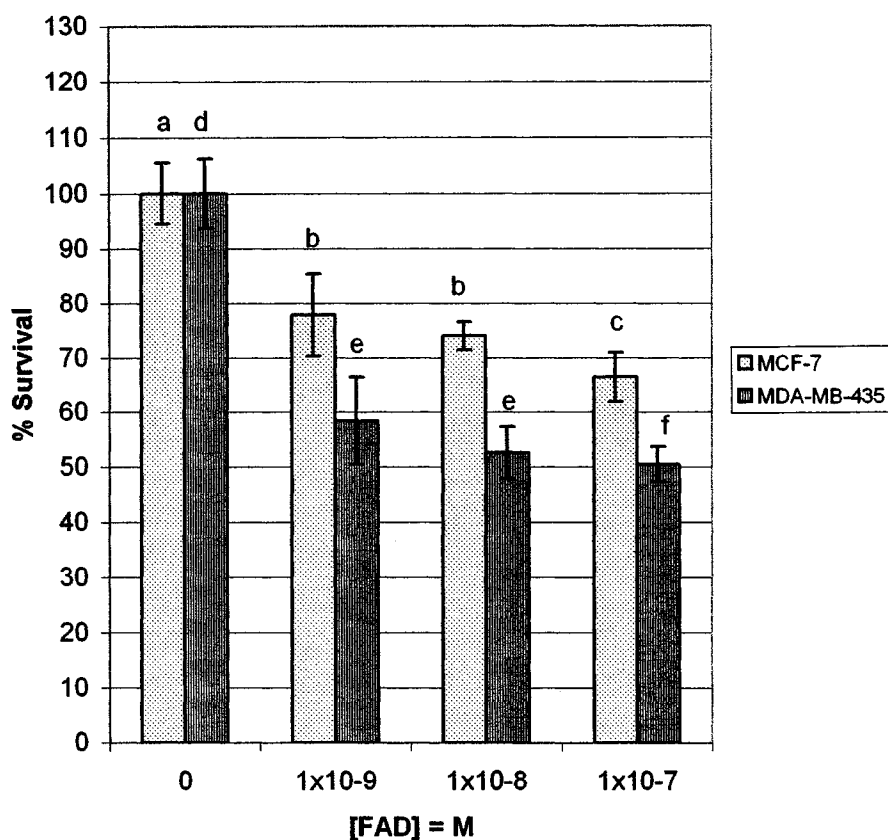


FIG. 9. FAD toxicity observed in MCF-7 and MDA-MB-435 cell lines. A response to FAD treatment is observed in both cell lines with the lowest survival occurring at the highest FAD concentration. (Different letters indicate significantly different means, $p < 0.05$). In both cell lines a significant difference exists between T0 (control) and treatments T1 (1×10^{-9} M), T2 (1×10^{-8} M) and T3 (1×10^{-7} M). In MCF-7 no significant difference exists between T1 and T2, but a significance difference is between T3 and T1. In MDA-MB-435, T2 is not significantly different from T1 but T3 is significantly different from T1. The cell lines are significantly different and significance of treatment to cell type response depends on the cell type.

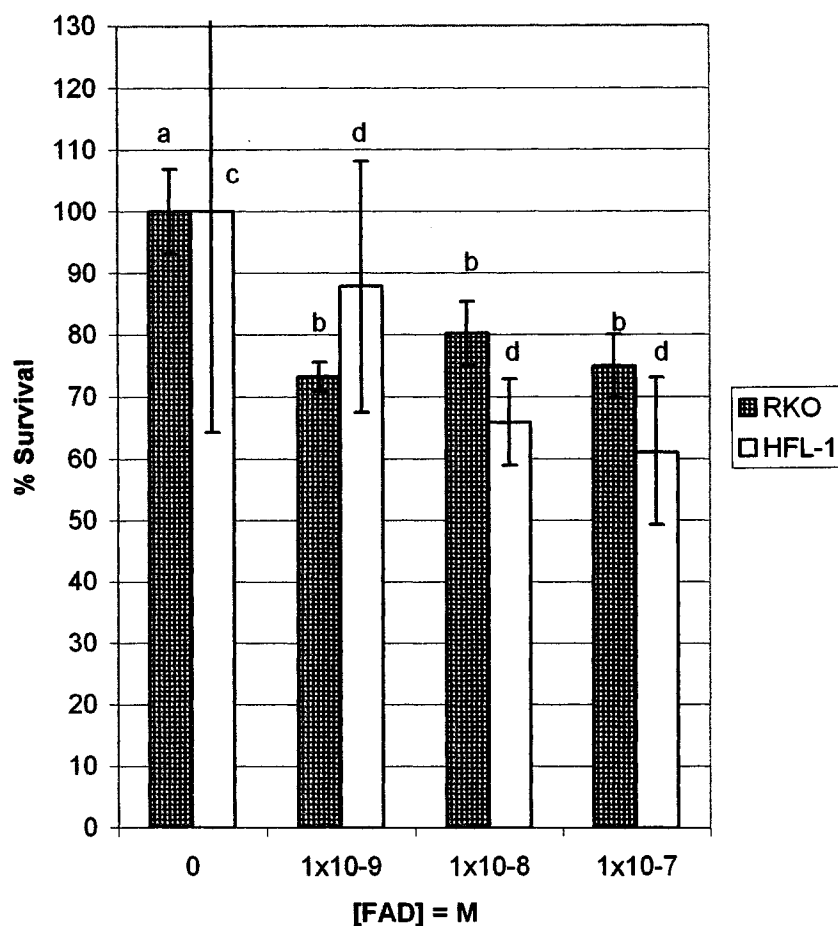


FIG. 10. FAD toxicity observed in cell lines RKO and HFL1. FAD has effects on both cell lines. RKO responds to incubation with FAD but holds its survival rate steady above 70%. Incubation of HFL1 with FAD shows a response as well. The lowest cell survival is observed at the highest concentration of FAD. (Different letters indicate significantly different means, $p < 0.05$). A significant difference ($p < 0.05$) was found to exist only between T0 and T1, T2, T3. No significant differences ($p > 0.05$) exist between the three treatments. The cell lines are significantly different ($p < 0.05$); therefore, treatment to cell response is only significant between the cell lines not within the cell lines. (Error bars with respect to T0 and T1 in HFL1 are large because of loss to mold).

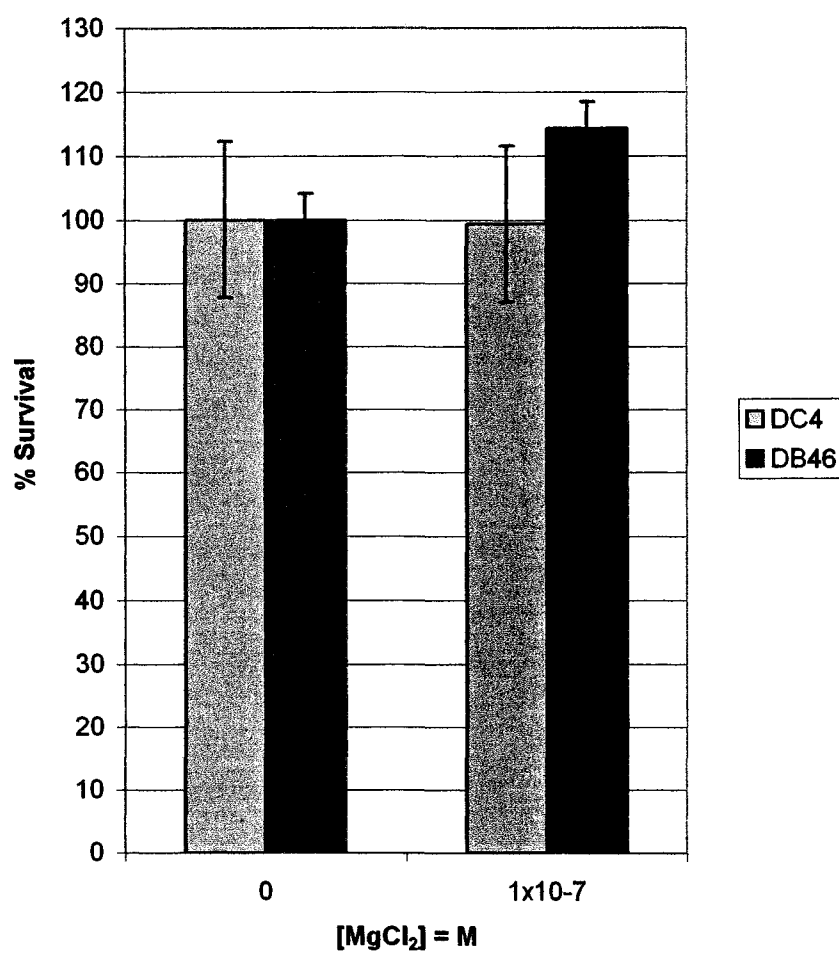


FIG. 11. MgCl₂ effects observed in DC4 and DB46 cell lines. MgCl₂ was set up as a control to ensure that it did not have an effect on cell survival or cell kill when applied in solution with ATP. No effects were observed with DC4 and some cell proliferation was seen in DB46.

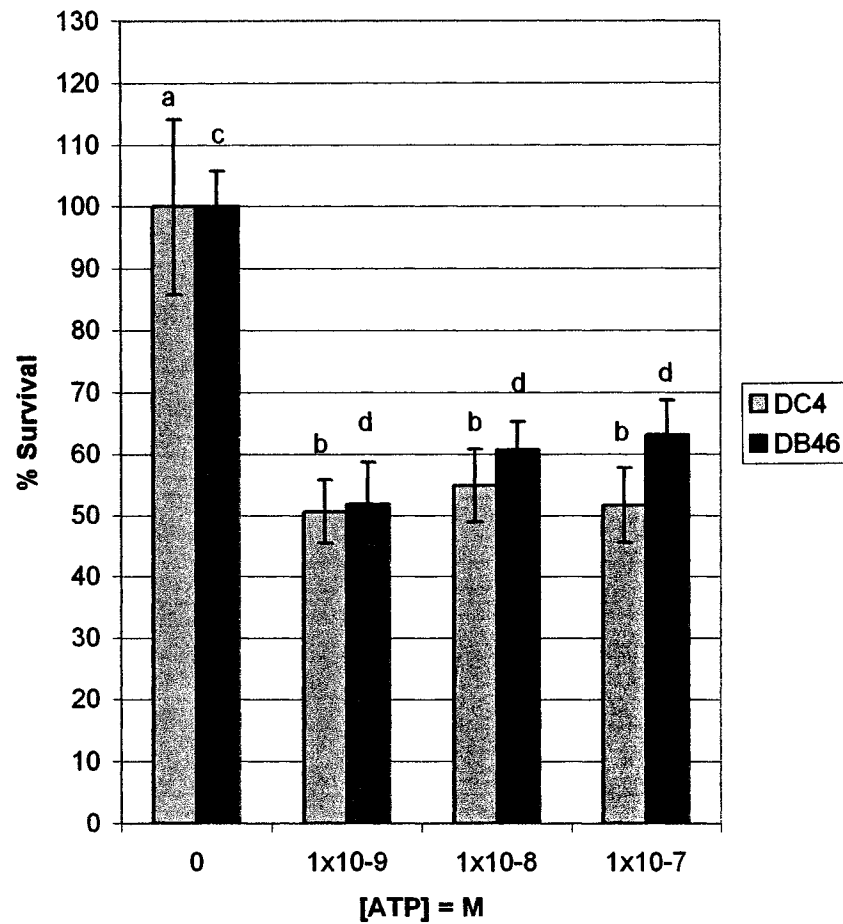


FIG. 12. ATP toxicity observed in DC4 and DB46 cell lines. DB46 and DC4 respond to ATP treatments. A step function is observed in both cell lines as well as similar responses to treatments. (Different letters indicate significantly different means, $p < 0.05$). Significance regarding ATP treatment effects ($p < 0.05$) exists only between T0 (control) and T1 ($1 \times 10^{-9} \text{M}$), T2 ($1 \times 10^{-8} \text{M}$), T3 ($1 \times 10^{-7} \text{M}$). No significant differences ($p > 0.05$) are found between treatments. The cell lines do not respond significantly different to treatment ($p > 0.05$), therefore no significant differences are found within cell lines or between cell lines regarding treatment with ATP.

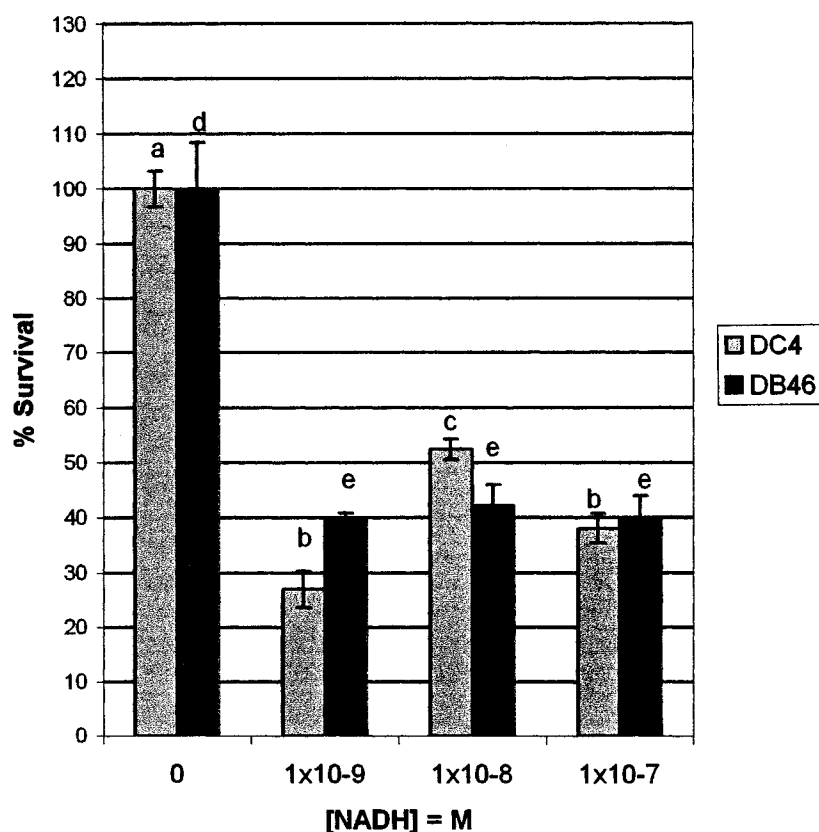


FIG. 13. NADH toxicity observed in DC4 and DB46 cell lines. NADH treatments result in cell death in DC4 and DB46 cell lines. A dose response is not observed in either cell line. (Different letters indicate significantly different means, $p < 0.05$). The cell lines do not respond significantly different ($p > 0.05$) to NADH treatment. Two significant differences are observed. The first significant difference is between T0 (control) and the three treatments T1 ($1 \times 10^{-9} \text{M}$), T2 ($1 \times 10^{-9} \text{M}$), T3 ($1 \times 10^{-9} \text{M}$). The second significant difference is between DC4 T1 and DC4 T2. Treatment response to cell type shows two significant effects regarding NADH treatment within the DC4 cell line indicated by the letters a, b and c, but no significant effects within the cell line DB46 or overall between the cell lines.

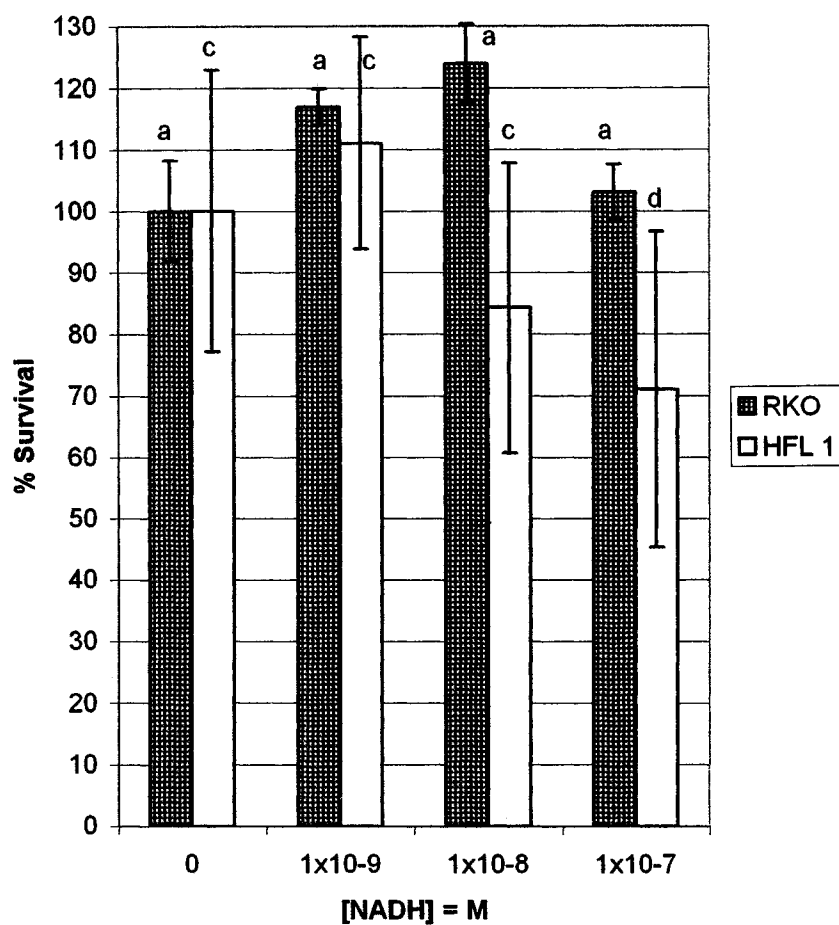


FIG. 14. NADH effects observed in RKO and HFL1 cell lines. Percent survival increases in response to NADH treatments in the RKO cell line but shows percent survival decrease in the HFL1 cell line. No significant differences ($p > 0.05$) regarding cell death are found within cell line RKO with respect to NADH treatments. A significant difference is found in HFL1 in T3 with respect to T0 ($p < 0.05$). Treatment response to cell type showed a significant difference in T3 to T1 within the HFL1 cell line. Between the cell lines treatments T1 and T2 (RKO) are significantly different from T3 (HFL1) and T2 (HFL1) is significantly different from T2 (RKO). (Large error bars are due to decreased sample size because of mold contamination).

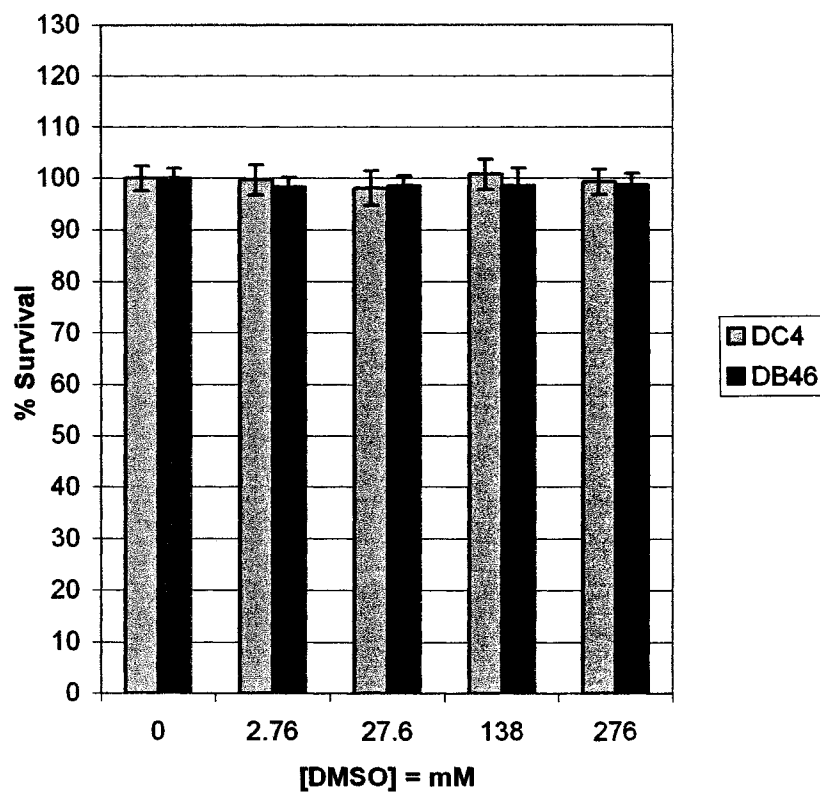


FIG. 15. Incubation of DC4 and DB46 cell lines with DMSO. DMSO treatments did not have an effect on either cell line.

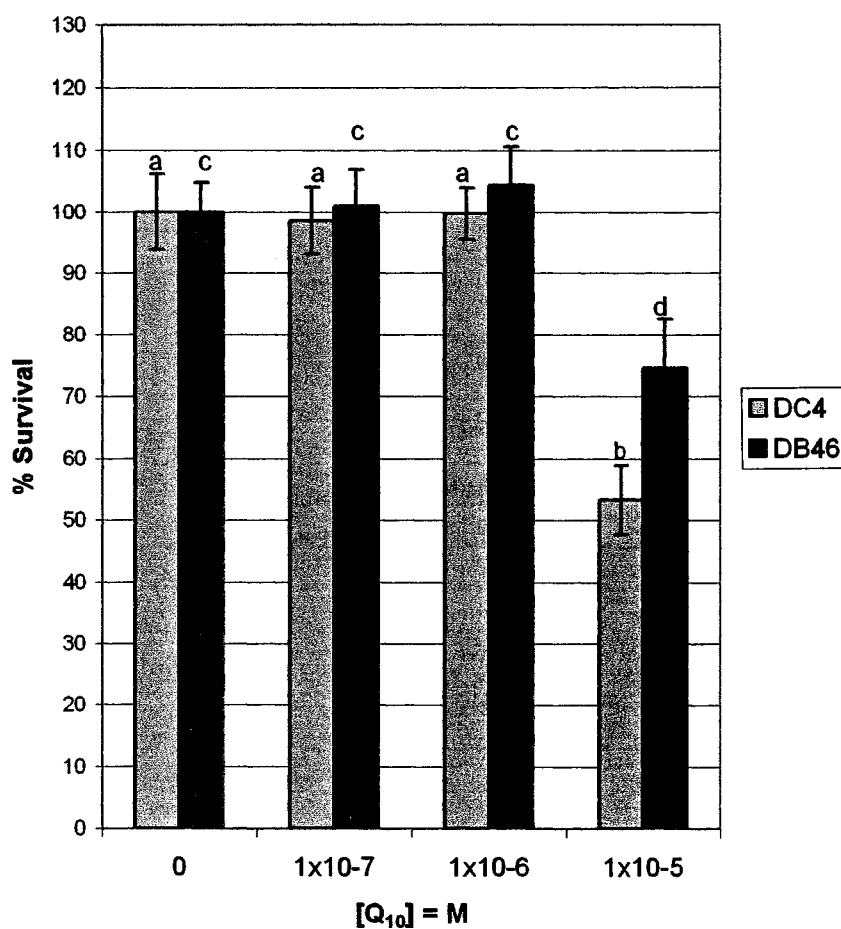


FIG. 16. Q₁₀ toxicity observed in DC4 and DB46 cell lines. Q₁₀ showed an effect only at the highest concentration in both cell lines. A dose response is not observed. (Different letters indicate significantly different means, $p < 0.05$). A significant difference in treatment ($p < 0.05$) exists between T3 ($1 \times 10^{-5} \text{M}$) and T0 (0), T1 ($1 \times 10^{-7} \text{M}$), T2 ($1 \times 10^{-6} \text{M}$). A significant difference ($p < 0.05$) exists between the cell lines at T3. Treatment response with regard to cell type indicates that no significant differences exist within or between the two cell lines except at T3.

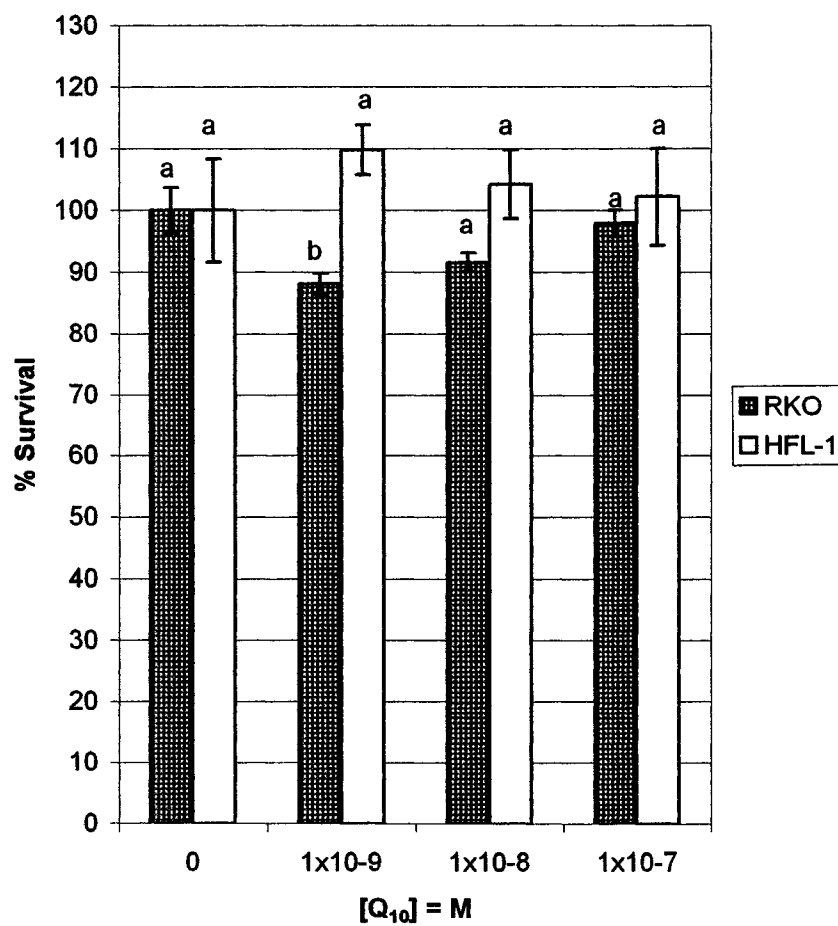
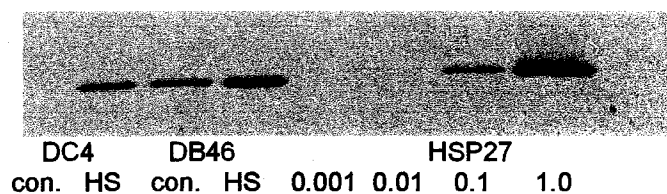


FIG. 17. Q_{10} toxicity observed in RKO and HFL1 cell lines. Q_{10} has no effect on HFL1. RKO is sensitive at the lowest concentration only. No significant treatment effects ($p > 0.05$) are found in either cell line. No significant differences ($p > 0.05$) exist between the two cell lines with regard to treatment response and no significant differences exist with regard to treatment and cell type within or between cell types.

A



B

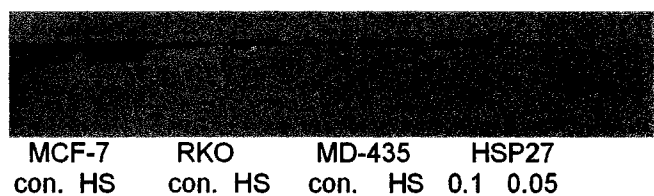


Fig. 18. HSP27 induction by heat shock (HS) observed in cell lines. *A*, breast cancer cell lines DC4 and DB46 express higher levels of HSP27 after heat shock. HSP27 expression (0.001-1.0 μg) is seen at 0.1 μg and 1.0 μg. HS DC4 shows the same HSP27 expression as control (con.) DB46. HS DB46 shows about a 4-fold increase of HSP27 expression after (HS). *B*, MCF-7 expression of HSP27 does not change after HS. RKO HSP27 expression is reduced after HS. MDA-MB-435 (MD-435) shows about a 3-fold increase of HSP27 expression following HS. HSP27 (0.1 and 0.05 μg) expression is seen at 0.1 μg.

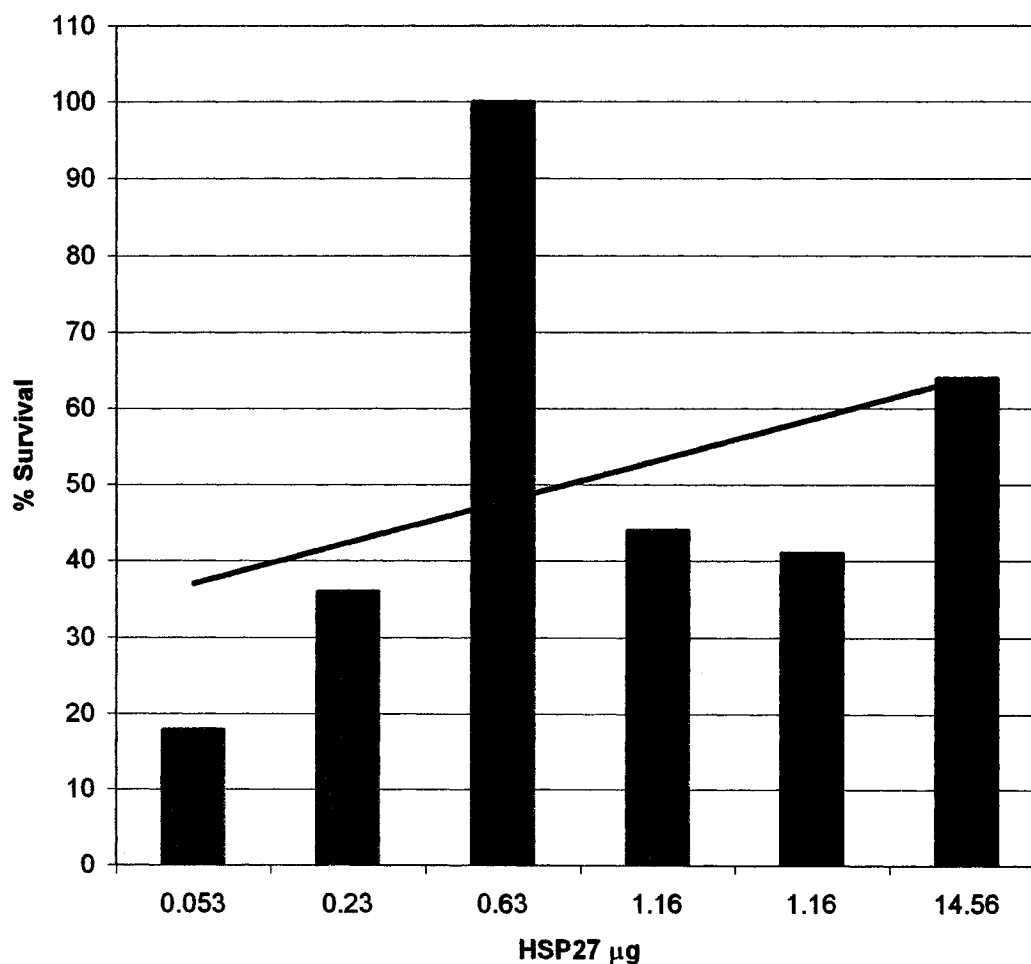


Fig. 19. Cytochrome c toxicity and percent survival relative to HSP27 expression. This graph shows quantification numbers relative to 0.1 μg of HSP27 as seen in Fig. 18 A and B (p. 65). The values correspond to the following cell lines: DC4 - 0.053, MDA-MB-435 - 0.23, RKO - 0.63, HS DC4 - 1.16, DB46 - 1.16 and MCF-7 - 14.56. A trend line shows that the more HSP27 expression is present in a breast cancer cell line, the more protection is provided against cytochrome c toxicity. What mechanism of protection the RKO cell line (colon cancer) has against cytochrome c toxicity is not clear. The level of HSP27 is not enough to provide as much protection as is observed. The percent survivals are at T3 from Table IV, p. 49.

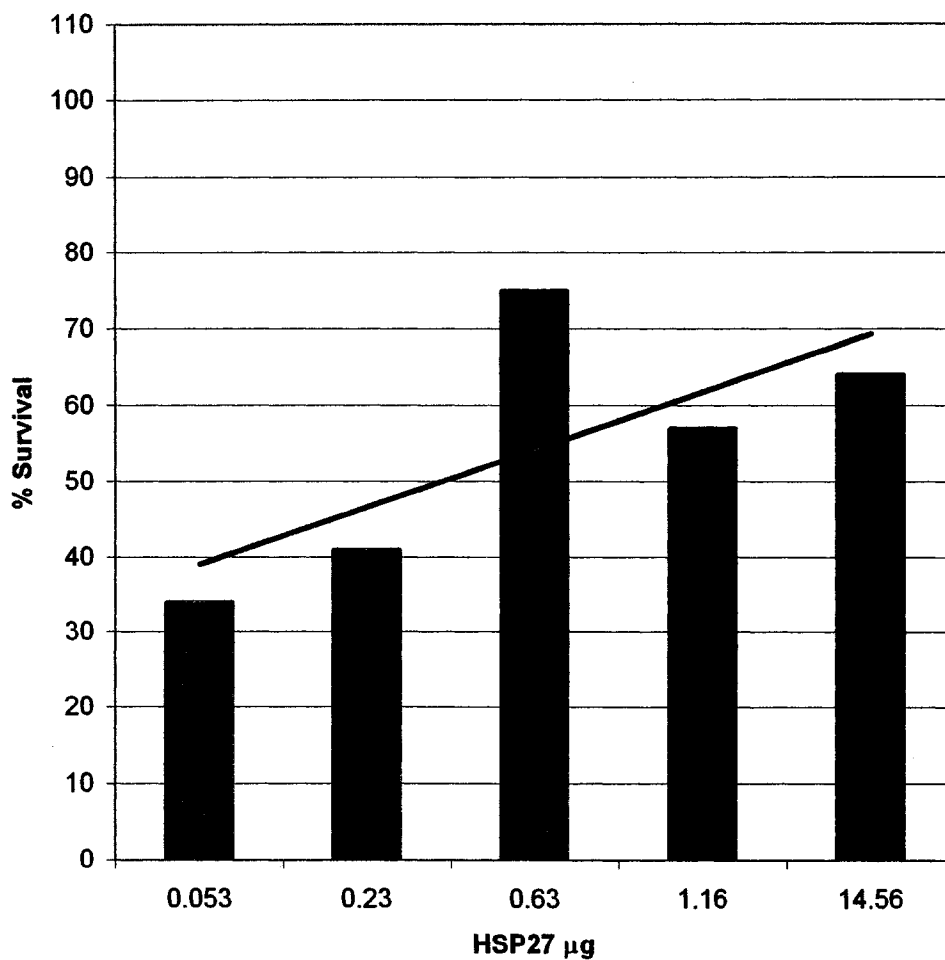


Fig. 20. FAD toxicity and percent survival relative to levels of HSP27 expression. This graph shows quantification numbers relative to 0.1 μg of HSP27 as seen in Fig. 18 A and B (p. 65). The values correspond to the following cell lines: DC4 - 0.53, MDA-MB-435 - 0.23, RKO - 0.63, DB46 - 1.16 and MCF-7 - 14.56. A trend line shows that the more HSP27 expression is present in a breast cancer cell line, the more protection is provided against FAD toxicity. What mechanism of protection against FAD toxicity is present in the RKO cell line is not clear. The level of HSP27 is not enough to provide as much protection as is observed. The percent survivals are at T3 from Table IV, p. 49.

CHAPTER 4

DISCUSSION

Previous research has given evidence that HSP27 binds to cytochrome c (5). This binding prevents the formation of the apoptosome and thus inhibits apoptosis. The goal of this thesis is to investigate treatment effects of exogenous addition of cytochrome c to cells in various concentrations. Can cytochrome c overcome the protection that HSP27 provides against cell death and therefore reduce cell proliferation? Given that cytochrome c is an electron carrier involved in the mitochondrial electron transport chain, can other electron carriers and components of this mitochondrial electron transport chain, overcome this protection provided by HSP27 as well? How do different cell lines respond to these treatment agents? Does HSP27 protect breast cancer cell lines more than other cell lines against different treatment agents? To answer these questions the following experiments were conducted and the results of these experiments are discussed in the following paragraphs.

Cytochrome c

Cytochrome c toxicity was investigated using the cell lines DC4, DB46, MCF-7, MDA-MB-435, RKO and HFL1. The first experiment was set up with DC4, HS DC4 (heat shocked DC4) and DB46 (Fig. 4, p. 51). DC4 was heat shocked, which results in the induction of many heat shock proteins that are involved in cell rescue, including

HSP27. The elevated expression of HSP27 in HS DC4 was confirmed by Western blotting (Fig. 18 A, p. 65). Cytochrome c treatments were applied in increasing concentrations ($T_0 = 0$, $T_1 = 5 \times 10^{-7}$ M, $T_2 = 5 \times 10^{-6}$ M, $T_3 = 5 \times 10^{-5}$ M) and a dose response was observed. The results show that HS DC4 responds similar to DB46 in cell survival rate, which is explained by the elevated expression of HSP27. Observed total cell survival at T3 is 41% and 44%, respectively. DC4 cell survival is 18% at T3. This result shows that DC4 is not able to tolerate cytochrome c toxicity as well as HS DC4 or DB46. The reduced survival rate can be explained by the low basal levels of HSP27, which are not enough to protect the cells from the toxic effects of cytochrome c (refer to quantification results in Chapter 3, p. 38). The low basal levels of HSP27 in DC4 control (con.) were also confirmed with Western blotting (Fig. 18 A, p. 65). A statistical analysis of the increasing concentrations of cytochrome c was performed. A significant effect is seen in cytochrome c treatments ($p < 0.0001$) on mean cell survival across each of the cell lines DC4, HS DC4 and DB46. Elevated levels of HSP27, as confirmed by Western blotting (Fig. 18 A, p. 65), are able to protect against the cytotoxic effects of cytochrome c. This protection gradually declines with increasing concentrations of cytochrome c with the least amount of protection by HSP27 against cell death observed at the highest concentration applied in T3. Statistical analysis shows that there are no significant differences ($p > 0.05$) between T0 and T1; however, significant differences ($p < 0.05$) exist between T2 with respect to T0, T1 and T3. Significant differences also exist between T3 with respect to T0, T1 and T2. Additionally, statistical analysis shows that the cell lines DC4 and DB46 do respond significantly different to cytochrome c treatments, thus confirming the protection that HSP27 provides in DB46. In summary, it can be said that

increasing concentrations of cytochrome c induce more cell death in these two breast cancer cell lines with DB46 displaying more protection against cell death than DC4 because of its elevated levels of HSP27.

The next cell lines exposed to exogenous cytochrome c were MCF-7 and MDA-MB-435. These two cell lines also responded in a dose dependent manner (Fig. 5, p. 52). MCF-7 shows more resistance to cytochrome c treatment than MDA-MB-435. At the highest concentration, T3, MCF-7 has 65% cell survival, which is about 30% more than the MDA-MB-435 cell line at that concentration. Western blotting (Fig. 18 B, p. 65) showed that MCF-7 had much more HSP27 expression than MDA-MB-435 (refer to Chapter 3, p. 38, for quantification numbers), which resulted in more protection from the cytotoxic effects of cytochrome c. Statistical analysis ($p < 0.0001$) also confirmed a significant effect of cytochrome c treatment regarding mean cell survivals in these two cell lines. The cell lines showed similar patterns to the DC4 and DB46 cell lines. Again no significant differences ($p > 0.05$) are found between T0 and T1 but T2 is significantly different from T3 ($p < 0.05$). T2 and T3 are significantly different from T0 and T1 as well. The cell types, MCF-7 and MDA-MB-435, are not significantly different and the treatment to cell type response shows that T3 had the most significant effect on both cell lines. HSP27 was able to give more protection against cell death in the cell line that expressed higher levels of this protein, namely MCF-7.

RKO was resistant to all cytochrome c concentrations (T0 = 0, T1 = 5×10^{-8} M, T2 = 5×10^{-7} M and T3 = 5×10^{-6} M) with 100% survival at all concentrations. HFL1 was sensitive to cytochrome c only at the highest concentration (T3) with 55% cell survival (Fig. 6, p. 53). Western blotting showed that RKO does not have a significant expression

of HSP27. It is not clear if other HSPs such as HSP100 or HSP70 play a role in cell survival in this cell line. What protects RKO cells against cytochrome c toxicity it not clear. It is not clear why only the highest dose of cytochrome c had an effect on the HFL1 cell line. A statistical analysis ($p=0.2566$) shows that there are no significant differences across the mean cell survivals of both cell lines. Furthermore it was found that the cell lines are not significantly different ($p>0.05$) and the treatment to cell type response is only significant with respect to T3 in the cell line HFL1.

To summarize the above results it was found that cytochrome c is toxic. Breast cancer cells lines appear to be more sensitive to cytochrome c treatments than the other cell lines used in the experiments. This sensitivity may be related to HSP27 levels at least in breast cancer cell lines. HSP27 provides protection against cytochrome c toxicity and increasing cytochrome c concentrations can reduce the protection that HSP27 provides.

FAD

FAD is involved in the mitochondrial electron transport chain and therefore was investigated for toxicity in DC4 & DB46 (Fig. 7, p. 54) as well as in the other four cell lines. The concentrations in the following treatments were $T_0 = 0$, $T_1 = 1 \times 10^{-9}$ M, $T_2 = 1 \times 10^{-8}$ M and $T_3 = 1 \times 10^{-7}$ M. At the highest concentration, T3, DB46 has a 57% cell survival, which is about 27% more than the DC4 cell line. The cytochrome c experiment showed that with increasing concentrations this electron carrier can overcome the protection that HSP27 provides against cell death. The same observation is made in the FAD experiments. DC4 has the least protection against the cytotoxic effects of FAD,

which again can be explained by the low levels of HSP27 in this cell line. Statistical analysis ($p < 0.0001$) shows high significance to mean cell survival regarding FAD treatments. The main effect ($p < 0.05$) is seen in T1, T2 and T3. All three treatments are significantly different from T0. T2 is significantly different from T3 but not from T1. The cell types show significant differences ($p < 0.05$) but treatment to cell type response shows significant differences exist between the cell lines and not within the cell lines. Thus the elevated levels of HSP27 in DB46 account for the difference between the cell lines. Because of the elevated levels of HSP27 in DB46 and the low levels of HSP27 in DC4, the three treatments had about the same effects within each cell line but the cell lines each responded differently to treatment.

Given that FAD was this toxic at small concentrations, it was of interest to see what the effects of treatments at the same concentrations as cytochrome c ($T0 = 0$, $T1 = 5 \times 10^{-7}$ M, $T2 = 5 \times 10^{-6}$ M, $T3 = 5 \times 10^{-5}$ M) are (Fig. 8, p. 55). Results show that these treatments overwhelm the protection HSP27 can provide. A dose response is observed. DC4 shows about 14% and DB46 about 17% cell survival at T1. The highest concentration shows no survival in the DC4 cell line and the DB46 cell line shows only 0.33% cell survival. Statistical analysis ($p < 0.0001$) confirmed that there is a highly significant effect of FAD treatment on mean cell survivals averaging across both cell lines and treatment effects are significantly ($p < 0.05$) different at increasing concentrations. This confirmed that all three treatments have significant effects on cell survivals.

MCF-7 and MDA-MB-435 also showed a response to this electron carrier (Fig. 9, p.56). The concentrations of FAD were the same as in the first FAD experiment ($T0 = 0$, $T1 = 1 \times 10^{-9}$ M, $T2 = 1 \times 10^{-8}$ M, $T3 = 1 \times 10^{-7}$ M) and at the highest concentration, T3,

MCF-7 had 64% cell survival, which was about 25% more cell survival than what was observed for MDA-MB-435. It can be speculated that HSP27 may have provided the protection against cell death in MCF-7. Statistical analysis ($p < 0.0001$) shows significance across the mean cell survivals of these two cell lines. Further analysis of cell type ($p < 0.05$) shows the cell types to be significantly different. Treatment analysis ($p < 0.05$) in the MCF-7 cell line shows a significant difference between the three treatments (T1, T2, T3) with respect to T0. In this cell line T1 and T2 are not significantly different from one another but T3 is significantly different from T1. In the MDA-MB-435 cell line T2 is not significantly different from T1 but T3 is significantly different from T1. Thus treatment to cell type ($p < 0.05$) shows that there are significant between the cell lines and significant treatment effect depends on the cell line.

FAD was observed to have an effect on RKO and HFL1 cell lines (Fig. 10, p. 57). Cell survival was 75% and 61%, respectively. FAD shows a significant effect ($p < 0.0001$) in these two cell lines on mean cell survival. Treatment analysis ($p < 0.05$) shows the treatments (T1, T2, T3) not to be significantly different from one another. The cell types are significantly different ($p < 0.05$) and treatment to cell type shows the treatments to be significantly different between the cell types not within the cell types and both cell lines show a similar pattern to FAD treatment.

To summarize the results, FAD is cytotoxic to all cell lines tested. HSP27 is able to provide some protection to breast cancer cell lines. FAD is more cytotoxic than cytochrome c. The cytotoxic effect of FAD is novel since we have not found any studies that have reported FAD cell toxicity.

ATP

The unexpected results of FAD toxicity lead to the investigation of ATP toxicity. It has been shown that many different cell types experience cell death after prolonged exposure to 1 - 5 mM concentrations of extracellular ATP (48). The plasma membrane receptor P2X was found to be involved in this process (48). It is not known if stimulation of this receptor causes apoptosis or necrosis (48). Another discovery was that ATP can elicit a biphasic dose response (49). It was shown that at 800 μ M, ATP is cytotoxic in a dose dependent manner, but between 800 - 2500 μ M cell count increases markedly despite higher ATP concentrations (49). It is stated that the intracellular pathways are not well known and it appears that the perturbation in intracellular ion homeostasis plays a major role (48). Given that FAD and ATP both have an attached AMP moiety lead to the reasoning, that perhaps, FAD was utilizing an extracellular ATP receptor. In this experiment, DC4 and DB46 cell lines were treated with the same concentrations ($T_0 = 0$, $T_1 = 1 \times 10^{-9}$ M, $T_2 = 1 \times 10^{-8}$ M, $T_3 = 1 \times 10^{-7}$ M) as in the FAD experiments except ATP/MgCl₂ was used (Fig. 12, p. 59). As mentioned in Chapter 3 (Results) a control experiment was set up with MgCl₂ to verify that it did not have any effect on these two cell lines because ATP has to have MgCl₂ so the reaction can go forward. The experiment (Fig. 11, p. 58) showed that this was the case. The following experiment, which involved ATP in solution with MgCl₂ did not show a dose response to ATP. The highest concentration (T_3) showed a cell survival of 52% in DC4 and 63% in DB46. DC4 showed 50% cell survival at T_1 and 55% at T_2 whereas DB46 showed cell survival of 52% at T_1 and 61% survival at T_2 . Statistical analysis ($p < 0.0015$) showed that the three treatments are significantly different from T_0 , the cell lines are not ($p > 0.05$)

significantly different from one another with regard to mean cell survival and treatment to cell type response shows that the results are non-significantly different.

In summary, we did not observe any biphasic results, which seems to be due to the much smaller concentrations that we used compared to what was listed in the literature above. We did find that ATP is cytotoxic to the breast cancer cell lines tested. ATP is as cytotoxic as FAD at low doses. HSP27 could provide some protection against ATP cytotoxicity. ATP and FAD both have an AMP moiety and display about the same cytotoxic effects on cell on breast cancer cell lines; however ATP is not an electron carrier, hence there must be a common mechanism through which ATP and FAD are able to induce cell death.

NADH

The next electron carrier involved in the mitochondrial electron transport chain with an attached AMP moiety is NADH. For the following experiments, concentrations were held the same as in the FAD and ATP experiments ($T_0 = 0$, $T_1 = 1 \times 10^{-9}$ M, $T_2 = 1 \times 10^{-8}$ M, $T_3 = 1 \times 10^{-7}$ M). The exogenous addition of NADH to cell lines DC4 and DB46 resulted in cell death (Fig. 13, p. 60). It is of interest that the lowest concentration, T_1 , also yielded the lowest cell survival of 27%. The next concentration, T_2 , almost doubled the cell survival to 52% and the highest concentration, T_3 , resulted in survival reduction to 38%. DB46 had 40% cell survival at T_1 , 42% cell survival at T_2 and 40% cell survival at T_3 . Increasing NADH concentrations could not influence the survival rates in DB46. Statistical analysis shows an effect of NADH ($p < 0.0001$) on the mean cell survival of the two cell lines. Treatment analysis ($p < 0.05$) shows the three treatments not

to be significantly different from one another but to be significantly different from T0. The cell lines are not significantly different ($p>0.05$) and treatment to cell type analysis shows no significant differences within or between the cell lines with the exception of the relation of T0 to the three treatments.

RKO and HFL1 were treated with the same concentrations of NADH (Fig. 14, p. 61) as the two breast cancer cell lines. RKO responded to NADH concentrations with an increase of cell proliferation at T1 to 117% and T2 to 124% and down at T3 to 103%. A non-significant dose response was observed for HFL1 where cell survival was observed from T0 - 100%, T1 - 111%, T2 - 84% to T3 - 71% as concentrations increased. Statistical analysis shows that there is no significant effect ($p=0.2313$) of NADH treatment on the two cell lines. Treatment analysis shows that the only significance is between T1 and T3, cell type analysis shows that cell types to be significantly different ($p<0.05$) but treatment to cell type response shows that even though the cell types are significantly different the treatments do not have any significant effects within the RKO cell line with respect to cell death induction, significance is only observed between cell lines RKO and HFL1 with regard to T2 and T3, and as mentioned earlier within the HFL1 cell line only between T1 and T3.

To summarize the results of the NADH experiments, NADH is cytotoxic in the breast cancer cell lines tested. NADH is more cytotoxic at T1 than FAD or ATP. HSP27 is not able to provide as much protection against cell death as it did in the FAD and ATP experiments. NADH like FAD and ATP has an attached AMP moiety. Observing that all three molecules, FAD, NADH and ATP, have about the same cytotoxic effects in breast cancer cell lines suggests for a common mechanism through which they reduce

cell proliferation exists. We have not found any studies that have investigated NADH cell toxicity.

Q₁₀

Observing that the three electron carriers that are involved in the respiratory chain effect cell survival, the fourth electron carrier that is part of this chain remained to be investigated as well.

Since Q₁₀ had to be dissolved in DMSO (Chapter 2, p. 33), DMSO was set up as a control to show that it was not able to induce cell death (Fig. 15, p. 62). The results of the experiment were as expected. DMSO did not induce cell death. With this confirmation, Q₁₀ was added exogenously to cell lines DC4 and DB46 in the following increasing concentrations: T0 = 0, T1 = 1x10⁻⁷ M, T2 = 1x10⁻⁶ M and T3 = 1x10⁻⁵ M (Fig. 16, p. 63). Only the highest concentration, T3, was observed to have an effect on DC4 and DB46 with cell survival being 53% and 74% respectively. Statistical analysis (p<0.0017) shows significant treatment effects of Q₁₀ with regard to T3. Analysis of cell type showed that the cell lines are not significantly different (p>0.05) and treatment to cell type analysis shows that even though the cell lines are not significantly different, T3 with Q₁₀ is significant between and within the cell lines.

The RKO and HFL1 cell lines were also checked for Q₁₀ toxicity (Fig. 17, p. 64). The concentrations, T1 = 1x10⁻⁹ M, T2 = 1x10⁻⁸ M and T3 = 1x10⁻⁷ M, were not able to induce any significant cell death in RKO. At T1 - 88% cell, T2 - 92% and T3 - 98% cell survival was observed. These results indicate that RKO was resistant to incubation with Q₁₀. HFL1 showed resistance to all concentrations. Statistical analysis (p>0.9972)

shows that no significant effects of Q₁₀ treatment were observed, the cell types are not significantly different ($p > 0.05$) and effects of treatment to cell type are insignificant.

To summarize the above results, it appears that Q₁₀ is the least effective of the four electron carriers of the mitochondrial electron transport chain to induce cell death in breast cancer cells as well as other cell lines tested. One of the reasons may be that it was challenging to get Q₁₀ into solution with DMSO. As mentioned in Chapter 2 (Materials and Methods, p. 26), a 15 ml centrifuge tube containing Q₁₀ in solution with DMSO was submerged halfway into beaker containing hot water. It may be that this heating denatured the electron carrier and therefore it was not as effective. This; however, does not explain the sudden drop in cell survival at T3 in DC4 and DB46. HSP27 provides protection against the cytotoxic effects of the electron carrier. Unlike the other electron carriers, Q₁₀ does not have an attached AMP moiety. More experiments need to be performed with this electron carrier.

Hypotheses

The first hypothesis in Chapter 1 was found to be true. Components of the electron transport chain, cytochrome c, FAD, ATP and NADH were able to reduce cell proliferation in human cancer and “normal” cell lines. However, not all cell lines responded the same way. The second hypothesis was found to be true only in breast cancer cell lines that either overexpressed HSP27 or could have thermal induction thereby increasing expression of HSP27. In the HS DC4, DB46, MCF-7 and MDA-MB-

435 cell lines HSP27 was able to give protection against the toxic effects of these electron carriers.

Table V (p.81) gives a summary of the results.

Concluding Remarks

Of all tested electron carriers in these experiments it was observed that exogenous cytochrome c (Figure 4, p. 51) and FAD (Fig. 8, p. 55) were the only two electron carriers that were able to elicit a dose response at increasing concentrations in breast cancer cells. FAD (Fig. 7, p. 54), at lower concentrations showed a step response. What these experiments did not show was whether FAD induces apoptosis or necrosis and how or if it enters the cell.

To conclude the cytochrome c experiments, we observed a dose response in breast cancer cell lines following treatment with exogenous cytochrome c as the concentrations of exogenous cytochrome c were increased. Cytochrome c is shown to be cytotoxic in cell lines that constitutively express HSP27 or express elevated levels of HSP27 following heat shock (Figures 4, 5, pp. 51, 52). What these experiments did not show was how or if exogenous cytochrome c enters the cell. If cytochrome c does not get into the cell, is there an extracellular receptor that this electron carrier binds to, donates electrons to and thereby initiates a pathway leading to cell death? Is this cell death then apoptosis or necrosis?

Future Work

It would be of interest to determine whether necrosis or apoptosis was induced in the

performed experiments. There are several ways to identify apoptotic events in a cell. A flow cytometer can do a light scatter analysis. This application takes advantage of the refractive index. The refractive index of the internal cytoplasm of a dying cell becomes more similar to that of the extracellular medium. The addition of propidium iodide can identify those cells that have become permeable. A cell membrane analysis can be performed with a flow cytometer. Under normal circumstances phosphatidylserine (PS) residues are located in the inner membrane of the cytoplasmic membrane. These residues are translocated in the membrane and are externalized during apoptosis. Annexin -V is a specific PS-binding protein that can be conjugated to fluorochromes. A fluorochrome can be combined with propidium iodide to identify dead cells. Nucleases are activated during apoptosis that degrade DNA. One way to detect these DNA fragments would be to use strand break labeling (TUNEL). TUNEL stands for Terminal deoxynucleotidyl transferase mediated dUTP Nick End Labelling. An enzyme is used to add dUTPs to the broken ends of DNA, which can then be detected by antibodies with fluorochrome labels. Staining with propidium iodide makes it possible to tell during which phase of the cell cycle the cells are exhibiting strand breaks.

It is not understood how exogenous cytochrome c treatments induce cell death. Therefore, determination of where exogenous cytochrome c binds outside of the cell or if exogenous cytochrome c binds to HSP27 could be answered by using fluorescent tagging.

Table V

*Summary of electron transport chain components, their effects and HSP27 protection
observed*

Cell Line	e ⁻ Carrier	Cell Death Induced	HSP27 Protection
DC4	Cytochrome c	Yes	No
	FAD	Yes	No
	ATP	Yes	No
	NADH	Yes	No
	Q ₁₀	Yes	No
DB46	Cytochrome c	Yes	Yes
	FAD	Yes	Yes
	ATP	Yes	a
	NADH	Yes	a
	Q ₁₀	Yes	Yes
MCF-7	Cytochrome c	Yes	Yes
	FAD	Yes	Yes
	ATP	N/A	N/A
	NADH	N/A	N/A
	Q ₁₀	N/A	N/A
MDA-MB-435	Cytochrome c	Yes	b
	FAD	Yes	b
	ATP	N/A	N/A
	NADH	N/A	N/A
	Q ₁₀	N/A	N/A

N/A = not applicable

a = no protection observed at dose applied

b = results are consistent with HSP27 protection but does not prove that other factors may
not have contributed to cell survival

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