UNIVERSITÉ DU QUÉBEC À MONTRÉAL

# MÉCANISMES MOLÉCULAIRES DE L'INDUCTION DE L'APOPTOSE PAR LE CHOC THERMIQUE CHEZ LES CELLULES DE L'OVAIRE DU HAMSTER CHINOIS SUREXPRIMANT LA P-GLYCOPROTÉINE

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# MOLECULAR MECHANISMS OF APOPTOSIS ACTIVATION BY HEAT SHOCK IN MULTIDRUG-RESISTANT CHINESE HAMSTER CELLS OVEREXPRESSING P-GLYCOPROTEIN

THESIS

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## DEDICATION

*The real voyage of discovery lies not in seeking new landscapes,*
*but in having new eyes*

To my parents, Grazyna and Wlodzimierz Wrzal. The most courageous and selfless individuals I will ever know. With infinite patience, they have taught me to face life's challenges with my chin up and when I faltered they provided a solid foundation to catch my balance and press on.

To my sisters, Marta and Barbara. Their love, laughter and support are what lights the way for each new adventure I embark on.

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# LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| **ABC** | : | ATP-binding cassette |
| **Ac** | : | Acetyl-L |
| **Ac-DEVD-AMC** | : | Ac-Asp-Glu-Val-Asp-AMC |
| **Ac-LEHD-AFC** | : | Ac-Leu-Glu-His-Asp-AFC |
| **Ac-VEID-AMC** | : | Ac-Val-Glu-Ile-Asp-AMC |
| **AFC** | : | 7-Amino-4-trifluoromethyl coumarin |
| **AIF** | : | Apoptosis inducing factor |
| **Akt** | : | Protein tyrosine kinase |
| **AMC** | : | 7-Amino-4-methyl coumarin |
| **AMP** | : | Adenosine monophosphate |
| **AP-1** | : | Activator protein 1 |
| **Apaf** | : | Apoptosis protease activating factor |
| **ASK** | : | Apoptosis stimulating kinase |
| **ATF** | : | Activating transcription factor |
| **ATP** | : | Adenosine triphosphate |
| **Bax** | : | Bcl-2 associated X protein |
| **Bcl** | : | B-cell lymphoma |
| **BCNU** | : | 1,3-bis(2-chloroethyl)-1-nitrosourea |
| **BH** | : | Bcl-2 homology |
| **Bid** | : | BH-3-interacting-domain death agonist |
| **Bip** | : | Luminal binding protein precursor |
| **BSA** | : | Bovine serum albumin |
| **CAD** | : | Caspase-activated DNase/DFF40 |
| **CAPK** | : | Ceramide-activated protein kinase |
| **CARD** | : | Caspase recruitment domain |
| **Caspase** | : | Cysteinyl aspartate protease |
| **CD** | : | Cell death |
| **Ced** | : | "Cænorhabdtidis elegans death". |

| | | |
|---|---|---|
| **cFLIP** | : | Cellular Fas-associated DD-like interleukin (IL)-1-converting enzyme inhibitory protein |
| **CHAPS** | : | 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate |
| **CHO** | : | Ovary hamster cell |
| **cMOAT** | : | Cannalicular multispecific organic anion transporter |
| **CREB** | : | Cyclic AMP-responsive element binding protein |
| **dATP** | : | Deoxyadenosine triphosphate |
| **DAXX** | : | Death associated protein |
| **DD** | : | Death domain |
| **DED** | : | Death effector domain |
| **DIABLO** | : | Direct IAP-binding protein with low pI |
| **DISC** | : | Death-inducing signal complex |
| **DMSO** | : | Dimethyl sulfoxide |
| **DNA** | : | Deoxyribonucleic acid |
| **DNA-PK** | : | DNA-dependent protein kinase |
| **DOX** | : | Doxorubicin |
| **DR** | : | Death receptor |
| **DRM** | : | Detergent resistant membrane |
| **DTT** | : | Dithiothreitol |
| **DVED-AMC** | : | Asp-Glu-val-Asp peptide bound to 7-amino-4-methyl-coumarin |
| **EDTA** | : | Ethylenediaminetetraacetic acid |
| **EGF** | : | Epidermal growth factor |
| **EGTA** | : | Ethyleneglycotetraacetic acid |
| **ER** | : | Endoplasmic reticulum |
| **ERK** | : | Extra cellular regulated kinase |
| **FAD** | : | Flavine adenine dinucleotide. |
| **FADD** | : | Fas-associated death domain |
| **FasL** | : | Fas ligand |
| **FBS** | : | Fetal bovine serum |
| **FCCP** | : | p-(trifluoro-methoxy) phenylhydrazone |
| **Gadd** | : | Growth arrest- and DNA damage-inducible gene |

| | | |
|---|---|---|
| **GRP** | : | Glucose-regulated protein |
| **GSH** | : | Gluthation (reduced form) |
| **GST** | : | Glutathione S-transferase |
| **$H_2O_2$** | : | Hydrogen peroxide |
| **HBV** | : | Hepatitis B virus |
| **HCl** | : | Hydrochloric acid |
| **HCV** | : | Hepatitis C virus |
| **HEPES** | : | N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid |
| **HRP** | : | Horseradish peroxide |
| **HSE** | : | Heat shock element |
| **HSF** | : | Heat shock factor |
| **HSPs** | : | Heat shock proteins |
| **HtrA2** | : | High temperature requirement protein A2 |
| **IAP** | : | Inhibitor of apoptosis protein |
| **ICAD** | : | Inhibitor of caspase-activated deoxyribonuclease |
| **ICE** | : | Interleukine converting enzyme |
| **IGFs** | : | Insulin-like growth factor |
| **IKK** | : | I-kappa B kinase |
| **IRE** | : | Inositol requiring enzyme |
| **JNK** | : | C-jun $NH_2$-terminal protein kinase |
| **kDa** | : | Kilodalton |
| **L-PAM** | : | L-phenylalanine mustard |
| **Mab** | : | Monoclonal antibody |
| **MADD** | : | Mitogen-activated kinase activating death domain protein |
| **MAPK** | : | Mitogen-activated protein kinase/ERK |
| **MAPKAK** | : | MAPK-activated protein kinase |
| **MAPKAP** | : | MAPK-activated protein |
| **MCA** | : | 4-Methyl-coumaryl-7-amide |
| **MCA-VDQVDGWK (DNP)-$NH_2$** | : | MCA-Val-Asp-Gln-Val-Asp-Gly-Trp-Lys-(2,4-dinitrophenyl)-L-Lys-amide |
| **MDR** | : | Multi-drug resistance |
| **MEK** | : | MAP/ERK kinase |

| | | |
|---|---|---|
| **MEKK** | : | MAPK/ERK kinase kinase |
| **MKK** | : | MAPK kinase |
| **MKKK** | : | MAPK kinase kinases |
| **MLK** | : | Mixed-lineage kinases |
| **MOPS** | : | 3-(N-Morpholino)propanesulfonic acid |
| **MRP** | : | Multi-drug resistance protein |
| **MSK** | : | Mitogen- and stress-activated protein kinase |
| **Mst** | : | Mammalian Ste20-like kinases |
| **NF-κB** | : | Nuclear factor kappa B |
| **NFAT** | : | Nuclear factor of activated T-cells |
| **NHEJ** | : | Non-homologous DNA end-joining |
| **NuMA** | : | Nuclear mitotic apparatus protein |
| **Omi** | : | Oocyte maturating factor |
| **PAK** | : | p21-activated kinase |
| **PARP** | : | Poly(ADP-ribose) polymerase |
| **PBS** | : | Phosphate buffered saline |
| **PERK** | : | Double-stranded RNA-activated protein kinase-like ER kinase |
| **P-GP** | : | P-glycoprotein |
| **PIPES** | : | Piperazine-N,N'-bis(2-ethanesulfonic acid) |
| **PKB** | : | Protein kinase B |
| **PKC** | : | Protein kinase C |
| **PMA** | : | Phorbol 12-myristate 13-acetate |
| **PMSF** | : | Phenylmethylsulfonyl fluoride |
| **PTP** | : | Permeability trasition pore |
| **PVDF** | : | Polyvinylidene difluoride |
| **Raf 1** | : | v-raf-1 murine leukemia viral oncogene homolog 1 |
| **RIP** | : | Receptor interacting protein |
| **RNA** | : | Ribonucleic acid |
| **ROCKI** | : | Rho-associated coiled coil forming kinase I |
| **ROS** | : | Reactive oxygen species |
| **RT-PCR** | | Reverse transcription polymerase chain reaction |
| **SAPK** | : | Stress activated protein kinase |

| | | |
|---|---|---|
| **SDS-PAGE** | : | Sodium dodecyl sulfate-polyacrylamide gel electrophoresis |
| **SEK** | : | SAPK/ERK kinase |
| **Smac** | : | Second mitochondria-derived activator of caspases/Diablo |
| **SREBP** | : | Sterol response element binding protein |
| **STAT** | : | Signal transducers and activators of transcription proteins |
| **TAJ** | : | Toxicity and JNK inducer |
| **t-Bid** | : | Truncated BH-3-interacting-domain death agonist |
| **TBS-T** | : | Tris-buffered saline containing Tween 20 |
| **TCR** | : | T-cell receptor |
| **TGF** | : | Transforming growth factor |
| **TNF** | : | Tumor necrosis factor |
| **TNF-R** | : | Tumor necrosis factor receptor |
| **TRADD** | : | TNFR1-associated death domain |
| **TRAIL** | : | TNF-related apoptosis inducing ligand |
| **TRAIL-R** | : | TNF-related apoptosis inducing ligand receptor |
| **TROY** | : | TNFR super family expressed on the mouse embryo |
| **U1-70 kDa** | : | 70 kDa subunit of the U1 small ribonucleoprotein |
| **UPR** | : | Unfolded protein response |
| **UV** | : | Ultraviolet light |
| **VDAC** | : | voltage dependent anion channel |
| **Z-IETD-AFC** | : | Z-Ile-Glu-Thr-Asp-AFC |
| **α-MEM** | : | Alpha minimum essential medium |

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## RESUME

La résistance pléïotropique aux médicaments est l'un des obstacles majeurs limitant l'utilisation de la chimiothérapie comme possibilité thérapeutique anticancéreuse. Bien que non totalement élucidé, ce phénomène de résistance observé a été associé à la surexpression d'une protéine de 170kDa, appartenant à la classe des protéines de transport ABC, ATP dépendante. Il a été démontré que les cellules surexprimant la MDR développent également un phénomène de résistance à l'induction de l'apoptose rendant ainsi les essais de sensibilisation des cellules résistantes aux médicaments et les recherches menées pour cette fin très avantageux et louables pour la thérapie anti-cancer.

Dans la présente étude nous avons investigué la possibilité de l'induction de l'apoptose chez des cellules de l'ovaire de hamster chinois surexprimants la protéine MDR (CHO MDR $CH^RC5$) et des cellules parentales ne développant pas la résistance pléïotropique aux médicaments (CHO AuxB1) par le choc thermique. Pour mieux comprendre ce phénomène de résistance, nous nous sommes intéressé en particulier, à la voie du récepteur de mort Fas et à la voie intrinsèque médiée par la mitochondrie. Nos résultats montrent que le choc thermique (40-43°C) active les caspases initiatrices 9 et 8 ainsi que les caspases effectrices 3, et 7. Contrairement aux cellules AuxB1, les cellules $CH^RC5$ exhibent une résistance à l'induction de la voie du récepteur de mort Fas par le choc thermique. La voie intrinsèque, quant à elle, est induite pareillement chez les deux lignées cellulaires. De plus, la translocation de Bax et du Bid tronqué (tBid) à la mitochondrie était responsable de la perméabilisation de la membrane mitochondriale et par conséquent la libération du cytochrome c et l'activation des caspases 9 et 3 chez les deux lignées cellulaires. Le clivage des substrats de la caspase 3 (ICAD et PARP) était similaire chez les deux modèles cellulaires, suggérant que les cellules MDR $CH^RC5$ sont non résistantes au choc thermique. Toutefois, cette étude est la première à investiguer les mécanismes moléculaires de l'apoptose induite par le choc thermique chez des cellules surexprimants la protéine MDR et à conjecturer le rôle de l'hyperthermie dans la lutte contre la résistance pléïotropique aux médicaments en médicine clinique.



**Mots-clefs:** Hyperthermie, Résistance pléiotropique, P-glycoproteine, Apoptose, Cellules.

## ABSTRACT

Multidrug resistance (MDR) is a major obstacle limiting the use of chemotherapy in cancer treatment. The MDR phenotype has been associated with overexpression of an ATP-dependent membrane efflux pump, P-glycoprotein. It has been suggested that MDR cells, aside from resistance to chemotherapy, might also inhibit apoptosis at a variety of levels within the death signalling pathways. Thus, reversal of resistance or sensitization of the MDR cells to chemotherapeutic drugs as well as the induction of apoptosis in MDR cells are essential parts of the search for more successful treatments of cancer.

This study investigates the induction of apoptosis in MDR CHRC5 cells in comparison to their parental, drug sensitive AuxB1 Chinese hamster ovary cells, using heat shock to sensitize the cells. We assessed the responses of MDR and drug sensitive cells to death receptor and mitochondria mediated signalling pathways of apoptosis in order to investigate possible mechanisms of resistance to heat shock induced cell death. Our findings showed that in CHRC5 cells the receptor mediated pathway appears to confer some resistance to heat shock (40-43°C) induced apoptosis with decresed levels of the Fas-associated death domain (FADD) protein, increased levels of antiapoptotic protein c-FLIP and a low level of caspase-8 activation by heat. Heat shock induced apoptosis to a similar extent in CHRC5 cells via the mitochondria mediated signalling pathway with Bax translocation to the mitochondria, mitochondrial membrane depolarization, release of cytochrome c and activation of caspase-9 and -3. Also, similar levels of cleavage of downstream substrates of caspase-3 were observed in the two cell lines suggesting that overall, MDR CHRC5 cells are not resistant to heat shock induced apoptosis. This study reveals, for the first time, the major molecular mechanisms of heat shock induced apoptosis in MDR tumor. The results imply that hyperthermia is a promising method used to irradicate MDR tumor cells in the clinic.



**Keywords:** Multidrug resistance, P-glycoprotein, Heat shock, Apoptosis, Caspases, Cell.

# CHAPTER 1: INTRODUCTION

## 1.1. CANCER

### 1.1.1. OVERVIEW

Cancer is a disease characterized by an uncontrollable proliferation of cells and the ability of those cells to invade other tissues, either by direct growth into adjacent tissues (invasion) or by migration of cells to distant sites (metastasis) (Rubin, 2001). The unregulated growth of cancer cells can be caused by a number of factors, which ultimately result in the sequential acquisition of somatic mutations in genes that control cell growth and differentiation or are involved in DNA repair. Mutations may be present in the germline, produced by environmental mutagens or arise as a consequence of normal cellular metabolism. Genes involved in cancer pathogenesis can be grouped into three categories: oncogenes, tumor suppressor genes and DNA repair genes. Oncogenes are the altered versions of normal proto-oncogenes, which regulate normal cell growth and differentiation. Tumor suppressor genes are normal genes whose products inhibit cellular proliferation. DNA repair genes function to maintain the integrity of the genome and fidelity of DNA replication. Mutations in these genes offset the mechanisms controlling cell division and repair and usually lead to the formation of a tumor (Bannasch, 1998).

A tumor refers to any abnormal mass of tissue and may be either benign (non-cancerous) or malignant (cancerous). Benign tumors do not penetrate adjacent tissue borders and do not spread or metastasize to distant sites. They also more closely resemble their tissue of origin. In contrast, malignant tumors have the added property of invading surrounding tissues and metastasizing to distant sites, where subpopulations of malignant cells take up residence, grow anew and invade again (Rubin, 2001).

### 1.1.2. CANADIAN AND QUEBEC CANCER STATISTICS

Each year since 1987, the National Cancer Institute of Canada (NCIC) publishes and interprets current statistics about cancer in Canada. These statistics are compiled through a collaboration of NCIC, Health Canada Statistics, Canada provincial/territorial cancer registries and university-based research. According to these statistics, in the year 2006, an estimated 153 100 new cases of cancer will be diagnosed in Canada: 74 700 in women and 78 400 in men; and 38 300 new cases in Quebec: 19 100 women and 19 200 men. There will be an estimated total of 70 400 cancer deaths in Canada with 19 100 of them attributed to Quebec. Across the country, breast cancer remains the leading cause of death for women with 22 200 new cases in Canada and 6 000 in Quebec, and prostate cancer remains the leading cause of cancer death for men with 20 700 new cases in Canada and 3 600 in Quebec. Lung cancer remains the leading cause of cancer death, when women and men are considered together, with colorectal cancer following in a close second. According to current incidence rates, 38 percent of Canadian women and 44 percent of men risk developing cancer during their lifetime. Approximately 2 of every 5 men and 1 in 2.6 women will develop cancer in their life, and 1 in 3.5 men and 1 in 4.3 women, or approximately 1 in 4 of all Canadians, will die of cancer. The current estimates for death due to cancer according to current mortality rates are 24 percent for women and 29 percent for men. There are 40 percent of new cases and 60 percent of deaths due to cancer occurring among those who are 70 years of age or older (Canadian Cancer Society www.cancer.ca). These statistics clearly show the importance of continuing cancer research and pushing forward to find accurate methods of early diagnosis and effective methods of treatment.

### 1.1.3. CAUSES OF CANCER

#### 1.1.3.1. CHEMICAL CARCINOGENS

The importance of chemicals as a cause of cancer has always been recognized and is emphasized by the epidemic of tobacco-related lung cancer in recent years (Martinez et al., 1995). Chemical carcinogens are considered mutagens, since they are agents that can permanently alter the genetic material of the cell. Chemicals cause cancer either directly or after metabolic activation. The direct-acting carcinogens are inherently sufficiently reactive to bind covalently to cellular macromolecules. These chemicals include organic compounds such as nitrogen mustard, benzyl chloride and certain metals like cadmium and nickel. However, the great majority of organic compounds require conversion to an ultimate, more reactive compound. This conversion is enzymatic and regulated by cellular systems involved in drug metabolism and detoxification, which are present in many of the cells in the body and particularly in liver cells (Reddy, 1992). Chemicals that belong to this group of carcinogens include aflatoxin, nitrosamines, aromatic amines and azo dyes, as well as some compounds found in tobacco smoke, like polycyclic aromatic hydrocarbons (Rubin, 2001). Other than metabolism of carcinogens, sex, hormonal status and diet all influence chemical carcinogenesis.

#### 1.1.3.2. PHYSICAL CARCINOGENS

Physical agents of carcinogenesis include ionizing radiation, ultraviolet light (UV) and asbestos. Ionizing radiation and ultraviolet radiation exhibit similar effects on cells, which include enzyme inactivation, inhibition of cell division, mutagenesis, cell death and tumorigenesis. The importance of DNA repair in protecting against the harmful effects of UV radiation can be seen in xeroderma pigmentosum, an autosomal recessive disease to which both neoplastic and non-neoplastic disorders of the skin are attributed to impairment in excision of UV radiation-damaged DNA

(Rubin, 2001). Cancers associated with exposure to asbestos fibers through inhalation are mostly an occupational hazard of heavily exposed workers, either in asbestos manufacturing, mining, installation or demolition. The latent period for developing an asbestos characteristic tumor, called malignant mesothelioma, is anytime between 20 and 40 years after exposure (Rubin, 2001). Cancers can also develop in response to a foreign object implanted in the body such as a plastic or metal film, various fibers, glass spheres and dextran polymers. However, humans appear to be resistant to foreign body carcinogenesis and few cancers arise following implants.

### 1.1.3.3. VIRUSES

The strongest association between viruses and the development of cancer in humans are: (1) the RNA retrovirus Human T-Cell Leukemia Virus (HTLV-1) and human T-cell leukemia/lymphoma, (2) the human papillomavirus (HPV) and squamous carcinoma of the cervix (Munoz and Bosch, 1996), (3) the hepatitis B and C viruses (HBV/HCV) and primary hepatocellular carcinoma (Beasley, 1988), (4) the Epstein-Barr virus (EBV) and certain forms of lymphoma and nasopharyngeal carcinoma (Magrath and Bathia, 1999), and (5) human herpesvirus 8 and Kaposi sarcoma (Landau et al., 2001). The HTLV-1 has been associated with the rare T-cell leukemias endemic in parts of Japan, Africa, the Caribbean and southeastern United States. HTLV-1 contains a genome with no known oncogene and does not integrate at specific sites within the host genome. It is analogous to other retroviruses such as the human immunodeficiency viruses (HIV)-1 and -2. The four DNA viruses, human papillomavirus (HPV), Epstein-Barr virus (EBV), hepatitis B (HBV), and herpesvirus 8 contain genes that encode proteins, which bind to specific host proteins, the products of tumor suppressor genes, involved in regulation of cell proliferation and decouple cell proliferation from inhibitory control. The transforming genes of

oncogenic DNA viruses exhibit almost no homology with cellular genes (Rubin, 2001).

### 1.1.3.4. GENETIC ALTERATIONS

As discussed earlier in section I.3., the alterations in the genetic material of the cell can result in the development of cancer. Mutations in oncogenes, tumor suppressor genes and genes responsible for DNA repair throw off the delicate balance of cell proliferation and death. Genetic alterations in oncogenes result in overproduction of a normal gene product or synthesis of an abnormally active mutant protein. Another mechanism of a genetic alteration that contributes to carcinogenesis is a mutation that creates a deficiency in a normal gene product that suppresses tumor formation; such is the case for tumor suppressor gene mutations. Among the most extensively studied tumor suppressor genes are retinoblastoma (Rb) and p53 gene products. They serve to restrain cell division in many tissues and their absence or inactivation is linked to the development of tumors (Rubin, 2001). Oncogenic DNA viruses encode products that interact with these suppressor proteins, thus inactivating their function. Genes involved in DNA repair respond to DNA damage and thus play a role in surveillance over the integrity of all genetic information. Alterations, which with time may lead to loss of function in these house-keeping genes, render DNA susceptible to the progressive accumulation of other mutations. It is important to consider hereditary syndromes such as hereditary non-polyposis colon cancer (HNPCC), which displays a heterozygous germline mutation in at least one of four genes involved in DNA mismatch repair. Another hereditary syndrome to consider is ataxia telangiectasia (AT), which involves the ATM gene whose product participates in multiple responses to DNA damage, including control of checkpoints in the cell cycle, activation of DNA repair enzymes, and regulation of apoptosis (Rubin, 2001; Stanley, 1995).

#### 1.1.3.5. TELOMERASE AND HUMAN CANCER

As cells divide, their telomeres, the tips of the chromosomes, progressively shorten. Because somatic cells do not normally express telomerase, an enzyme which maintains the length of the telomere, the telomere progressively shortens with each replication, thus acting as a molecular clock that determines the lifespan of a replicating cell. However, cancer cells express telomerase, and reactivation of this enzyme is associated with immortalization of cancer cells (Rubin, 2001; Kim et al., 1994).

### 1.1.4. THE FIGHT AGAINST CANCER

Cancer treatment varies depending on the nature and stage of the cancer, variables pertaining to the patient, as well as the goals of the treatment. The most common treatments of cancer include surgery, chemotherapy and radiation therapy. These may be employed alone or in combination. Alternative methods in cancer treatment that have emerged over the recent years include immunotherapy, gene therapy, enzymotherapy, herbal therapy and hyperthermia. A novel and promising new approach in cancer treatment is the use of angiogenic agents which act to inhibit tumor blood vessel growth.

#### 1.1.4.1. SURGERY

Surgery is the oldest form of cancer treatment. It remains the primary treatment for a variety of solid tumors such as breast, colon, head and neck, and uterine cancer. It can be used to diagnose or treat cancer. Several types of surgical procedures exist depending on the stage and type of cancer. Preventive surgery is performed to remove non-malignant tumor masses that are likely to become malignant, such as polyps in the colon. Diagnostic surgery is used to obtain a tissue

sample for analysis so a biopsy and cancer staging can be done. Curative surgery deals with the removal of a contained tumor mass. However, there are other types of surgery employed in cancer treatment. These include: cytoreductive, palliative, supportive and reconstructive surgery (Fleming et al., 2001, Pollock et al., 2003).

### 1.1.4.2. CHEMOTHERAPY

Chemotherapy is a systemic therapy, which affects the whole body by going through the bloodstream (Burke et al., 1996). The purpose of chemotherapy and other systemic treatments is to get rid of any cancer cells that may have spread from the primary tumour. Chemotherapy refers to an agent that attacks the cell directly, in an attempt to induce cell death. Chemotherapy is used to eradicate cancer, maintain long-term remission, to increase the effectiveness of surgery or radiotherapy and to help control pain and other symptoms associated with the particular cancer (Aiba, 2004; Trotice et al., 1997). Chemotherapy is an area of treatment that is constantly changing and developing. These developments include new anticancer drugs, new combinations of drugs, and "old" combinations of drugs on untested types of cancer, as well as various combination treatments involving drugs with surgery and/or radiation. Chemotherapy is most effective against cancers that divide rapidly and have good blood supply. Chemotherapy may be given as a single drug or a combination of drugs. Combinations have been developed for several reasons: different drugs attack the cancer cells in different ways; some drugs may make other drugs more potent; combinations help to avoid the problem of cancer cells becoming "immune" or resistant to a certain drug; for many kinds of cancer, a combination of drugs (each one of which is effective in attacking that kind of cancer) provides more effective cancer-kill with fewer harmful effects on healthy tissues.

Alkylating agents such as nitrogen mustards and ethylamines are the most commonly used in chemotherapy. Secondly, plant (vinca) alkaloids are anti-tumour

agents derived from plants. The best known of this category are vincristine and vinblastine (Sui and Fan, 2005). Thirdly, taxanes are groups of drugs that include paclitaxel and docetaxel (Bunn, 2000; Earhart, 1999), which are widely used to treat advanced ovarian and breast cancers (Khayat et al., 2000; Lamb and Wiseman, 1998). Finally, anti-metabolites, nitrosoureas and anti-tumour antibiotics are among the well known categories of chemotherapeutic agents. Anti-tumour antibiotics are a group of structurally unrelated antimicrobial compounds produced by streptomyces species in culture. They are cell cycle non-specific. They are distinct from the antibiotics used to treat bacterial infections. Rather, these drugs affect the structure and function of nucleic acids by intercalation between base pairs and by causing DNA strand fragmentation or DNA cross-linking. However, they lack the specificity of the antimicrobial antibiotics and thus produce significant toxicity. A number of anti-tumour antibiotics such as Adriamycin, dactinomycin, bleomycin and mithramycin are used to treat a variety of cancers.

### 1.1.4.3. RADIOTHERAPY

Radiation therapy is a highly specific and effective modality for cancer treatment and is often used after surgery. Radiotherapy is usually seen as a direct attack on cells, similar to chemotherapy. It uses high energy radiation forms, such as X-rays, gamma, neutrons as well as other sources to kill cancer cells. Radiation has its greatest effect on tissues that divide rapidly. The dose of radiation is determined by the size, extent, type and stage of tumour along with its response to radiation therapy. Calculations are performed to determine the dose and timing of radiation in treatment planning. Often, the treatment is given over several different angles in order to deliver the maximum amount of radiation to the tumour and the minimum amount to normal tissues (Perez and Brandy, 2002, Hof and Debus, 2005).

## 1.2. APOPTOSIS

### 1.2.1. OVERVIEW

Apoptosis refers to a continuous physiological process of genetically programmed cell death defined by distinct morphological and biochemical changes. Regulation of the homeostatic balance between cell proliferation and cell death is essential for development and maintenance of multi-cellular organisms. The importance of apoptosis is evident in a wide variety of different biological systems, including normal cell turnover, the immune system, embryonic development, metamorphosis, and hormone dependent atrophy, as well as in chemical-induced cell death (Arends and Wyllie, 1991; Ellis et al., 1991; Cohen et al., 1992). The regulatory mechanisms controlling programmed cell death are as fundamental, and as complex, as those regulating cell proliferation. Changes in the signalling cascades regulating apoptosis, whether by extra-cellular triggers, acquired or germ line genetic mutation, or viral mimicry of signalling molecules, can result in an array of diseases, including neurodegenerative diseases, ischemic damage, autoimmune disorders and several forms of cancer (Thompson, 1995; Nicholson, 1996). Thus, the study of the mechanisms of apoptosis is one of the most active fields of medical research today.

Apoptosis differs significantly from cell death by necrosis. Death by necrosis occurs due to a failure to control cellular homeostasis after undergoing damage (Schultz and Harrington, 2003). Morphological and biochemical characteristics of necrosis include: swelling of the cytoplasm and organelles (particularly mitochondria), organelle dissolution, rupture of the plasma membranes and leakage of cellular contents into extra-cellular space. Necrosis typically affects groups of contiguous cells and an inflammatory reaction usually develops in adjacent viable tissue in response to the released cellular debris. Apoptosis is distinguished from death by necrosis in that it is a closely regulated process induced by specific stimuli, including cytokines, hormones, viruses and toxic insults. It occurs without the release of inflammatory mediators and affects mostly single cells or small groups of

cells in an asynchronous fashion. In a morphological sense, it differs from necrosis in that condensation and fragmentation of nuclear chromatin occurs, as well as compaction of cytoplasmic organelles, dilatation of the endoplasmic reticulum, a decrease in cell volume and alterations to the plasma membrane resulting in the recognition and phagocytosis of apoptotic cells (Figure 1.1) (Arends and Wyllie, 1991).

The morphological changes which occur during apoptosis are accompanied by a number of biochemical changes. Some of these changes occur at the cell surface and promote recognition by phagocytes (Homburg et al., 1995; Martin et al., 1995; Pradhan et al., 1997; Savill, 1997). Intracellular changes include the degradation of chromosomal DNA into high molecular weight (Canman et al., 1992; Brown et al., 1993; Oberhammer et al., 1993) and oligonucleosomal fragments (Wyllie et al., 1980), as well as cleavage of a specific subset of cellular polypeptides (Kaufmann, 1989; Ucker et al., 1992; Lazebnik et al., 1993). This cleavage is known to be accomplished by a specialized family of cystein-dependent aspartate-directed proteases (Lazebnik et al., 1994) termed caspases (Alnemri et al., 1996).

Two major apoptotic pathways exist: the extrinsic pathway (receptor-mediated apoptosis) and the intrinsic pathway (mitochondria-associated apoptosis). Both apoptotic signalling pathways converge at the level of specific proteases – the caspases.

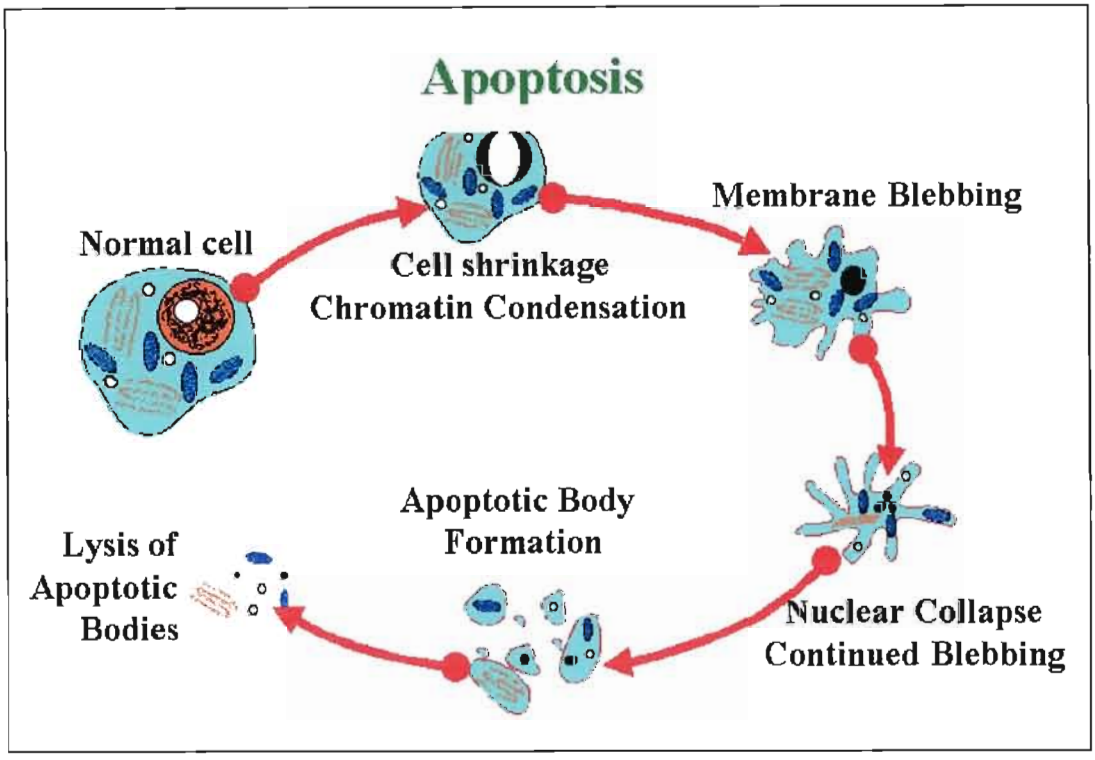



**Fig. 1.1 Morphological changes during apoptosis** Apoptosis is defined by a distinct set of morphological changes observed during cell death including loss of focal adhesions. Cells undergoing apoptosis usually exhibit a characteristic morphology, including fragmentation of the cell into membrane-bound apoptotic bodies, nuclear and cytoplasmic condensation and endolytic cleavage of the DNA into small oligonucleosomal fragments. The cells or fragments are then phagocyted by macrophages.

(http://www.bendermedsystems.com/112.html).

### 1.2.2 EXTRINSIC PATHWAY

The extrinsic pathway involves the activation of apoptotic signalling cascades by the binding of a specific protein ligand to a cell surface trans-membrane receptor (Figure 1.2) (Schultz and Harrington, 2003). Death receptors involved in this process belong to the tumour necrosis factor receptor (TNF-R) family and include death receptors like Fas, TNFR1, death receptor 3 (DR3), TNF-related apoptosis-inducing

ligand receptor 1 (TRAIL-R1 or DR4), TRAIL-R2 (DR5) and DR6. These receptors are generally composed of 3 identical polypeptide chains and along with individual characteristics also have binding and signalling features in common (Ashkenazi and Dixit, 1998; Rath and Aggerwal, 1999). Each of the 6 DRs contains an intracellular cytosolic death domain (DD), that functions as a protein-protein binding module after recruiting various cytosolic signalling molecules that comprise the specific apoptotic pathways (Schultz and Harrington, 2003). The receptor-induced pathway leads to the recruitment of procaspase-8 or -10 (initiator caspases) to the death inducing signalling complex (DISC). The activated caspase is capable of directly activating effector caspases, such as caspase-3, -6 and -7.

One of the best characterized surface receptors is Fas (Apo-1 or CD95), a 319-amino acid type 1 trans-membrane glycoprotein, with broad distribution on both lymphoid and non-lymphoid cells (Itoh et al., 1991). Fas is essential in activation of apoptosis, as well as in the activation of the transcription factor nuclear factor (NF)-κB (Tartaglia et al., 1993; Lawen, 2003). NF-κB protects cells from apoptosis by promoting expression of survival factors, such as members of the inhibitor of apoptosis (IAP) family. An apoptotic signal results in target cells when FasR is bound by its natural ligand FasL or by agonistic antibodies. FasL is expressed by activated CD4(+) and CD8(+) T cells. By binding to FasR expressed on target cells, these activated T cells can kill target cells. Furthermore, it is possible for cells to activate their own Fas receptor in an autocrine fashion and commit suicide (Wallach et al., 1999). The binding of FasL to FasR leads to receptor trimerization (Ashkenazi and Dixit, 1998; Chinnaiyan et al., 1996). Adaptor proteins like Fas-associated death domain protein (FADD) then bind to the cytosolic death domain of FasR via their DDs. FADD also contains a second protein-protein interaction domain, the death effector domain (DED) (Muzio et al., 1996; Enari et al., 1998). The DED of FADD binds to the DED or prodomain of caspase-8. The complex of Fas, FasL, FADD and procaspase-8 is called the DISC. The procaspase-8 molecules are brought into close

proximity in the DISC, thus they trans-activate each other. Activated caspase-8 then activates downstream caspases such as caspase-3, -6, -7 and -9, resulting in apoptosis.

However, there are other members of the TNF-R family that can signal for apoptosis via their DDs. For instance, tumour necrosis factor-α (TNF-α) interacts with two receptors, TNFR1 (CD120a or p55) and TNFR2 (CD120b or p75) to stimulate multiple biologic responses including apoptosis (Ashkenazi and Dixit, 1998). Although TNFR1 has no DD, intracellularly, TNFR1 associates with TNFR1-associated DD (TRADD), which, via its DD, binds to FADD, and results in transmission of a death signal. As in the Fas signalling cascade, activation of caspase-8 via the recruitment to the DISC leads to the cleavage of downstream caspases, such as caspase-3, an endogenous endonuclease which targets the inhibitor of caspase-activated deoxyribonuclease (CAD) (Sakahira et al., 1998). The inhibitor of CAD (ICAD) and CAD exist as an inactive complex. ICAD is cleaved by activated caspase-3 to release CAD, which can then enter the nucleus and degrade the cell's chromosomal DNA (Irmler et al., 1997), leading to DNA fragmentation and cell death. Death receptors (DR) 4 and 5 bind to and are activated by TNF-related apoptosis-inducing ligand (TRAIL). Through their cytosolic DD, they recruit FADD and form a DISC as described for Fas and TNFR1 and 2. DISC signalling can be inhibited by the expression of the cellular Fas-associated DD-like interleukin (IL)-1-converting enzyme inhibitory protein (c-FLIP). c-FLIP is a dominant negative caspase-8 that leads to the formation of a signalling inactive DISC (Thome et al., 1997; Scaffidi et al., 1999; Tepper and Seldin, 1999; Scaffidi et al., 1998).

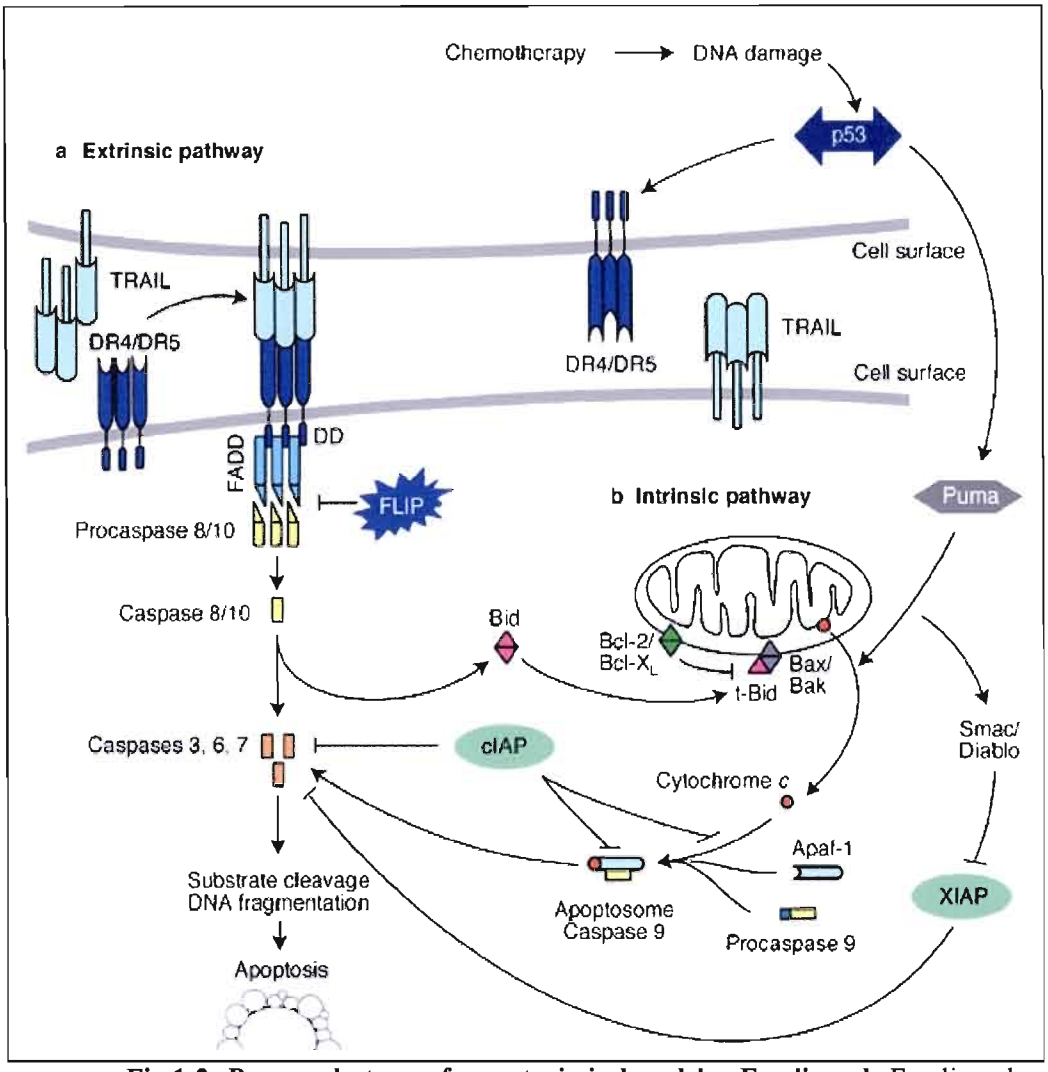



**Fig.1.2. Proposed steps of apoptosis induced by Fas ligand**: Fas ligands, which usually exist as trimers, bind and activate their receptors by inducing receptor trimerization. Activated receptors recruit adaptor molecules such as Fas-associating protein with death domain (FADD), which recruit pro-caspase 8 to the receptor complex, where it undergoes autocatalytic activation. Activated caspase 8 activates caspase 3 through two pathways; The complex one is that caspase 8 cleaves Bcl-2 interacting protein (Bid) and its COOH-terminal part translocates to mitochondria where it triggers cytochrome c release. The released cytochrome c binds to apoptotic protease activating factor-1 (Apaf-1) together with dATP and pro-caspase 9 and activates caspase 9. The caspase 9 cleaves pro-caspase 3 and activates caspase 3. The other pathway is that caspase 8 cleaves pro-caspase 3 directly and activates it (http://www.expertreviews.org).

### 1.2.3. INTRINSIC PATHWAY

The intrinsic apoptotic pathway is associated with the mitochondria. It is activated by a variety of extra- and intracellular stresses, including oxidative stress and cytotoxic drugs (Figure 1.3). As previously described for the extrinsic pathway, the TNF receptors, when bound to their appropriate ligand, can initiate a cascade of death signals. One outcome of this is the activation of caspase-8 and in turn cleavage and activation of caspase-3. In the presence of caspase-3, the quantity of active caspase-8 produced at the DISC determines whether a mitochondrial-dependent (low quantity, type II) or –independent (high quantity, type I) apoptotic pathway is used (Li et al., 1998). Caspase-8 also cleaves and activates a promoter of apoptosis in the Bcl-2 family called Bid (Ghafourifar et al., 1999), which becomes truncated (tBid) and translocates to the mitochondria resulting in the release of cytochrome *c* from the mitochondrial inter-membrane space into the cytosol (Figure 1.2). Ceramide is another factor that induces cytochrome *c* release from the mitochondria, generated by hydrolysis of sphingomyelin, followed by activation of acidic sphingomyelin (Waterhouse et al., 2002).

Early loss of mitochondrial membrane potential and the opening of the mitochondrial permeability pore are thought to be necessary for the release of cytochrome *c*, although both events are not needed in all instances (Ly et al., 2003; van Loo et al., 2002). A range of other apoptotic molecules in addition to cytochrome *c* is released from the mitochondria (Figure 1.3) (Joza, 2001). One such molecule is an apoptosis-inducing factor (AIF), which has been shown to induce apoptosis independently of caspases (Hunot and Flavell, 2001; Chai et al., 2000). Another molecule that is released is second mitochondria-derived activator of caspases (Smac)/DIABLO, which is a protein that eliminates the inhibitory effect of IAPs and interferes with their protective effect against ultraviolet radiation-induced cell death (Susin et al., 1996). After release from the mitochondria, cytochrome *c* binds to the apoptosis protease-activating factor (Apaf)-1, which leads to the

activation of caspase-9 and -3 and the release of Smac/DIABLO (Susin et al., 1997; Li et al., 1997).

Cytochrome *c* is a required cofactor and forms a complex with Apaf-1, procaspase-9 and deoxyadenosine monophosphate (dATP) (Kelekar and Thompson, 1998). This large complex is called the apoptosome and leads to activation of caspases. The apoptosome consists of seven Apaf-1, seven cytochrome c, seven dATP, and seven procaspase-9 molecules. The apoptosome-bound procaspase-9 is auto-activated and goes on to activate effector caspases such as caspase-3, which leads to the cleavage of a number of cellular substrates involved in apoptosis and finally endonuclease-dependent fragmentation of the cell's DNA (Schultz and Harrington, 2003).

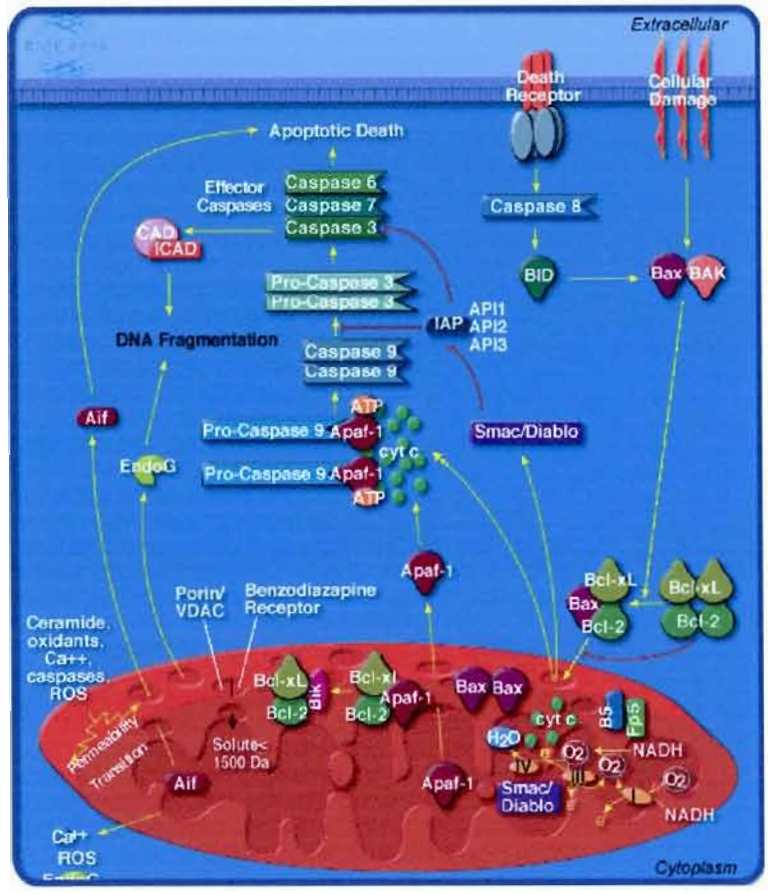



**Fig.1.3. Mitochondrial pathway of apoptosis.** Mitochondria participate in apoptotic signalling pathways through the release of mitochondrial proteins into the cytoplasm. Cytochrome c is released from mitochondria in response to apoptotic signals, and activates Apaf-1, a protease released from mitochondria. Activated Apaf-1 activates caspase-9 and the rest of the caspase pathway. Smac/DIABLO is released from mitochondria and inhibits IAP proteins that normally interact with caspase-9 to inhibit apoptosis. Apoptosis regulation by Bcl-2 family proteins occurs as family members form complexes that enter the mitochondrial membrane, regulating the release of cytochrome c and other proteins. Apoptosis inducing factor (AIF) is another mitochondrial factor that is released into the cytoplasm to induce apoptosis. AIF-induced apoptosis is important during development but is not caspase dependent (http://www.biocarta.com/pathfiles/h_mitochondriaPathway.asp).

### 1.2.3.1. Bcl-2 FAMILY PROTEINS

The release of apoptotic factors from the mitochondria is closely controlled by a group of proteins, which belong to the Bcl-2 family of proteins (Figure 1.3). The Bcl-2 family shares homology within specific regions, called Bcl-2 homology (BH) domains, designated BH1, BH2, BH3 and BH4 (Adams and Cory, 1998; Borner et al., 1994). However, the overall amino acid sequence homology among members is low (Yin et al., 1994; Farrow and Brown, 1996; Chittenden et al., 1995; Wang et al., 1996; Huang et al., 1998; McDonnell et al., 1999; Korsmeyer, 1995; Enari et al., 1998). Family members are classified based on function and BH-domain organization. The proteins, via their BH domains, interact specifically to form either homo- or heterodimers, ultimately expressing their pro- or anti-apoptotic influence. The family is divided into three main groups: (1) the anti-apoptotic proteins, most of which contain a C-terminal membrane anchor and the four BH domains, like Bcl-2, Bcl-$x_L$, Mcl-1, A1 and Bcl-w, (2) the pro-apoptotic members (which lack some of the four BH domains), like Bax, Bak, Bok and Bcl-$x_S$ and (3) the BH3-only proteins (that only contain the 3$^{rd}$ BH domain, an amphipathic helical structure, and are all pro-apoptotic), which include Bik, Hrk, Bim, Blk, Bad and Bid (Seshagiri and Miller, 1997). The relative levels of pro- and anti-apoptotic proteins determine a cell's susceptibility to apoptosis (Chinnaiyan et al., 1997). Bcl-2 family members also regulate caspase activation by altering the release of cytochrome *c* and changing the ability of scaffolding proteins such as Apaf-1 to recruit pro-caspase (Spector et al., 1997; Wu et al., 1997; Strasser et al., 2000).

### 1.2.4. CASPASES

Both the extrinsic and intrinsic apoptotic pathways converge at the level of specific proteases – the caspases. There are 14 mammalian caspases identified to date (Earnshaw et al., 1999). They are synthesized as pro-enzymes, which usually

undergo proteolysis and activation by other caspases in a cascade (Garcia-Calvo et al., 1998). Exceptions to this are procaspase-8 and -9, which can undergo autocleavage (Figure 1.4). They have a general organization in which the N-terminal prodomain is followed by a sequence encoding first a large and then a small subunit. In some pro-caspase, these subunits are separated by a small spacer that is excised from the pro-enzyme during its maturation (Garcia-Calvo et al., 1998). Peptide caspase inhibitors can inhibit downstream caspase activation and subsequently apoptosis (Grutter, 2000). Caspases are divided into three groups: (1) the initiator caspases that are characterized by long prodomains (>90 amino acids) containing either death effector domains (DED) domains (caspase-8 and caspase-10) or a caspase recruitment domain (CARD) (caspase-2 and caspase-9; CED-3); (2) the executioner or effector caspases containing short prodomains (caspase-3, caspase-6 and caspase-7) and (3) the remaining caspases whose main role lies in cytokine maturation rather than apoptosis (Walker et al., 1994). Each active caspase is derived from processing and self-association of two caspase pro-enzymes (Figure 1.4). During this conversion a minimum of two cleavages take place, one separating the prodomain from the large subunit and another separating the large subunit from the small subunits (Garcia-Calvo et al., 1998). The resulting tetrameric enzymes contain two active sites at opposite ends of the molecule. The large and small subunits within each heterodimer interdigitate to form a core composed of six-stranded β-sheets flanked by α-helices, a quaternary structure unique to proteases (Mittl et al., 1997; Rotonda et al., 1996; Wilson et al., 1994; Coleman et al., 2001).

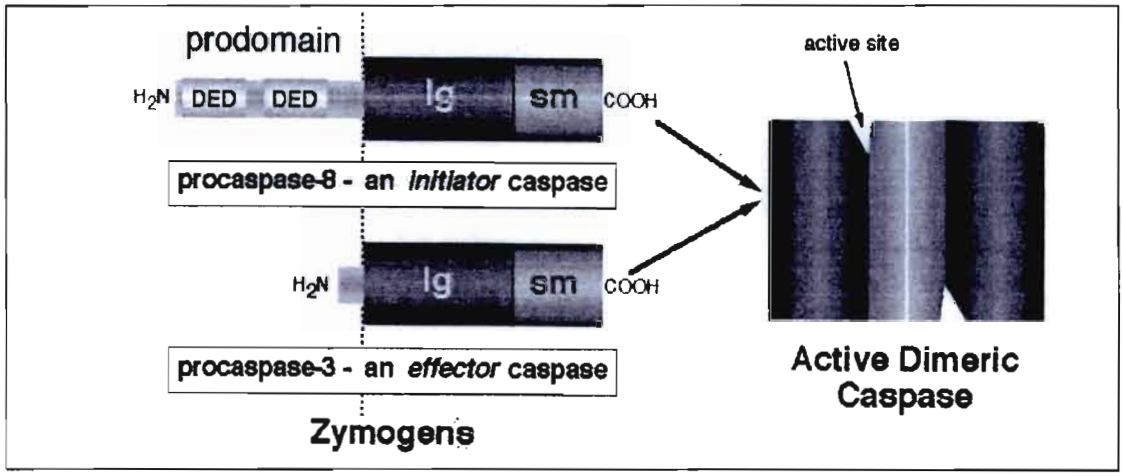



**Fig.1.4. Structures of procaspase-8:** an initiator caspase, and procaspase-3, an effector caspase. The heterotetrameric enzyme shown on the right results from proteolytic activation, as described in the text (Garcia-Calvo et al., 1998).

All caspases hydrolyze peptide bonds on the carboxyl side of an aspartate residue (Garcia-Calvo et al., 1998). Initiator caspases cleave and activate effector caspases. These then cleave cellular substrates, which leads to all phenomena of the apoptotic morphology. The unique individual structural features contribute to the different substrate specificities among caspases. For instance one effector caspase, caspase-3, when activated, is capable of cleaving many important cellular substrates, including ICAD, Rho-associated coiled coil forming kinase I (ROCKI), poly (ADP-ribose) polymerase (PARP, a DNA repair enzyme), actin, fodrin and lamin involved in maintaining cellular integrity. Other caspase-3 substrates include protein kinases like mitogen-activated protein kinase/extracellular regulated kinase (MAPK/ERK) kinase kinase (MEKK-1), p21-activated kinase (PAK2), mammalian Ste20-like kinases 1 and 2 (Mst1/Krs, Mst2), v-raf-1 murine leukemia viral oncogene homolog 1 (Raf1) and other proteins involved in signal transduction pathways. Active caspase-3 can cause membrane blebbing via ROCKI cleavage, which leaves the kinase constitutively active, permanently phosphorylating myosin light chains (Sebbagh and Renvoize, 2001; Leverrier and Ridley, 2001; Nagata, 2000). It also causes

causes disassembly of the cell structure and DNA fragmentation via ICAD cleavage that, as previously described, sets CAD free to move into the nucleus (Birnbaum et al., 1994).

Caspase activation and activity can be regulated by interactions with several cellular proteins (Figure 1.3). One such group is the inhibitor of apoptosis (IAP) protein group, which includes five polypeptides. Four of these, the cIAP-1, cIAP-2, XIAP and NAIP, each consist of an N-terminal domain containing multiple copies of a baculovirus IAP repeat (BIR) motif (Lovering et al., 1993) and a C-terminal zinc-containing protein-protein interaction domain (RING finger) (Liston et al., 1996). The fifth, survivin, contains only a BIR domain. Each of the IAPs can inhibit apoptosis induced by a variety of stimuli (Duckett et al., 1996; Ambrosini et al., 1997; Deveraux et al., 1997). XIAP, cIAP-1, and cIAP-2 bind to and inhibit active caspase-3 and -7 (Roy et al., 1997; Deveraux et al., 1998). They also bind to procaspase-9 and prevent its activation (Salvesen and Duckett, 2002). In addition to cellular inhibitors of apoptosis, many viruses also contain viral IAPs, viral anti-apoptotic Bcl-2 proteins or other inhibitors of apoptosis in order to prevent infected host cells from dying (Moodie and Wolfrnan, 1994).

### 1.2.5. MITOGEN ACTIVATED PROTEIN KINASES

As previously discussed, many cellular molecules are activated or deactivated during apoptosis. One important group of cellular factors included in this process is the protein kinases. Three parallel mitogen activated protein kinase (MAPK) cascades occur in mammalian cells. They are characterized by their analogous effectors: extracellular signal-regulated kinases (Erk), stress activated protein kinases/c-Jun-N-terminal (SAPK/Jnk), and $p38^{MAPK}$ (Figure 1.5). The Erk pathway is triggered by a variety of growth factors, including serum. Binding of the ligand to its G-protein receptor tyrosine kinase initiates the signalling cascade, which is

highlighted by the recruitment and activation of the Raf protein via Ras. This triggers the Erk pathway (Marshall, 1994). The activated form of Raf phosphorylates the dual-specificity kinases, MAPK/ERK kinase 1 and 2 (MEK 1/2), which in turn phosphorylate the Thr and Tyr residues of the Thr-Glu-Tyr (TEY) activation motif of ERK1/2. The phosphorylated ERK1/2 translocates to the nucleus, where it phosphorylates multiple substrates including activators of transcription like p90RSK S6 kinase (Frodin and Gammeltoft, 1999), MAPK-activated protein kinase (MAPKAK)-1, phospholipase $A_2$ and mitogen- and stress-activated protein kinase (MSK), as well as transcription factors Elk-1, Ats-1, Sap1a, m-Myc, signal transducers and activators of transcription proteins (STAT) such as Stat3, adapter proteins such as Sos, growth factor receptors such as the epidermal growth factor receptor (EGF), and estrogen receptors (Denhardt, 1996; Cahill, 1996). Generally, activation of the ERK signalling pathway has a role in mediating cell division, migration and survival.

The SAPK/Jnk pathway is stimulated by stressors like cytokines (TNF and FasL), heat shock, oxidative stress and UV light (Yan, 1994). The pathway is understood to become activated through the phosphorylation of the MAPK/ERK kinase kinase (MEKK) -1, a kinase analogous to the Raf protein in the Erk pathway (Figure 1.5) (Hirai et al., 1996). At this level in the cascade there are also other MAPK kinase kinases (MKKKs), which include apoptosis-inducing kinase (ASK) 1 and a member of the mixed-lineage kinases (MLK) (Teramoto et al., 1996). The MKKKs are activated by GTP-binding proteins of the Rho family (Racs, Rhos, and the Cdc42s) (Yuasa et al., 1998), but may occur independently of GTPases (Yao, 1997). The MEKK-1 phosphorylates SAPK/ERK kinase (SEK) -1 and MAPK kinase (MKK) -7, which dually activate SAPK via phosphorylation of the Thr and Tyr residues of the Thr-Pro-Tyr (TPY) activation motif (Shaulian and Karin, 2002). Similarly to Erk, the activated SAPK translocates to the nucleus where it phosphorylates c-Jun, leading to increased activity of activator protein-1 (AP-1)

transcription factor (Atfi et al., 1997). Other transcription factors are also known to be targets including the nuclear activating transcription factor (ATF) -2, Elk-1, Myc, Smad3, tumour suppressor p53, nuclear factor of activated T-cells (NFAT) -4, DPC4, a human MAD related protein, and mitogen-activated kinase activating death domain protein (MADD), a cellular death domain protein (Zhang et al., 1998; Hoeflich and Woodgett, 2001; Dougherty et al., 2002). Activation of the SAPK/JNK signalling cascade generally results in apoptosis, although it has been shown to promote cell survival under certain conditions, and is involved in tumorigenesis and inflammation (Chaung, 2000).

The $p38^{MAPK}$ pathway is activated by a variety of stressors and cellular insults such as heat, oxidative stress, inflammatory cytokines and TNF receptor signalling, but not by mitogenic stimuli (Figure 5) (Rogalla et al., 1999). Although SAPK and $p38^{MAPK}$ pathways appear to share the same initiating G-protein, further investigation into the initial steps of this cascade is needed. It has been found that MLK-3 activates MKK-3/6, which subsequently activates the $p38^{MAPK}$ route by phosphorylating the Thr and Tyr residues of the Thr-Gly-Tyr (TGY) activation motif (Yan, 1994). It has also been found that SEK-1 can activate $p38^{MAPK}$, thus linking it to the SAPK pathway (Stein et al., 1997). Once activated, $p38^{MAPK}$ can phosphorylate and activate several transcription factors and modulate gene expression. The $p38^{MAPK}$ is known to phosphorylate and activate the mitogen activated protein kinase-activated protein kinase-2/3 (MK-2/3) *in vivo,* which then phosphorylates mammalian heat shock protein HSP27 and MAPK-activated protein-2 (MAPKAP-2) (Farrow and Brown, 1996). In addition, p38MAPK can also activate ATF2, Sap-1a (Kyriakis and Avruch, 2001), Stat, Max/Myc complexes, MEF-2A/C, Elk-1 and cyclic AMP-responsive element binding protein (CREB). Thus, it appears to be important in cell motility, transcription and chromatin remodelling as well as apoptosis (Cardone et al., 1997).

The protein kinases involved in apoptosis are highly regulated during this process. Many of them are cleaved in or near their regulatory domains, producing

catalytically competent fragments with increased activity. These include the MEKK1 (Deak et al., 1998; Widmann et al., 1998), p21-activated kinase (PAK) 2 (Rudel and Bockoch, 1997; Lee et al., 1997), mammalian Ste20-like kinases (Mst1/Krs and Mst2) (Graves et al., 1998; Lee et al., 1998). It has been shown that in certain cases the expression of such deregulated kinases in cells actively promotes apoptosis (Graves et al., 1998; Xia et al., 1995). Cleavage of three of these kinases may have a similar effect in cells. MEKK1, PAK2, and Mst1 are all capable of activating the SAPK/JNK pathway (Deak et al., 1998; Widmann et al., 1998; Graves et al., 1998; Lee et al., 1998), which leads to heightened transcription of genes under control of the transcription factor c-Jun. These three kinases all constitutively activate SAPK/JNK, a kinase that is widely implicated in apoptosis, whereas the antagonistic pathway (Earnshaw et al., 1999) involving the classical MAP kinases ERK-1 and -2, which promote cell survival, is extensively disrupted during apoptosis (Kuhl and Rensing, 2000).

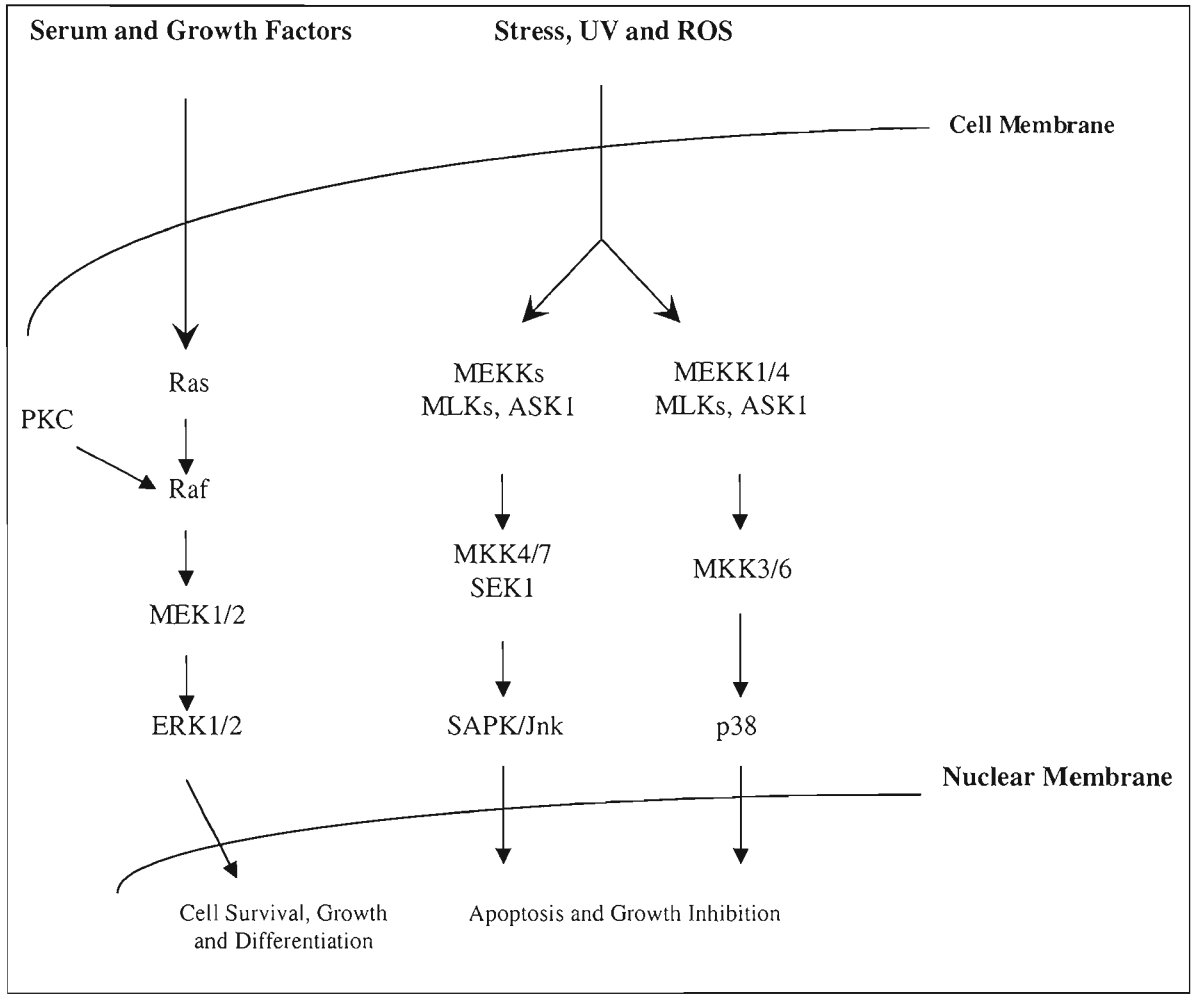



**Fig.1.5. Overview of the Mitogen-Activated Protein Kinase (MAPK) signalling pathways.** The best described activators and downstream targets are shown. The trans-acting factors mediating effects of the ultimate kinases are not shown. Mitogen-activated protein kinase (MAPK) pathways (ERK, JNK and p38) are a family of serine/threonine protein kinase that have been shown to regulate several physiological and pathological cellular phenomena, including inflammation, apoptotic cell death, oncogenic transformation, tumour cell invasion and metastasis. The ERK1,2 pathway has been shown to stimulate cell growth and oppose the apoptotic effects of JNK/SAPK and p38 pathways. The JNKs and p38 signalling pathways are responsive to stress stimuli, such as cytokines, ultraviolet irradiation, heat shock, and osmotic shock, and are involved in cell differentiation and apoptosis. (Figure adapted from DM. Templeton)

### 1.2.6. HEAT SHOCK PROTEINS

Injured or stressed cells may undergo either necrosis or apoptosis, depending on the level of stress. However, if the stress level is very low, cells attempt to survive and activate a stress response system. Once the stress stimulus is removed the cells can recover their normal cellular function. Briefly, the effects of stress/heat shock response include: 1) inhibition of DNA synthesis, transcription, RNA processing and translation; 2) inhibition of progression through the cell cycle; 3) denaturation and misaggregation of proteins; 4) increased degradation of proteins through both proteasomal and lysosomal pathways; 5) disruption of cytoskeletal components; 6) alterations in metabolism that lead to net reduction in cellular ATP; and 7) changes in membrane permeability that lead to an increase in intracellular $Na^+$, $H^+$ and $Ca^{2+}$ (Lindquist, 1986; Sonna et al., 2002; Jaattela, 1999). In mammalian cells, non-lethal heat shock produces changes in gene expression and in the activity of expressed proteins in a stress/heat shock response (Sonna et al., 2002; Parsell and Lindquist, 1993). This response characteristically includes an increase in thermo-tolerance, the ability to survive subsequent, more severe heat stresses, as well as cross-tolerance to other stressors (Creagh et al., 2000). These characteristics of the stress/heat shock response earned it the well known name of the chaperone system, which protects the cell from damage and in extreme stress situations leads to apoptosis and even necrosis (Ishikawa et al., 1999).

In order to understand the important role of heat shock proteins (HSPs) and their relevance to apoptosis, one must appreciate their function in the stress/heat shock response in the cell. HSPs are transcribed using the heat shock element (HSE), a stretch of DNA located in the promoter region of heat shock proteins (HSPs) and a number of other genes (Morimoto, 1998). It functions as a binding site for transcription factors called heat shock factors (HSFs), namely HSF-1, HSF-2, and HSF-4 as well as HSPs (Pirkkala et al., 2001). HSF-1 is involved in the acute response to heat shock, while the other two participate in a number of different

regulatory and developmental processes (Pirkkala et al., 2001; Sarge et al., 1993). Prior to heat induced activation, HSF-1 exists as a monomer localized in the cytoplasm. It is thought to be bound to HSPs in unstressed cells and activated by the increase in concentration of denatured proteins which present exposed hydrophobic domains to which HSPs bind, releasing HSF-1 (Pirkkala et al., 2001). After activation by thermal stress, HSF-1 is found in the nucleus in trimeric form, concentrated in granules (Georgopoulos and Welch, 1993). It is this activated trimeric form of HSF-1 that binds to the HSE and is involved in increased HSP gene transcription during heat stress (Georgopoulos and Welch, 1993).

HSPs play an important role in stabilization of proteins by helping to prevent misaggregation of denatured proteins and assisting in their refolding back into native conformations (Creagh et al., 2000; Otterbein and Choi, 2000). They also regulate cellular redox state and protein turnover (Creagh et al., 2000; Sreedhar and Csermely, 2004). Although the intensity and duration of the heat stimulus needed to induce HSP expression vary considerably from tissue to tissue, a typical *in vitro* exposure involves heating mammalian cells to 42-45°C for 20-60 minutes and then reverting them to normothermic temperatures (37°C). Several studies have shown that during the period of hyperthermia and shortly thereafter, HSPs become the predominant proteins synthesized by cells (Feige and Polla, 1994).

### 1.2.6.1. HEAT SHOCK PROTEINS: ROLE IN APOPTOSIS

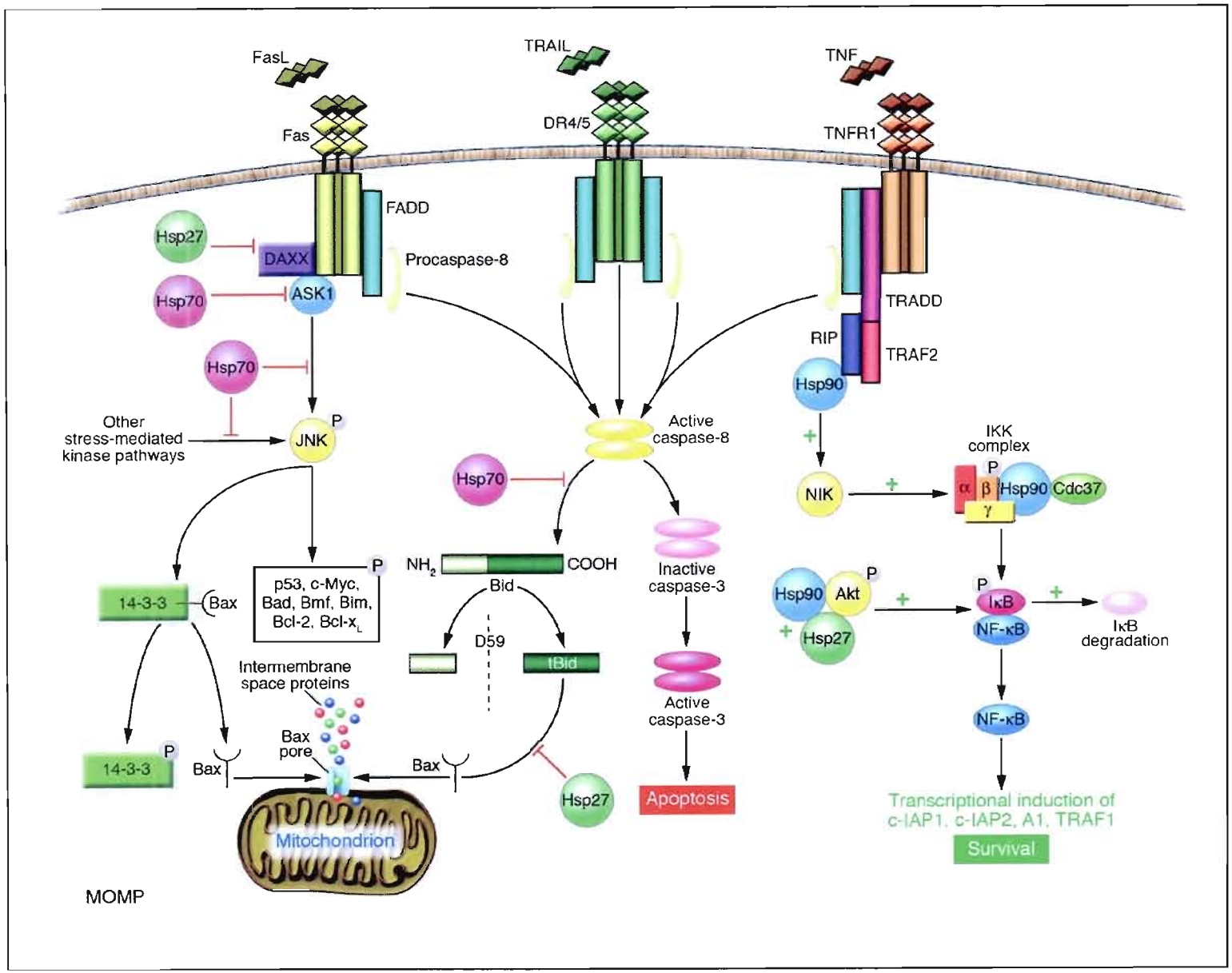
Heat shock proteins can inhibit or aid the apoptotic machinery through their chaperone functions by affecting protein assembly and folding, ubiquitin degradation pathways and protein translation. Chaperone-independent regulation of apoptosis by heat shock proteins is also reported in the case of the JNK and AIF proteins. HSP 70, HSP 27 and HSP 90 represent the three main groups of HSPs, which have been implicated in the process of apoptosis (Figure 1.6). HSP 70 belongs to the HSP 70

family of proteins, which function mainly as chaperones, interacting transiently with many proteins in an ATP-dependent manner (Liossis et al., 1997). It is present at basal levels in the unstressed cell, but following stress it becomes strongly induced and is present in the cytosol and nucleus (Chant et al., 1995). However, its exact role in apoptosis is difficult to define as many contradictory findings have been observed in regards to its apoptotic and anti-apoptotic functions. HSP 70-induced thermotolerance has been observed to cause leukaemic T cells to become more susceptible to receptor-mediated Fas and T-cell/cell death-3 antigen receptor (TCR/CD3)-induced apoptosis (Filippovich et al., 1994). Several studies have reported enhanced apoptosis when HSP 70 levels are increased in various other cells (Murdoch, 1995; Furlini et al., 1994; Liossis et al., 1997; Gao et al., 2001)

HSP 70 has been shown to have an overall inhibitory role in stress kinase pathways. HSP 70 proteins bind and stabilize protein kinase C (PKC) and Akt, also known as protein kinase B (PKB), both of which have anti-apoptotic properties (Konishi et al., 1997). HSP 27 also increases Akt activity by an unknown mechanism (Mosser et al., 2000). HSP 70 inhibits stress signal-induced cell death by suppressing JNK activation, which is an important factor in stress induced apoptosis. HSP 70 also appears to participate in a Bid-dependent cell death pathway. HSP 70 appears to affect the Bid-dependent apoptotic pathway negatively by inhibiting JNK activation by a mechanism that is not fully understood (Gabai et al., 2000; Gabai et al., 2002; Beere et al., 2000). HSP 70 has also been observed to directly interact with Apaf-1 and inhibit apoptosome formation (Saleh et al., 2000; Ravagnan et al., 2001) as well as to directly bind to AIF, inhibiting AIF-dependent apoptosis (Jaattela et al., 1998). In addition, HSP 70 overexpression can inhibit caspase-dependent events that occur much later in apoptosis, such as activation of cytosolic phospholipase $A_2$ and changes in nuclear morphology. HSP 70 could also protect cells from overexpression of caspase-3 (Sakahira and Nagata, 2002). ICAD seems to recognize an intermediate folding state conferred by HSP70-HSP40, suggesting that they may promote the

formation of the CAD-ICAD complex during protein translation (Chretien and Landry, 1988).

A


FasL
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stress-mediated
kinase pathways
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TRADD
RIP
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D59
tBid
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caspase-3
14-3-3
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c-IAP1, c-IAP2, A1, TRAF1
Survival
MOMP

**B**

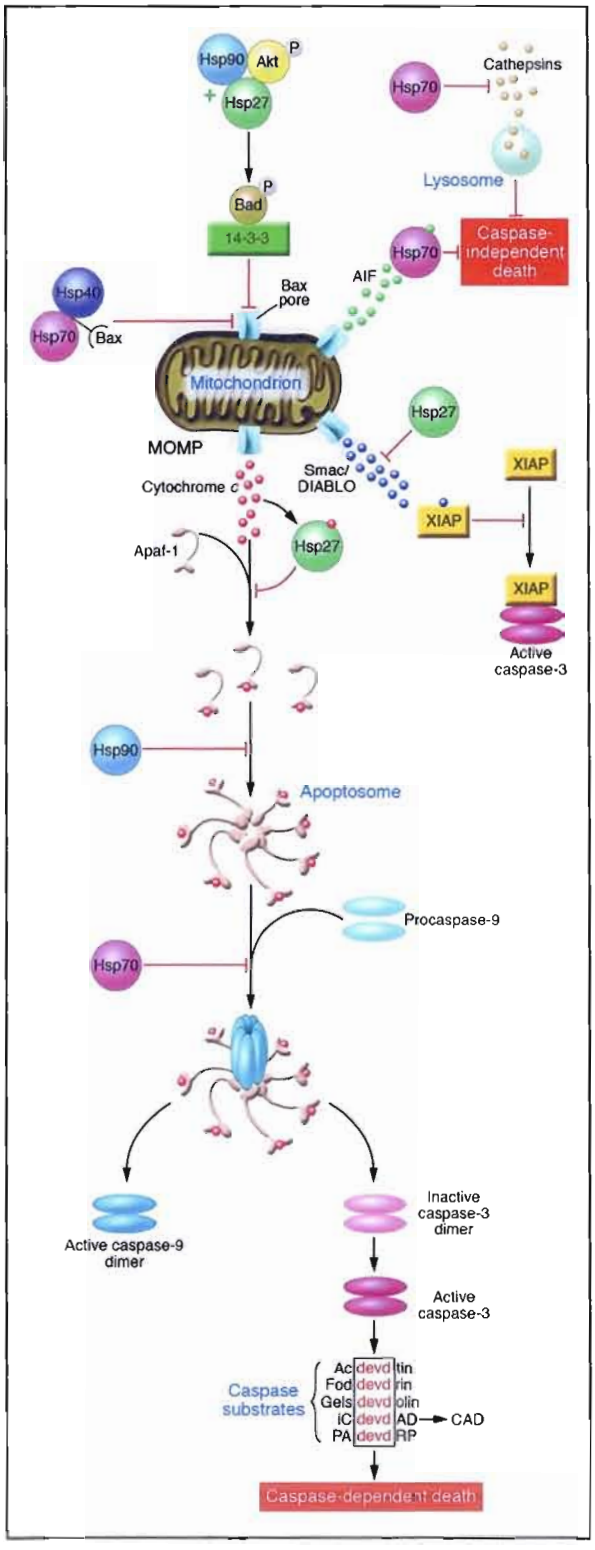


**Fig. 1.6. Heat shock proteins (HSPs) in the regulation of the major events in apoptosis.** Members of 3 of the major HSP families (HSP 27, HSP 70 and HSP 90) are shown to be involved. (A) Regulation of the extrinsic pathway by Hsps. Hsps regulate at multiple points within the signalling pathways activated by ligation of a cell surface death receptor by the appropriate ligand. These include the maintenance of prosurvival signals generated via TNF-mediated activation of NF-κB and suppression of proapoptotic signalling events, e.g., JNK activity and Bid cleavage. Integration of the extrinsic and intrinsic pathways is mediated via the caspase-8–mediated cleavage and activation of Bid as well as activation of JNK, which can impact on numerous molecules that regulate mitochondrial integrity (shown in the shaded area). (B) Regulation of the intrinsic pathway by Hsps. Hsps regulate several aspects of the intrinsic apoptotic pathway. These include both direct mediators such as Bax and indirect regulators such as Akt of Mitochondrial membrane permeabilization to prevent MOMP as well as events downstream of mitochondrial disruption to regulate apoptosome assembly. Caspase-independent cell death may also be affected via HSP-mediated suppression of AIF activity and inhibition of lysosome permeabilization and cathepsin release (Beere, 2005; Feige and Polla, 1994).

The stress inducible expression of HSP 27, a member of a small HSP family, has a significant role in protection against apoptosis (Figure 1.6) (Mehlen et al., 1995; Mehlen et al., 1996). HSP 27 appears to inhibit apoptosis mediated through Fas and other receptor-mediated pathways. It has been shown to block apoptosis induced by Fas, staurosporine, monocytes, hydrogen peroxide and anti-cancer drugs (Mehlen et al., 1996; Garrido et al., 1997; Samali and Cotter, 1996; Jaattela and Wissing, 1993; Richards et al., 1996; Arrigo, 1998). Upon ligation, the adaptor protein DAXX (death associated protein) is reportedly recruited to Fas receptor C-terminus, which is followed by eventual JNK activation (Mehlen et al., 1996). HSP 27 may interact with DAXX to prevent its translocation from the nucleus to the cytosol. HSP 27 is also thought to regulate apoptosis by maintaining the redox equilibrium of the cell (Takayama et al., 2003) and has been demonstrated to inhibit apoptosis by increasing the intracellular level of the antioxidant glutathione (GSH) (Takayama et al., 2003). In oxidative stress-induced apoptosis, HSP 27 has been shown to play a protective role. The inhibition of HSP 27 phosphorylation discourages the formation of high molecular weight complexes of HSP 27 and in turn decreases its protective role (Bruey et al., 2000). Furthermore, overexpression of HSP 27 increases the resistance of cells to various apoptotic stimuli (Takayama et al., 2003). One mechanism by which HSP 27 could interfere with apoptosis is by directly binding to cytosolic cytochrome *c* and sequestering it from Apaf-1 (Bruey et al., 2000). High molecular weight complexes of HSP 27 appear competent to inhibit apoptosome formation (Lewis et al., 2000).

Finally, HSP 90 has also been implicated in evasion of apoptosis. HSP 90 plays a role in modulating TNF-receptor signalling. Upon ligation of TNFR-1, the receptor interacting protein (RIP) is recruited to the receptor and promotes the activation of NF-κB and JNK. HSP 90 interacts with RIP, resulting in its stabilization (Chen et al., 2002). Otherwise, RIP is rapidly degraded and causes decreased TNF-induced NF-κB activity and increased cell vulnerability (Sato et al.,

2000). HSP 90 is equally important for the correct association of the I-κB kinase (IKK) complex which affects TNF-induced NF-κB activity (Basso et al., 2002). It has been reported to directly interact with Akt. When this interaction was prevented with HSP 90 inhibitors, Akt was dephosphorylated and destabilized, which increased the likelihood of apoptosis (Samali et al., 1999; Xanthoudakis et al., 1999).

In addition to the three main HSP families, the involvement of HSP 40 and 60 has also been recognized for their role in apoptosis. HSP 60 has been reported to play a role in caspase-3 maturation (Gupta and Knowlton, 2002; Ling, 1992). Both have been shown to complex with the proapoptotic protein Bax (Ling and Thompson, 1973, Gotoh et al., 2004). Under hypoxic conditions, this complex dissociates and Bax translocates to the mitochondria and participates in apoptosis. However, their exact role in apoptosis is not yet fully understood.

### 1.2.6.2. HYPERTHERMIA: HEAT SHOCK AS A CANCER TREATMENT

Hyperthermia (HT) is a relatively recent clinical procedure in which body tissues are exposed to elevated temperatures (40-44°C). This procedure induces tumour cell death by a spectrum of molecular, metabolic, cellular and tumour tissue changes, including conformational changes of cellular proteins, stimulation of immune response, as well as alterations of tumour microenvironment (Streffer, 1995; Song et al., 1995; Vaupel and Keller, 1995; Issels, 1999; Feyerabend et al., 1999). Clinical hyperthermia can be divided into three categories: local hyperthermia (including superficial local and interstitial local hyperthermia) (LHT), regional hyperthermia (RHT), and whole body hyperthermia (WBH) (Falk and Issels, 2001). The application of heat can be induced by electromagnetic field technique, ultrasound, or perfusion methods. Local hyperthermia refers to heat that is applied to a very small area, such as a tumour. The area may be heated externally with high-frequency waves aimed at a tumour from a device outside the body (single microwave or ultrasound).

To achieve internal heating, one of several types of sterile probes may be used, including thin, heated wires or hollow tubes filled with warm water; implanted microwave antennae; and radiofrequency electrodes. In regional hyperthermia, an organ or a limb is heated. Magnets and microwave antennae that produce high energy are placed over the region to be heated. In perfusion, the patient's blood is removed, heated, and then pumped into the region that is to be heated internally. Whole-body heating is used to treat metastatic cancer that has spread throughout the body. It can be accomplished using warm-water blankets, hot wax, inductive coils, or thermal chambers and refers to the procedure of raising a patients' body-core temperature to 40°C for 3-6 hours (Hildebrandt et al. 2005).

Although, HT alone does not achieve valuable clinical effects in terms of long lasting tumour remission (Liu and Wilson, 1998), there is an increasing amount of preclinical and clinical data suggesting that HT could be beneficial as a subsidiary treatment to chemotherapy or radiotherapy (Bates and Mackillop, 1986). The major advantage of combining HT with chemotherapy is to potentate the cytotoxic effects of anticancer drugs in the targeted region of tumour, without increasing toxicity to surrounding tissues. Consequently, this could lead to an increase in therapeutic index and a decrease in the side effects caused by drugs (Streffer, 1995; Feyerabend et al., 1999; Liu and Wilson, 1998; van der Zee, 2002). There are at least 18 randomized studies which have demonstrated that the combination of HT with radiotherapy, chemotherapy or both, led to improved clinical outcome [Dahl, 1994; Dewhirst et al., 2005]. This was demonstrated for tumours of the head and neck, brain, rectum, cervix, breast, lung, oesophagus, vulva/vagina and for melanoma (Jones et al., 2005).

Hyperthermia has been shown to increase the cytotoxic effect of many anticancer drugs such as Adriamycin, bleomycin, BCNU, cisplatin, and the alkylating agent melphalan, from both *in vitro* and *in vivo* studies (Li, 1984; Bates et al., 1987; Orlandi et al., 1995; Bates and Mackillop, 1990; Honess and Bleehen, 1988; Hahn, 1979; Wallach, 1977). This could be partly explained by membrane damage (Bates et

al., 1985) or by altered membrane permeability (Towle, 1994) caused by heat, therefore allowing larger quantities of drugs to enter the cell. It has been previously shown in our lab that multidrug resistant (MDR) cells overexpressing P-glycoprotein (P-GP) did not exhibit cross resistance to heat suggesting that heat shock could be useful in elimination of MDR cells. Moreover, HT has been observed to overcome primary resistance to drugs such as cisplatin, methotrexate and mitomycin c (Larrivee and Averill, 1999). It has been documented that MDR due to overexpression of P-GP can be reversed *in vitro* by combining HT with the anticancer drug melphalan (Honess and Bleehen, 1988). Hyperthermia was more effective in reversing MDR involving P-GP when melphalan or Adriamycin was combined with P-GP modulators such as cyclosporin A (Larrivee and Averill, 2000; Averill and Larrivee, 1998; Averill and Su, 1999), verapamil (Averill-Bates and Courtemanche, 1995; Turcotte and Averill-Bates, 2001), or with the glutathione S-transferase (GST) inhibitor ethacrynic acid (Dumontet et al., 1998). The P-GP modulator PSC 833 reduced resistance to vinblastine in MDR K562 and MESSA cells at 43°C (IARC, 1989).

### 1.2.7. MULTIDRUG RESISTANCE

Resistance to anti-neoplastic agents constitutes a major obstacle in the treatment of many types of cancer. The development of resistance to one particular drug often confers cross-resistance to a wide range of structurally and functionally unrelated drugs (Fajo et al., 1985). The underlying mechanisms of multidrug resistance (MDR) are not well understood. However, the phenotype has been associated with overexpression of a 170 kDa ATP-dependent membrane transport protein, P-glycoprotein (P-GP) (Skovsgaard, 1978; Kertner et al., 1983; Krishna and Mayer, 2000). P-GP is part of the ATP-binding cassette (ABC) family of membrane transport ATPases, which are of considerable clinical importance. In general, the transport ATPases are comprised of four structural domains, two that span the membrane, each containing several transmembrane segments, and two that remain in

the cytoplasm. The last two units, termed the nucleotide binding domains, play a role in cleaving ATP to derive energy necessary for transporting cell nutrients (Figure 1.7) (Borst et al., 2000). P-GP pumps substrates out of tumour cells through an energy-dependent mechanism in a unidirectional fashion. Therefore, it reduces intracellular drug concentrations and in turn decreases the cytotoxicity of anti-tumour drugs which include anthracyclines, vinca alkaloids, podophyllotoxins and taxanes. P-GP is present in healthy human, mouse and hamster cells (Roninson et al., 1986; Van der Bliek et al., 1987; Croop et al., 1989; de Bruijn et al., 1986; Gros et al., 1988). It has also been detected in human leukemias, lymphomas, sarcomas and neuroblastomas (Borst et al., 2000).

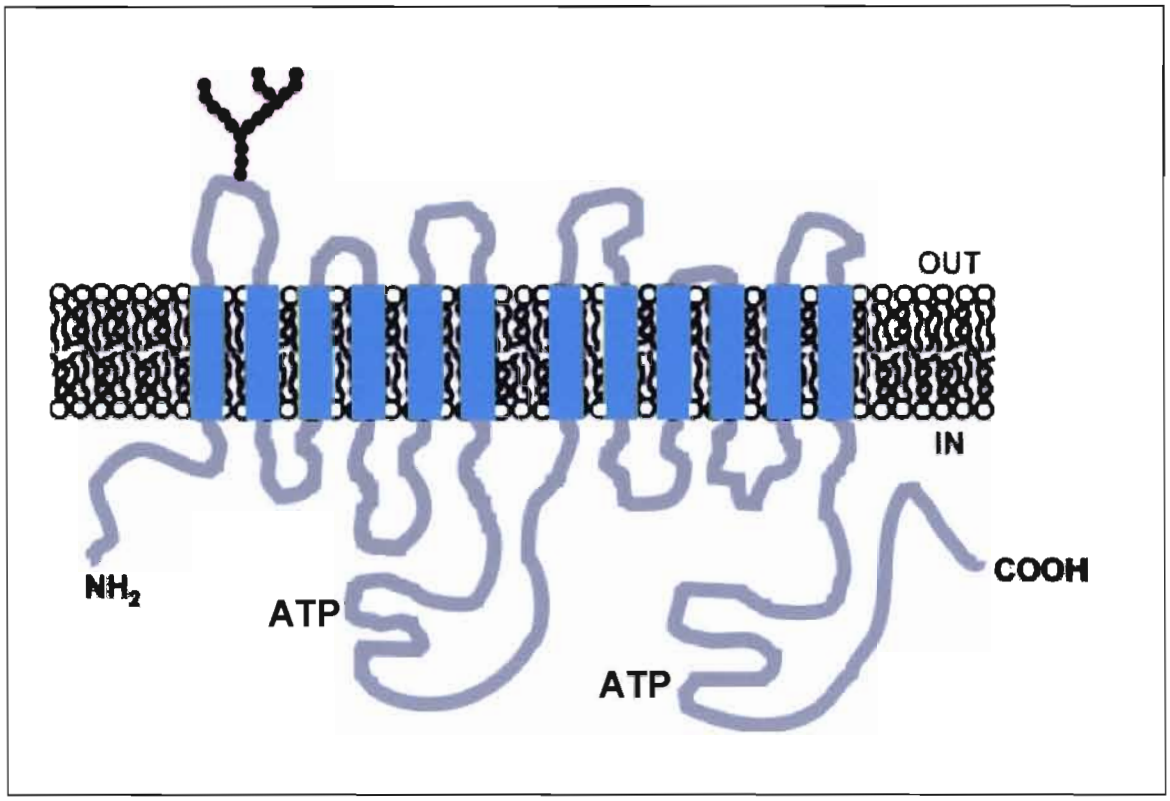


**Fig.1.7. Structure of P-glycoprotein.** The transport ATPase is comprised of 4 structural units, two of them span the membrane, each containing several transmembrane segments, and two remain in the cytoplasm and play a role in cleaving ATP to derive energy needed for transporting cell nutrients. (Courtesy of www. chembio.uoguelph.ca/sharom/Pgptopol2.jpg)

Another member of the ABC family, which has been implicated in MDR of cancer cells is the multidrug resistance associated protein (MRP). MRP is a 190 kDa membrane protein and has been described as a GS-X pump capable of transporting organic anion drug conjugates and intact anticancer drugs (Cole et al., 1992; Krishnamachary and Center, 1993; Barrand et al., 1994; Grant et al., 1994; Borst et al., 1997). MRP substrates include anthracyclines such as doxorubicin (DOX), vinca alkaloids and etoposide. Although MRP is very similar to P-GP in terms of substrate

specificity and action, only 15% amino acid homology exists between the two proteins. Several homologs of MRP have been identified, namely MRP-1 and MRP-2 (cannalicular multispecific organic anion transporter or cMOAT) as well as MRP3-7 (Dahl, 1995). The clinical cancers exhibiting MRP expression include hematological, lung, acute lymphoblastic leukemia and chronic leukemia (Borst et al., 2000). However, MRP is also expressed in normal human tissue, such as muscle, lung, spleen, bladder, adrenal gland and gall bladder. Reduced glutathione (GSH) has been suggested as an important component of MRP-mediated MDR and drug transport (Borst et al., 2000).

Moreover, there are non-transport based mechanisms that affect multiple drug classes. These types of resistance can be caused by altered activity of specific enzyme systems such as glutathione S-transferase (GST) and topoisomerase, which can decrease the cytotoxic effect of chemotherapeutic drugs in a manner independent of intracellular drug concentrations. GST is an enzyme system involved in drug and xenobiotic detoxification. It is extensively involved in the metabolic biotrans-formation of many anti-cancer drugs. Among these are nitrogen mustards, such as BCNU and cyclophosphamides. Several resistant cell lines have been shown to overexpress GST (Borst et al., 2000). Further, the cellular regulation of GSH has also been shown to play a key role in detoxification and cellular repair following the damaging effects of DOX and alkylating agents. Increases in GSH levels have been observed in many alkylating agent-resistant cell lines (Borst et al., 2000). This suggests that reduced levels of intracellular GSH will result in chemosensitization of drug resistant cells. Reduction of GSH with buthionine sulfoxime (BSO) has been successfully used in the clinic to sensitize tumour cells to treatments with chemotherapy and radiotherapy (Bates et al., 1995; Britten et al., 1992).

As mentioned earlier topoisomerase activity can also be involved in the MDR phenotype. Two types of topoisomerase exist in eukaryotic cells. Type I topoisomerase serves to alter DNA topology via single strand breaks, while type II

topoisomerase alters DNA topology by causing transient double strand breaks. Both enzyme classes are involved in DNA replication. Consequently, these enzymes constitute therapeutic targets for anti-cancer drugs in rapidly dividing tumour cells (Borst et al., 2000). Cells have been shown to become resistant to topoisomerase II inhibitors such as DOX and etoposide due either to the underexpression of topoisomerase II or topoisomerase II gene mutations. Resistance may occur alone or along with P-GP overexpression. A compensatory overexpression of topoisomerase I has been observed in cells resistant to topoisomerase II inhibitors, where cells have reduced topoisomerase II expression (Borst et al., 2000).

In addition, changes in the balance of proteins that control apoptosis can also reduce chemosensitivity since most anticancer drugs are believed to exert their cytotoxic effects via apoptotic pathways. Thus, resistance may develop with loss of genes required for cell death such as p53 or overexpression of genes that block cell death such as proto-oncogenes like *c-myc* or *c-fos* (Solary et al., 2001). Therefore, several different approaches should be considered in order to effectively overcome MDR. A promising new approach is the implementation of hyperthermia discussed earlier.

## CHAPTER 2: OBJECTIVES AND HYPOTHESIS

It has been suggested that MDR cells, aside from resistance to chemotherapy, might also inhibit apoptosis at a variety of levels within the death signalling pathways. Therefore, reversal of MDR or sensitization of the MDR cells to chemotherapeutic drugs as well as the induction of apoptosis in MDR cells are essential parts of the search for successful treatments of cancer. Thus, there are three major objectives to this study.

1. The first objective is to compare basal levels of pro- and anti-apoptotic proteins in multidrug-resistant Chinese hamster ovary cells ($CH^RC5$), which overexpress P-GP in comparison to their respective parental drug-sensitive counterparts AuxB1 cells.

2. The second objective is to investigate the induction of apoptosis via the receptor-mediated pathway of apoptosis in $CH^RC5$ in comparison to AuxB1 cells using hyperthermia to sensitize the cells.

3. The third objective is to investigate the degree of involvement of the mitochondria-mediated pathway of apoptosis in response to heat shock in $CH^RC5$ versus AuxB1 cells.

The model of multidrug resistance used in this study utilizes Chinese hamster ovary cells ($CH^RC5$), which overexpress P-GP on their cellular membrane. It is important to note that these cells confer resistance to chemotherapeutic drugs like colchicines, Adriamycin and melphalan. Before beginning to investigate molecular mechanisms of heat shock induced apoptosis in $CH^RC5$ and their parental drug-sensitive counterparts, AuxB1 cells, it is crucial to establish whether these cells differ in their basal levels of pro- (Apaf-1, Bax, cytochrome c, FADD) and anti-apoptotic

proteins (c-FLIP) as well as other proteins involved in the apoptotic pathways, such as caspases, Fas receptor, ICAD and PARP. Our hypothesis is that there are differences between the $CH^RC5$ and AuxB1 cells in the basal level of these proteins, which could render $CH^RC5$ cells resistant to apoptotic stimuli.

In order to investigate the mechanisms of heat-induced apoptosis in $CH^RC5$ and AuxB1 cells, it is important to explore both the extrinsic and intrinsic apoptotic pathways. A variety of markers must be evaluated to assess any differences in apoptotic signalling pathways between the two cell lines. To begin, the most general markers of apoptosis include nuclear chromatin condensation, caspase activation and the fate of caspase substrates such as ICAD and PARP. Looking at the state of these proteins will show whether caspase-dependent pathways are involved. Further, the receptor-mediated apoptotic pathway markers include the expression of the Fas receptor on the membranes of both cell lines as well as the expression of anti-apoptotic proteins like FLIP and IAP. Previous studies have found that levels of FLIP are down regulated after exposing Jurkat and HeLa cells to hyperthermia (Notarbartolo et al., 2004), which is thought to sensitize these cells to Fas-mediated apoptosis. Meanwhile, overexpression of IAPs has been reported in MDR cells suggesting a possible route of inhibition of apoptosis in MDR cells (Ruefli et al., 2002). When investigating receptor-mediated apoptotic pathways one must look at the involvement of the DISC components such as the translocation of FADD to the cell membrane, the cleavage of procaspase-8 as well as the presence of the DISC complex itself as a whole. P-GP has been found to inhibit caspase-8 activation but not formation of the DISC during Fas-induced apoptosis in P-GP expressing tumour cells (Till et al., 1973).

It is also imperative to explore the involvement of the mitochondria in heat-induced apoptosis in the two cell lines. The assessment of the mitochondrial membrane potential and the release of cytochrome *c* into the cytosol would be necessary. The formation and presence of the apoptosome is a crucial apoptotic

event, thus the presence of Apaf-1 and cleavage of procaspase-9 should be investigated. We expect to find differences in the receptor- and mitochondria-mediated pathways of apoptosis between the multidrug-resistant Chinese hamster ovary cells ($CH^RC5$) and their respective parental drug-sensitive counterparts AuxB1 cells.

# CHAPTER 3: EXPERIMENTAL RESULTS

## 3.1. Preface

This chapter includes a manuscript presenting the results of the research project. This manuscript will be submitted in the near future to the scientific journal Experimental Cell Research. The article entitled "Molecular mechanisms of apoptosis activation by heat shock in multidrug-resistant Chinese hamster ovary cells" investigates the role of receptor- and mitochondria-mediated apoptotic pathways induced by heat shock in multidrug-resistant Chinese hamster ovary cells ($CH^RC5$) and their parental counterparts, drug-sensitive AuxB1 cells. The experiments and the composition of this manuscript were done by Paulina Wrzal under the supervision of Dr. Diana Averill.

## 3.2. Manuscript

# MOLECULAR MECHANISMS OF APOPTOSIS ACTIVATION BY HEAT SHOCK IN MULTIDRUG-RESISTANT CHINESE HAMSTER CELLS

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## ABSTRACT

Multidrug resistance (MDR) is a major obstacle limiting the use of chemotherapy in cancer treatment. The MDR phenotype has been associated with overexpression of P-glycoprotein. MDR cells, aside from resistance to chemotherapy, might also inhibit apoptosis at a variety of levels within the death signalling pathways. Thus, reversal of resistance or sensitization of the MDR cells to chemotherapeutic drugs as well as the induction of apoptosis in MDR cells are essential parts of the search for more successful treatments of cancer. This study investigates the induction of apoptosis in MDR $CH^RC5$ cells in comparison to their parental, drug sensitive AuxB1 Chinese hamster ovary cells, using heat shock. We assessed the responses of MDR and drug sensitive cells to death receptor and mitochondria mediated signalling pathways of apoptosis in order to investigate possible mechanisms of resistance to heat shock induced cell death. Our findings show that in the heat shock induced receptor mediated pathway of apoptosis $CH^RC5$ cells exhibit higher levels of cytosolic FADD and lower levels of caspase-8 activation. In the mitochondria mediated pathway of heat induced apoptosis $CH^RC5$ cells show higher levels of mitochondrial Bax and higher initial mitochondrial membrane depolarization. Also, similar levels of cleavage of downstream substrates of caspase-3 were observed in the two cell lines suggesting that overall, MDR $CH^RC5$ cells are not resistant to heat shock induced apoptosis, compared to drug sensitive cells. This study reveals, for the first time, the major molecular mechanisms of heat shock induced apoptosis in MDR tumor. The results imply that hyperthermia is a promising method used to irradicate MDR tumor cells in the clinic.

## INTRODUCTION

Multidrug resistance (MDR) represents a major setback limiting the use of chemotherapy in cancer treatment. The overexpression of a 170 kDa ATP-dependent membrane transport protein, P-glycoprotein (P-GP) represents one of the main mechanisms associated with the MDR phenotype (Juranka et al. 1989; Ling, 1997; Yakirevich et al., 2006). P-GP functions as an efflux pump, carrying substrates out of tumor cells, resulting in decreased intracellular drug levels. It has been suggested that MDR cells, aside from resistance to cytotoxic effects of chemotherapy, might also hinder apoptosis induced by drugs and other stressors at a variety of levels within the death signaling pathways (Dudley and Lamming, 1996; Aouali et al., 2003).

Apoptosis is a mode of regulated cell death, which can be induced mainly by three unique signaling pathways: the death receptor-, the mitochondria- and more recently, the endoplasmic reticulum (ER)-mediated signaling pathways. The death receptor-mediated (extrinsic) pathway of apoptosis is triggered by extracellular activation of death receptors such as Fas and tumor necrosis factor (TNF). This is followed by the recruitment of adaptor proteins such as Fas-associated death domain (FADD) to the receptor and subsequent binding of procaspase-8 and activation of caspase-8 (Salvesen and Dixit, 1999; Kim, 2002). The activated initiator caspase-8 then goes on to cleave and activate other substrates such as effector caspase-3 and pro-apoptotic Bcl-2 family protein Bid (Barnhart et al., 2003). Once activated, effector caspase-3 cleaves many of its substrates such as inhibitor of caspases activated DNase (ICAD) and poly (ADP-ribose) polymerase (PARP) (Nagata et al., 2000). These downstream effects, which include chromatin condensation and fragmentation and the formation of apoptotic bodies, lead to the phenomenon of apoptosis. Two other initiators, caspase-10 and -2 are similarly activated via cell surface receptors but through the TNRF1 and death receptor 5 (DR5) receptors. Like caspase-8, they cleave their substrates such as Bid and caspases-3 and -9, propelling cell death (Earnshaw et al., 1999).

On the other hand, mitochondria-mediated apoptosis can be triggered by chemotherapeutic drugs and a variety of environmental stresses. These stimuli cause a shift in the balance between pro- and anti-apoptotic Bcl-2 family members. For instance, the translocation of pro-apoptotic protein Bax from the cytoplasm to the mitochondrial membrane, where it participates in formation of pores in the outer mitochondrial membrane, leads to membrane depolarization and subsequent release of other pro-apoptotic proteins such as cytochrome *c* into the cytosol (Robertson et al., 2003). Once in the cytosol, cytochrome *c* associates with apoptosis protease activating factor (Apaf-1) and the initiator procaspase-9, forming the apoptosome. Under these circumstances, activation of caspase-9 is achieved followed by activation of effector caspases like caspase-3 and this eventually leads to apoptosis (Ringger et al., 2004). The two main apoptotic signaling pathways communicate via a pro-apoptotic protein Bid that, once cleaved by either caspase-8 or -10 into tBid, translocates from the cytosol to the outer mitochondrial membrane causing its permeabilization and either initiating or magnifying mitochondria-mediated apoptosis (Gross, 2006).

The more recently discovered ER signaling pathway appears to be mediated by several apoptotic pathways (Momoi, 2006). These include activation of the initiator caspase-12, which is thought to be activated by proteases such as caspase-7 and calcium-dependent calpain. The apoptosis signaling kinase/c-Jun N-terminal kinase (ASK/JNK) pathway leads to Bax-dependent cytochrome *c* release from mitochondria and caspase-9 activation. The ER membrane protein Bap31 and caspase-8 form a complex, which leads to activation of caspase-8, cleavage of Bid, cytochrome *c* release from mitochondria and caspase-9 activation.

Heat shock or hyperthermia is currently being used in the cancer clinic in combination with radiation therapy and/or chemotherapy. The aim is to sensitize tumor cells and render them more responsive to these cytotoxic treatments without increasing toxicity to normal tissues, resulting in a higher therapeutic index (Dahl,

1995; Issels, 1999; Feyeraband et al., 1999; Van de Zee, 2002). Several recent studies have reported improvement of tumor response to radiation and chemotherapy in combination with heat shock in the clinic for tumors of the head and neck, brain, rectum, cervix, breast, lung, esophagus, vulva/vagina, and for melanoma (Jones et al., 2005; De Wit et al., 1999; Van der Zee et al., 2000; Issels et al., 2001; Wendtner et al., 2001; Wust et al., 2002; Hindebrandt et al., 2004; Fraker, 2004).

Furthermore, several *in vitro* studies indicate that heat in combination with chemotherapy can sensitize MDR tumor cells and render them more responsive to this cytotoxic cancer treatment. We previously demonstrated that MDR cells overexpressing P-GP or MRP1 did not exhibit cross-resistance to heat (Bates and Mackillop, 1986; Souslova and Averill-Bates, 2004). These cells show similar sensitivity to heat induced cytotoxicity as their drug-sensitive counterparts. However, hyperthermia either alone, or combined with chemosensitizers such as cyclosporine A, PSC833 and verapamil, was able to reverse P-GP-mediated MDR to chemotherapeutic agents such as Adriamycin, melphalan, vinblastine and docetaxel (Bates and Mackillop, 1990; Averill and Larrivée, 1998; Averill and Su, 1999; Larrivée and Averill, 2000; Dumontet et al., 1998, Liu et al., 2001). Therefore, hyperthermia appears to be a promising means for eliminating MDR tumor cells and/or improving their sensitivity to cytotoxic agents. An advantage is that heat is essentially non-toxic compared to the toxicity of chemosensitizers such as cyclosporin A, verapamil or PSC833, which limits their clinical use. However, little is known about the mechanisms by which heat shock/hyperthermia causes cell death by apoptosis in MDR cells.

This study investigates the molecular mechanisms of heat shock-induced cell death by apoptosis in Chinese hamster ovary cells, assessing the differences in responses to heat shock that could occur between drug-sensitive AuxB1 and MDR $CH^RC5$ cells overexpressing P-GP. A comprehensive look into possible differences that may exist in major apoptotic signaling pathways will provide a glimpse into

mechanisms that not only confer MDR in these cells, but also help to maintain MDR by evading cell death by apoptosis. These findings will also offer ideas on the potential clinical use of heat shock to increase sensitivity of MDR cells to apoptosis.

## MATERIALS AND METHODS

### CELL CULTURE

The MDR cell line $CH^RC5$ was selected for resistance to colchicine from the drug-sensitive AuxB1 parent cell line (Ling and Thompson, 1973). The resistance factor to colchicine is approximately 300-fold, but $CH^RC5$ cells are also cross-resistant to other anticancer drugs including Vinca alkaloids, Adriamycin and melphalan (Elliott and Ling, 1981). The CHO cell lines AuxB1 and $CH^RC5$ were grown in monolayers in tissue culture flasks at 37°C under 5% $CO_2$ in minimum essential medium (MEM) Alpha, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin (50 U/ml)-streptomycin (5 μg/ml) (Invitrogen Canada, Burlington, ON). The culture medium for the cell line $CH^RC5$ contained colchicine (5 μg/ml) (Sigma Chemical Co., St. Louis, MO), which was removed for the final passage before experiments. Studies were carried out using cells grown to confluence and incubated for 24 h at 37°C with fresh culture medium prior to each experiment. Cells were harvested with phosphate-buffered saline (PBS) containing sodium citrate (0.015 M), washed by centrifugation, and resuspended in PBS containing 1% bovine serum albumin (BSA) and 10 mM glucose (Turcotte and Averill-Bates, 2001).

### DETERMINATION OF CASPASE ACTIVITY

Freshly harvested $CH^RC5$ and AuxB1 CHO cells (1 x $10^6$/ml) were resuspended in PBS-BSA 1%, 10 mM glucose and incubated in a final volume of 1.0 ml at the appropriate temperature ranging between 37-43°C in temperature-controlled water baths (Haake D8, Fisher Scientific, Montreal), with a temperature precision of ± 0.02°C. Under these conditions, 5 ml of cell suspension reached a temperature within 0.1°C of the water bath temperature within 5 min. The time for heat shock treatment was calculated after the 5 min heating period. After the appropriate time, the cells were washed once with cold PBS by centrifugation (1000g, 3 min) to stop

the heat treatment. The cells were resuspended in 75 µl of reaction buffer (20 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES), 100 mM NaCl, 10 mM dithiothreitol (DTT), 1 mM EDTA, 0.1% 3-[(3 cholamidopropyl) dimethylammonio]-2-hydroxy-1-propanesulfonic acid (CHAPS), 10% sucrose, pH 7.2) (Stennicke and Salvesen, 1997) and were deposited into 96-well plates and broken by freezing at -20$^0$C for 20 minutes. The kinetic reaction was started after addition of 25 µl of the appropriate caspase substrate (50 µM) at 37°C and measured using a spectrofluorimeter (Spectra Max Gemini, Molecular Devices, Sunnyvale, CA). Caspase-3 activity was measured by cleavage of the fluorogenic substrate *N*-acetyl-Asp-Glu-Val-Asp-amino-4-methylcoumarin to produce amino methylcoumarin (AMC) and caspase-6 activity was measured by cleavage of the substrate Ac-VEID-AMC, with λmax excitation at 380 nm and λmax emission at 460 nm for both (Calbiochem, La Jolla, CA). Caspase-7 activity was measured by cleavage of the fluorogenic substrate MCA-VDQVDGWK(DNP)-$NH_2$ with λmax excitation at 325 nm and λmax emission at 395 nm. Caspase-8 activity was measured by cleavage of Z-IETD-AFC to produce amino trifluorocoumarin (AFC) and caspase-9 activity was measured by cleavage Ac-LEHD-AFC, with λmax excitation at 415 nm and λmax emission at 490 nm for both (Bettaieb and Averill-Bates, 2005). Activities of caspases-2 and -10 were measured by cleavage of the substrates ZVDVAD-AFC and AEVD-AFC respectively, with λmax excitation at 400 nm and λmax emission at 505 nm for both. Activities of caspases are represented as Vmax of the kinetic reaction.

## ANALYSIS OF PROTEIN EXPRESSION BY WESTERN BLOT

*1. Relative expression of Procaspases, FasR, FADD, c-Flip, Bax, Cytochrome c, Apaf-1, Bid, IAPs, Survivin and HSPs in AuxB1 and CH$^R$C5 cells:*

To assess and compare total levels of protein expression for apoptotic proteins between MDR CH$^R$C5 and AuxB1 cells, freshly harvested CHO cells (10 x 10$^6$/ml)

were resuspended in PBS-BSA 1%, 10mM glucose and then centrifuged (1000g, 3 min). Cells were then resuspended in 500 $\mu$l of lysis buffer H (10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM $Na_4P_2O_7$, 2 mM $Na_3VO_4$, 0.1% sodium dodecyl (lauryl) sulfate (SDS), 0.5% sodium deoxycholate, 1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulphonylfluoride (PMSF) and 100 $\mu$l/10 ml of protease inhibitor cocktail) (Sigma-Aldrich, St. Louis, MO) and incubated on ice (0°C) for 2 h and mixed every 15 min. Debris, unbroken cells and nuclei were removed by centrifugation (2500g for 10 min) and the supernatant was collected. Proteins were quantified according to Bradford (Bradford, 1976), solubilised in Laemmli sample buffer and then boiled for 5 min at 95°C. Protein samples (30 $\mu$g) were separated by SDS-polyacrylamide gel elecrophoresis (SDS-PAGE) according to Laemmli (Laemmli, 1970). Electrophoresis was carried out at a constant voltage of 125 V. Cellular proteins were transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane using a MilliBlot Graphite Electroblotter I apparatus (Milli-pore, Bedford, MA). The transfer buffer contained 96 mM glycine, 10 mM Tris and 10% methanol. The transfer was carried out for 1.5 h at constant amperage of 80 mA/gel. Hydrophobic or nonspecific sites were blocked either for 1 h at RT or overnight at 4°C with 5% powdered skim milk in Tris-buffered saline (50 mM Tris and 150 mM NaCl) containing 0.1% Tween 20 (TBS-T). Membranes were washed four times for 15 min in TBS-T (Tanel and Averill-Bates, 2005). The blots were probed with primary antibodies (1:1000): anti-Fas Receptor (FasR), anti-c-Flip, anti-Bax, anti-c-IAP1/2 and anti-survivin (Santa Cruz, CA), anti-Apaf-1 (Chemicon, Temicula, CA), anti-cytochrome *c* (BD Biosciences, Mississauga, ON), anti-Bid, anti-HSP90, anti-HSP70, anti-FADD (1:4000) (Stressgen, Victoria, BC) and Hsp27 L2R3 (gift from Dr. Jacques Landry, Centre de recherche, Hotel-Dieu de Québec, QC, Canada). Secondary antibodies consisted of horseradish peroxidase (HRP)-conjugated goat anti-mouse, anti-rabbit and anti-goat IgG (Biosource, Camarillo, CA). The PVDF membranes were incubated with the primary antibody in TBS-T, 1% BSA for 1 h at room temperature. Membranes were washed four times

for 15 min and incubated for 1 h at RT with peroxidase-conjugated secondary antibody (1:1000) in TBS-T containing 5% milk powder. PVDF membranes were washed four times for 15 min and proteins were detected using the ECL plus chemiluminescence kit (PerkinElmer, Boston, MA). For verification of equal protein loading, the blots were probed with anti-GAPDH (Santa Cruz, CA) antibody and by coloration of the gels using Coomassie blue. Protein expression was quantified using a scanning laser densitometer, relative to GAPDH (Molecular Dynamics, Sunnyvale, CA).

*2. Cleavage of Procaspases, ICAD and PARP:*

Early (1 h) and late (2 h) apoptotic events were investigated by the assessment of cleavage of initiator caspase procaspase-8, as well as caspase substrates ICAD and PARP. Freshly harvested $CH^RC5$ and AuxB1 cells (10 x $10^6$/ml) were resuspended in PBS-BSA 1%, 10 mM glucose and incubated in a final volume of 5 ml at the appropriate temperature ranging between 37-43 °C in temperature-controlled water baths. After the appropriate time, cells were washed once with cold PBS by centrifugation (1000 g, 3 min) to stop the heat treatment, resuspended in 500 $\mu$l of lysis buffer H and incubated on ice (0 °C) for 2 h, and mixed using quick vortex every 15 min. Debris, unbroken cells and nuclei were removed by centrifugation (2500 g for 10 min) and the supernatant was collected. Protein samples (30 ug) were separated by SDS-PAGE and immunoblotted with primary antibodies (1:1000): anti-ICAD, anti-PARP and anti-caspase-8 (Santa Cruz, CA), and appropriate secondary antibodies, as described above.

*3. Translocation of FADD, Bax, tBid and Cytochrome c:*

In order to investigate the translocation of apoptotic proteins between cellular compartments, induced by heat shock, cellular fractionation was performed as

described previously with modifications (Jurkiewicz and Averill-Bates, 2004; Samali et al., 1999). Following heat shock treatment for 1 h at temperatures ranging between 37-43°C, $CH^RC5$ and AuxB1 cells (20 x $10^6$/ml) were washed once with cold buffer A (100 mM sucrose, 1 mM EGTA, 20 mM MOPS, pH 7.4) and resuspended in lysis buffer B (100 mM sucrose, 1 mM EGTA, 20 mM MOPS, 0.1 mM (DTT), 5% freshly added percoll, 0.01% digitonin, 1 mM PMSF and 100 ul/10 ml of cocktail of protease inhibitors, pH 7.4). Membranes were broken using a dounce homogeniser (50 strokes/sample). Debris, unbroken cells and nuclei were removed by centrifugation (2500 g for 10 min) and supernatants were then centrifuged (15,000 g for 15 min) to separate mitochondria. Supernatants were collected and centrifuged (100,000 g for 1h) to separate the cytosolic fraction from the nucleosomal fraction. Mitochondrial fractions were resuspended in lysis buffer H. Protein samples (30 $\mu$g) for mitochondrial, cytosolic and/or microsomal fractions were separated by SDS-PAGE and immunoblotted with primary antibodies: anti-FADD (1:4000), anti-Bax, anti-cytochrome *c* and anti-tBid (Stressgen, Victoria, BC) (1:1000). Purity of subcellular fractions was verified by $GST\pi_1$ (Calbiochem, La Jolla, CA), cytochrome oxidase and calnexin antibodies (Molecular Probes, Eugene, OR), specific to cytoplasmic, mitochondrial and microsomal fractions, respectively.

## DETERMINATION OF APOPTOSIS BY FLOW CYTOMETRY

*1. Mitochondrial Membrane Potential ($\Delta\psi m$):*

Following heating at different temperatures (37-43°C) for 1 h, MDR $CH^RC5$ and AuxB1 cells ($1x10^6$) were incubated with the lipophilic cationic dye 5,5,6,6'-tetrachloro-1,1',3,3'tetra-ethyl-benzimidazolyl-carbocyanine-iodide (JC-1) (5 $\mu$M) (Molecular Probes) for 30 min at 37°C (Mancini et al., 1997). Cells were washed three times by centrifugation (2,500g, 3 min) and resuspended in 1 ml of cold PBS. Prior to analysis, 10 $\mu$g/ml of propidium iodide (PI) was added to stain necrotic cells.

Data were collected using a FACS scan flow cytometer equipped with an argon laser emitting at 488 nm and analyzed using Cell Quest software (Becton-Dickinson, Menlo Park, CA). Forward and side scatters were used to establish size gates and exclude cellular debris from the analysis. JC-1 emission was collected on FL-1 and FL-2 channels at 530 (± 20) nm and 590 (± 20) nm, respectively. The PI emission was collected on the FL-3 channel (620 ± 15 nm). Ten thousand cells were analyzed for each sample. Under normal conditions, JC-1 exists in a monomeric form and stains as green fluorescence (FL-1). JC-1 forms J-aggregates in the presence of increasing mitochondrial potential. These J-aggregates give a red fluorescence (FL-2). By comparing the green/red fluorescence ratio, after removing PI-stained necrotic cells, one can determine the changes in mitochondrial membrane potential ($\Delta\psi m$).

*2. Labeling of Cell Membranes with Annexin V-FITC:*

Apoptosis-induced externalization of phosphotidylserine (PS) was measured by Annexin V-FITC staining, which was performed as follows: after heat shock (1 h at 40-43°C), $CH^RC5$ and AuxB1 cells (1 x $10^6$/ml) were washed once with cold PBS. Cells were then resuspended in binding buffer (0.1 M HEPES, pH 7.4; 1.4 M NaCl; 25 mM $CaCl_2$) at 37°C at a concentration of 1 x $10^6$ cells per ml. One hundred $\mu$l of the solution was transferred and 5 $\mu$l of Annexin V-FITC (BD Biosciences, Mississauga, ON) was added. The cells were then resuspended and incubated for 15 min at RT in the dark. Once the incubation was completed, 400 $\mu$l of binding buffer was added as well as 5 $\mu$l of PI (1 mg/ml). Samples were analyzed by flow cytometry as described previously (Averill-Bates et al., 2005). The intensities of fluorescence emitted by Annexin V and PI were collected on FL1 and FL3 channels, respectively.

## DETERMINATION OF APOPTOSIS BY FLUORESCENCE MICROSCOPY

*Hoechst Staining:*

$CH^RC5$ and AuxB1 cells were heated (37-43°C) for 2 h in temperature controlled water baths. The Fas/FasL antagonist, Kp7-6 (100 $\mu$M) was added 1 h before heat treatment, where appropriate. Thereafter, Hoechst 33258 (50$\mu$g/ml) (blue fluorescence) (Sigma Chemical Co., St. Louis, MO), which binds to condensed chromatin in the nucleus of apoptotic cells, was added for 15 min along with 5$\mu$M of cyclosporin A, to prevent efflux of the Hoechst probe by P-GP. PI (50$\mu$g/ml) was subsequently added to visualize necrotic cells (red fluorescence) by fluorescence microscopy (model IM, Carl Zeiss Canada Ltd, St Laurent, QC) and photographs were taken by digital camera (camera 3CCD, Sony DXC-950P, Empix Imaging Inc, Mississauga, ON). Images were analysed by Northern Eclipse software (Tanel and Averill-Bates, 2005). Cells were classified using the following criteria: (a) live cells (normal nuclei, pale blue chromatin with organized structure); (b) membrane-intact apoptotic cells (bright blue condensed or fragmented chromatin); (c) necrotic cells (red, enlarged nuclei with smooth normal structure) (Lee and Shacter, 1999). The fractions of apoptotic and necrotic cells were determined relative to the total cells (obtained using bright field illumination). At least 300 cells were counted for each condition.

## STATISTICS

Data are presented as means ± standard error of the mean (SEM) from at least 3 independent experiments performed with multiple estimations per point. Statistical analysis for comparisons between the two cell lines at each temperature was performed with one-way analysis of variance (ANOVA), paired Student's t-test, or

Fisher's least significant difference test, as appropriate. Statistical comparison between the heat shocked MDR $CH^RC5$ cells and non treated control MDR $CH^RC5$ cells was made using one-way ANOVA with Dunnett adjustment. Software used was JMP Statistical Discovery 4.0 (SAS Institute Inc., Cary, NC). Differences were considered statistically significant at $P < 0.05$.

## RESULTS

*Heat shock-induced apoptosis via the death receptor pathway*

The death receptor pathway of apoptosis is characterized by the activation of the initiator caspase-8 through association of procaspase-8 with death receptors and adaptor proteins like FADD. In AuxB1 cells, FADD protein levels showed a significant decrease in the cytosol (Fig. 1A-B) as the FADD protein was recruited to the DISC during heat shock induced apoptosis. In $CH^RC5$ cells, FADD protein levels in the cytosol were higher than in AuxB1 cells at all temperatures and showed a slight decrease only at 43°C relative to 37°C (Fig. 1B). Following the 1h heat shock treatment at 41-43°C, FADD protein levels significantly increased at the cellular membrane level in both AuxB1 and $CH^RC5$ cells (Fig. 1C-D). This increase in the plasma membrane levels of FADD with increasing temperatures was similar in both cell lines (Fig. 1C-D). Purity of cytoplasmic and microsomal fractions was 98 and 92%, respectively (Fig. 1E).

Cleavage of procaspase-8 was also investigated by assessing protein levels of procaspase-8 with increasing temperatures. It was found that, although, AuxB1 cells show a significant decrease in procaspase-8 levels with increasing temperatures from 41 to 43°C, this trend is also present in $CH^RC5$ cells (Fig. 2A-B) to a lesser extent. A significant increase in enzymatic activity of caspase-8 was detected in AuxB1 cells at 42-43°C (Fig. 2C). There was a 2–fold increase in activity at 43 °C. However, $CH^RC5$ cells responded differently to heat shock, with no initial activation of caspase-8 at temperatures of 40-42°C followed by a 2.5–fold increase in activity at 43°C (Fig. 2C). The activity of two other initiator caspases was also assessed, caspase-10 and -2, which both showed a significant increase in activity at 42-43°C in AuxB1 cells and at 43°C in $CH^RC5$ cells, compared to 37°C (Fig. 3A-B).

In order to assess the molecular mechanisms of heat shock induced apoptosis in MDR $CH^RC5$ compared to their drug sensitive counterparts AuxB1 cells, it is important to consider any differences that may exist between the two cell lines due to

selection for MDR. Thus, the relative expression of several pro- and anti-apoptotic proteins in the death receptor mediated pathway was measured and some variance was observed in total protein levels between the two cell lines. $CH^RC5$ cells show slightly higher mean levels of endogenous proteins for both c-$FLIP_S$ and c-$FLIP_L$ and slightly lower mean levels of endogenous FADD protein, compared to AuxB1 cells (Fig. 4A-E).

*Cleavage of pro-apoptotic protein Bid in heat shock-induced apoptosis*

A converging point exists between the extrinsic and intrinsic apoptotic pathways. This point involves the cleavage of pro-apoptotic protein Bid into tBid by active caspase-8. The tBid can then translocate to the mitochondrial membrane where it can induce or amplify mitochondria-mediated apoptosis. A gradual significant decrease in Bid protein levels was observed in the cytosol with increasing temperatures of 41-43°C in both cell lines (Fig. 5A-B). There was a corresponding significant increase in tBid in mitochondria of AuxB1 cells at 41-43°C, and a tendency to increase in $CH^RC5$ cells (Fig. 5C-D). Purity of cytoplasmic and mitochondrial fractions was 98 and 97%, respectively (Fig. 1E). $CH^RC5$ cells have significantly higher Bid levels in the cytosol at 37°C, compared to AuxB1 cells, and undergo a higher overall decrease in Bid levels with increasing temperatures (40-43°C) (Fig. 5B).

*Heat shock-induced apoptosis via the mitochondrial pathway*

The mitochondrial pathway of apoptosis involves the release of cytochrome *c*, among other pro-apoptotic molecules, from mitochondria into the cytosol, where it associates with Apaf-1 and procaspase-9 leading to activation of caspase-9. The release of pro-apoptotic proteins from the mitochondria is mediated either by other upstream pro-apoptotic proteins, such as Bax and/or by mitochondrial membrane depolarization via the opening of the permeability transition pore. As illustrated in Fig. 6A-D, there was significant induction of Bax translocation from the cytosol to

the outer mitochondrial membrane in both AuxB1 and $CH^RC5$ cells upon 1 h heat shock treatment at 41-43°C. At 37°C, the level of Bax appears to be higher in mitochondria in $CH^RC5$ than in AuxB1 cells. Depolarization of the mitochondrial membrane in both cell lines induced a shift in JC-1 fluorescence from the FL2 to FL1 channel in response to increasing temperatures of 40-43°C (Fig. 7A-E, 7G-K). The relative fluorescence indicating mitochondrial membrane potential showed a significant decrease in membrane potential in both cell lines in response to heat, using p-(trifluoro-methoxy) phenylhydrazone (FCCP) as a positive control (Fig. 7F,7L,7M). Depolarization of mitochondrial membrane potential was significant at temperatures of 42-43°C in $CH^RC5$ and 41-43°C in AuxB1 cells (Fig. 7M).

These events were followed by the release of cytochrome *c* from the mitochondria into the cytosol. A well-defined, significant decrease in cytochrome *c* levels occurred in the mitochondria at 41-43°C, with a corresponding increase in the protein in the cytosol in response to heat shock (Fig. 8A-D). Enzymatic activity of caspase-9 exhibited a similar increase in both cell lines at 42-43 °C (Fig. 9A). There was a 3 –fold increase in activity in AuxB1 and $CH^RC5$ cells at 43°C, relative to the control at 37°C. This was confirmed by a gradual and similar decrease in procaspase-9 protein levels in the cytosol with increasing temperatures of 41-43 °C, in AuxB1 and $CH^RC5$ cells (data not shown).

The relative expression of several pro- and anti-apoptotic proteins in the mitochondria mediated pathway was also measured and some variance was observed in total protein levels between the two cell lines. $CH^RC5$ cells show slightly lower mean levels of endogenous proteins of cytochrome *c* and procaspase 9 and higher mean levels endogenous Apaf-1 protein (Fig. 10A-E).

*Activation of effector caspases-3 and -7 and cleavage of their substrates in heat shock-induced apoptosis*

Effector caspases such as caspase-3 and -7 play a critical role in disassembly of the cell during later stages of apoptosis. Following exposure to heat shock, a

gradual increase in activity of caspase-3 was observed with increasing temperatures of 42-43°C in AuxB1 and $CH^RC5$ cells (Fig. 11A). A significant 4-fold increase in activity was observed in both cell lines at 43°C. There was a decrease in protein levels of procaspase-3 with increasing temperatures in AuxB1 cells, and to a lesser extent in $CH^RC5$ cells (data not shown). The activity of caspase-7 followed a somewhat different trend with a gradual, significant increase in activity in AuxB1 cells, yet, almost no activation with heat shock in $CH^RC5$ cells (Fig. 11B).

Once activated, effector caspases cleave many different substrates including other procaspases, propelling the process of apoptosis. Caspase-3, being the main effector caspase involved in apoptosis, cleaves many important substrates, which leads to the irreversible destruction of the cell. Two of these substrates, PARP and ICAD are involved in nuclear chromatin fragmentation and condensation. PARP (116 kDa) is cleaved into two fragments of 85 and 24 kDa by caspase-3. The cleavage of PARP was evaluated by measuring protein levels of PARP and its fragment in the cytosol. A significant decrease in full length PARP was found in response to 2 h heat shock temperatures of 41-43°C in $CH^RC5$ cells and 43°C in AuxB1 cells (Fig. 12A-B). ICAD (47 kDa) is normally an inhibitor of CAD and displays its function by binding CAD and rendering it unable to cross the nuclear membrane and carry out its pro-apoptotic effects on chromatin in the nucleus. The cleavage of ICAD by caspase-3 releases CAD and allows it to degrade DNA leading to apoptosis. Levels of ICAD in the cytosol significantly decreased with exposure to increasing temperatures of 41-43 °C for 2 h in both AuxB1 and $CH^RC5$ cells (Fig. 12C-D).

The relative expression of several pro- and anti-apoptotic proteins was measured and some variance was observed in total protein levels between the two cell lines. $CH^RC5$ cells display lower endogenous protein levels of cIAP-1 and –2, Survivin and HSP70; and higher endogenous protein levels of HSP27 (Fig. 13A-I).

*Apoptosis levels in AuxB1 and $CH^RC5$ cells*

Aside from the involvement of particular pro- and anti-apoptotic proteins in heat shock induced cell death, it is equally important to have a global view of the levels of apoptosis in both AuxB1 and $CH^RC5$ cells, confirming the event. For this purpose, the levels of apoptosis in both cell lines were measured using Annexin V-FITC as a marker of apoptosis. Both cell lines exhibit an increase in apoptosis at temperatures of 42-43°C, compared to 37°C (Fig. 14A-K). It is important to note that levels of apoptosis are significantly higher in $CH^RC5$ than in AuxB1 cells at all temperatures.

Further, nuclear chromatin condensation of apoptotic cells was investigated using Hoechst staining and visualized by fluorescent microscopy. Both cell lines exhibit a significant increase in apoptosis at temperatures of 41-43°C with no initial apoptosis in both cell lines at 37 and 40°C (Fig. 15A-F). In comparison to cells treated only with heat shock, the cells treated with Fas/FasL antagonist show lower levels of apoptosis at temperatures of 41-43°C (Fig. 15A-F). Low levels of necrosis were detected at 37 to 43°C compared to apoptosis, with no significant differences between the two cell types (Fig. 15G).

## DISCUSSION

This study shows the molecular mechanisms of heat shock induced apoptosis in MDR $CH^RC5$ cells relative to their drug-sensitive counterparts CHO AuxB1 cells. A detailed approach, encompassing the key players in both the extrinsic and intrinsic apoptotic pathways, was taken. The study shows that heat shock of 42 and 43°C for 1-2 h is sufficient to induce apoptosis involving receptor and mitochondrial pathways, activation of caspases and cleavage of their substrates. The study also illustrates that heat shock alone can induce apoptosis in MDR $CH^RC5$ cells. It is clear that there are differences between drug-sensitive and MDR cells in the induction of apoptosis with heat. These differences can be seen mainly in the extrinsic pathway as well as at the level of endogenous expression of several apoptotic proteins.

The effects of heat shock on key players in the receptor mediated pathway, mainly FADD and procaspase-8, differ between AuxB1 and $CH^RC5$ cells. In $CH^RC5$ cells, cytosolic levels of FADD and procaspase-8 proteins remain relatively high despite heat shock even at 43°C. This may point to some differences in the apoptotic machinery of the MDR $CH^RC5$ cells as it has been shown in the past that the cleavage of pro-caspase-8 was completely blocked in human leukemia T-cells resistant to treatment, although it was unperturbed in parental cells (Friesen et al., 1996). Nevertheless, despite higher trends in the cytosol of $CH^RC5$ cells, translocation of FADD to the plasma membrane is similar in the two cell lines, increasing gradually with increasing temperatures. $CH^RC5$ cells exhibit much lower caspase-8 activity than AuxB1 cells at 40-42°C. However, the main difference is that caspase-8 activity increases only at 43°C in $CH^RC5$ cells, thus the gradual heat induced increase in caspase activation seen at milder temperatures in AuxB1 cells is missing here. These results show that $CH^RC5$ cells are resistant to heat shock temperatures in terms of the receptor mediated pathway and show defects in receptor-mediated apoptotic machinery. Another important difference is the higher endogenous levels of c-FLIP protein in $CH^RC5$ cells, which may point to another mechanism of resistance in these

cells to heat shock induced receptor mediated pathway of apoptosis. c-FLIP functions as an inhibitor of caspase-8, preventing its activation at the DISC via binding site competition. This could explain the lower caspase-8 activity in $CH^RC5$ cells. However, recently it has been found that under certain conditions where cellular levels of $FLIP_L$ are high, it may act as an activator of caspase-8 (Chang et al., 2002; Micheau et al., 2002; Boatright et al., 2004). The involvement of the Fas death receptor pathway in heat shock induced apoptosis in both AuxB1 and $CH^RC5$ cells was assessed using the Fas/FasL antagonist by fluorescent microscopy using Hoechst 33258. It appears that the Fas receptor pathway plays an important role as levels of apoptosis in response to heat shock decrease in Fas/FasL antagonist treated cells.

Moreover, it has been previously shown that $CH^RC5$ cells have a higher content of P-GP in their plasma membrane than AuxB1 cells (Turcotte and Averill-Bates, 2001). P-GP is localized to membrane microdomains, which are detergent resistant (detergent resistant membranes, DRMs) and up-regulated in MDR cells (Lavie et al., 1998; Liscovitch and Lavie, 2000). DRMs are made of lipid rafts and caveolae, which are laterally separated into cholesterol- and sphingolipid-rich domains of the plasma membrane (Simons and Toomre, 2000; Razani et al., 2002; van Meer, 2002). These microdomains are relatively rigid and less fluid than the surrounding plasma membrane, which is partly caused by their cholesterol content (London and Brown, 2000). Lipid rafts are highly dynamic and prone to rapid assembly and disassembly, contributing to dynamic protein segregation (Simons and Toomre, 2000; Fasterberg et al., 2003). Raft localization has been shown to modulate an array of proteins such as receptor function and activity (Gimpl et al., 1997), and ATPase activity of P-GP (Rothnie et al., 2001; Garrigues et al., 2002). P-GP appears to exist in its more active state when localized inside lipid rafts (Ghetie et al., 2004) and membrane fluidization has been shown to impair P-GP function (Sinicrope et al., 1992; Ragev et al., 1999). Moreover, acute depletion of cholesterol impacts P-GP mediated drug transport in a substrate- and cell-type-specific manner (Luker et al., 2000). A recent study showed that cholesterol depletion removes P-GP from raft

membranes into non DRM fractions, whereas repletion fully reconstitutes raft localization. Furthermore, cholesterol depletion reduces P-GP function in MDR cells resulting in intracellular substrate accumulation (Troost et al., 2004). It has been shown by Elyassaki and Wu (2004) that lipid rafts are involved in mediating UV-induced Fas receptor aggregation, independently of Fas ligand. The study showed that stress by UV impacted the composition of lipid rafts by reducing cholesterol and increasing ceramide levels. The compositional change resulted in the clustering of lipid rafts, resulting in aggregation of FasR, which led to subsequent recruitment of FADD and activation of caspase-8.

It has been recently suggested that one mechanism of FasR-mediated apoptosis might be via change in membrane composition, due to an external stressor, particularly an increased clustering of lipid rafts. It may be that this is a major mechanism by which receptor-mediated apoptosis in response to stressors like UV and heat shock occurs in cells. Cellular membranes, particularly plasma membrane are known to undergo temperature-induced "disorganization" to a point where a lipid transition causes a membrane structural change, which results in cell death (Yatvin, 1977; Yatvin et al, 1987; Yatvin, 1987; Yatvin and Cramp, 1993). Endogenous levels of FasR protein do not differ between AuxB1 and $CH^RC5$ cells. However, since MDR $CH^RC5$ cells contain higher levels of P-GP in their plasma membrane than AuxB1 cells and that P-GP is localized and more active in lipid rafts, then perhaps high levels of P-GP present in lipid rafts in the plasma membrane of $CH^RC5$ cells could interfere in the recruitment of FasR into lipid rafts in response to heat shock and thus the activation of the receptor mediated pathway of apoptosis in these cells. Yet, a decrease in cholesterol, as seen with UV stress, should render P-GP non functional, sequestering it from the lipid rafts. It has previously been shown that the reduction in cholesterol is not dependent on UV-dosage, but it cannot be rebuked that it is not dependent on temperature. In the future, this avenue needs to be more closely investigated, in order to establish the role of heat shock in P-GP function and plasma membrane cholesterol levels.

It is equally important to note that FasR aggregation does not necessarily have to correlate with Fas-mediated apoptosis. It has been shown in the past that these events are not directly correlated and may even be inversely correlated (Lee and Shacter, 2001). Furthermore, one must consider the role of ceramide in induction of apoptosis in response to heat shock. Ceramide is extensively involved in apoptosis via several routes. One route involves initiation of apoptosis by permeabilization of the mitochondrial outer membrane to pro-apoptotic proteins, such as cytochrome *c*, AIF and Smac/DIABLO which are located in the intermembrane space. This permeabilization can be performed by ceramide directly because ceramides are able to form channels in planar phospholipid membranes (Siskind and Colombini, 2005) and in liposomes (Stiban et al., 2006).

Several pro-apoptotic changes were found in $CH^RC5$ cells, which may compensate for resistance observed in the receptor-mediated pathway in these cells. Differences in the mitochondria-mediated pathway of apoptosis in AuxB1 and $CH^RC5$ cells in response to heat shock can be observed. Bax protein levels in the mitochondria of $CH^RC5$ cells are higher than those in AuxB1 cells and only a slight increase with increasing temperature can be observed. Translocation of Bax from the cytosol to the mitochondria, where Bax oligomerizes into a high molecular weight complex, leads to the permeabilization of the mitochondrial outer membranes (Eskes et al., 2000; Antonsson et al., 2001; Kuwana et al., 2001). Bax was also shown to interact with voltage-dependent anion channel (VDAC) (Shimizu et al., 1999) and adenine nucleotide translocator (Marzo et al., 1998), which partially compose the mitochondrial permeability transition pore. Thus, Bax may function in mitochondria by facilitating the opening of the mitochondrial permeability transition pore eventually leading to mitochondrial swelling, rupture of the outer membrane and release of several pro-apoptotic proteins. Endogenous levels of proapoptotic protein Apaf-1, which normally constitutes the apoptosome and is crucial for the execution of the mitochondria mediated apoptotic pathway, were elevated in $CH^RC5$ compared to AuxB1 cells. These observations along with earlier results may point to the

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dominance of the mitochondria-mediated pathway in heat shock-induced apoptosis in these MDR cells. Further, a temperature dependent decrease in mitochondrial membrane potential can be seen in both AuxB1 and $CH^RC5$ cells.

Crosstalk between the receptors and the mitochondria occurs during apoptosis via the pro-apoptotic protein Bid that is normally cleaved to tBid by activated caspase-8. Despite lower activity of caspase-8 in $CH^RC5$ cells, Bid cleavage and tBid translocation to the mitochondria is similar to that in AuxB1 cells. Thus, the cleavage of Bid may be attributed to other proteases such as caspase-2 and -10 (Bonzon et al., 2006; Fischer et al., 2006; Milhas et al., 2005). The activation of these caspases, as well as caspase-8, with heat shock confirms their involvement in heat shock induced apoptosis in both AuxB1 and $CH^RC5$ cells. Although not significant, the mitochondrial membrane potential appears to be slightly lower in $CH^RC5$ than in AuxB1 decreasing even further with heat shock. This suggests that $CH^RC5$ cells have a pro-apoptotic tendency and this may be attributed to lower levels of anti-apoptotic proteins such as HSP70, Survivin and cIAPs.

Other differences between AuxB1 and $CH^RC5$ cells can be seen downstream of the heat shock induced initiation of either the receptor- and/or mitochondria-mediated pathways of apoptosis. Procaspase-3 protein levels are generally higher in $CH^RC5$ than in AuxB1 cells and remain fairly high even with heat shock temperatures of 42 and 43°C. However, caspase-3 activity correlates well between the two cell lines and in $CH^RC5$ cells does not reflect the lower cleavage of procaspase-3. This does not necessarily point to a mechanism of resistance in $CH^RC5$ cells as it has been previously shown that caspase activity does not always correlate with procaspase cleavage (Salvesen and Dixit, 1999). Further, caspase-7 activity remains relatively constant in $CH^RC5$ cells while it increases in AuxB1 cells with increasing temperature. However, like caspase-3, caspase-7 is an effector caspase and although it has many substrates for cleavage it shares most of them with caspase-3 (Earnshaw et al., 1999). Therefore, caspase-7 does not appear to play a role in downstream events of apoptosis in response to heat shock in $CH^RC5$ cells.

Finally, the difference in the percentage of apoptosis seen between AuxB1 and $CH^RC5$ cells using Annexin V binding implies that MDR $CH^RC5$ cells have much higher levels of apoptosis even under control conditions of 37°C. Annexin V is a fluorescent dye that binds externalized phosphatidylserine (PS). Phosphatidylserines normally reside on the inner leaflet of the plasma membrane bilayer, however, upon induction of apoptosis they flip to the outside of the plasma membrane and are able to selectively bind Annexin V. This is not possible in vital cells where PS is confined to the cytosolic face of the plasma membrane. It has recently been found that P-GP, in addition to its drug transporter function, can also act as a flippase of phospholipids, such as PS, and by remaining mobile in the phospholipid bilayer of the plasma membrane it contributes to the asymmetric lipid distribution in the outer and inner leaflet of the bilayer (Romsicki and Sharom, 2001; Pohl et al., 2002; van Meer, 2002). Thus, the high levels of AnnexinV-PS fluorescence in MDR $CH^RC5$ cells are misleading and appear to be due to the presence of increased amounts of P-GP in their plasma membrane and not necessarily to a higher percentage of apoptotic cells. Our results confirm that Annexin V cannot be used as an accurate indicator of apoptosis in P-GP overexpressing cells. Nevertheless, overall both cell lines respond identically to heat shock induced apoptosis.

In order to get a better indication of apoptotic levels in MDR $CH^RC5$ cells, fluorescence microscopy using Hoechst 33258 stain was used. Hoechst 33258 binds to condensed chromatin in the nucleus of apoptotic cells thus giving an assessment of apoptotic levels independent of P-GP function. The results showed no differences in apoptosis in either AuxB1 or $CH^RC5$ cells at temperatures of 37 and 40°C as was seen with Annexin V, and apoptosis was shown at 41-43°C. Apoptotic levels appear to be higher in $CH^RC5$ then in AuxB1 cells at 42-43°C. This may be attributed to lower endogenous levels of anti-apoptotic proteins such as IAPs and Survivin in these cells. The trend for heat shock induced apoptosis is remarkably similar in both cell lines, whether assessed by Hoechst staining or Annexin V and in both cases $CH^RC5$ cells

appear to compensate for their differences in importance of apoptotic pathways to give an overall nearly identical response as the parental cells.

In conclusion, there is variance in heat shock induced apoptosis between drug-sensitive AuxB1 and MDR $CH^RC5$ cells. It appears that $CH^RC5$ cells are resistant to heat shock via the receptor mediated pathway of apoptosis. Differences in endogenous expression of anti-apoptotic proteins such as c-Flip may play a role in the mechanism of receptor mediated resistance to heat shock induced apoptosis in these cells. The results suggest that the mitochondria mediated pathway of apoptosis is the main route of heat shock induced cell death in $CH^RC5$ cells. Downstream effects of heat induced apoptosis, such as the activation of caspase-3 and cleavage of ICAD and PARP, imply that $CH^RC5$ cells respond as equally to heat shock induced apoptosis as AuxB1 cells. Heat shock or hyperthermia is already being used in the clinic in combination with chemotherapy or radiotherapy aiming to once again render tumor cells sensitive to these cytotoxic treatments. This has been relatively successful, however, the molecular mechanisms by which heat may sensitize tumor cells to cell death is not well understood. A useful benefit of hyperthermia in the cancer clinic is that it has the potential to directly kill MDR cells. The consequences of the presence of P-GP in MDR cells have not all been elucidated. Understanding of the mechanisms of P-GP-dependent MDR in response to heat would be highly beneficial when designing patient treatments. This is the first *in vitro* study which shows the molecular mechanisms of heat shock induced apoptosis that differ between drug sensitive AuxB1cells and MDR $CH^RC5$ cells overexpressing P-GP.

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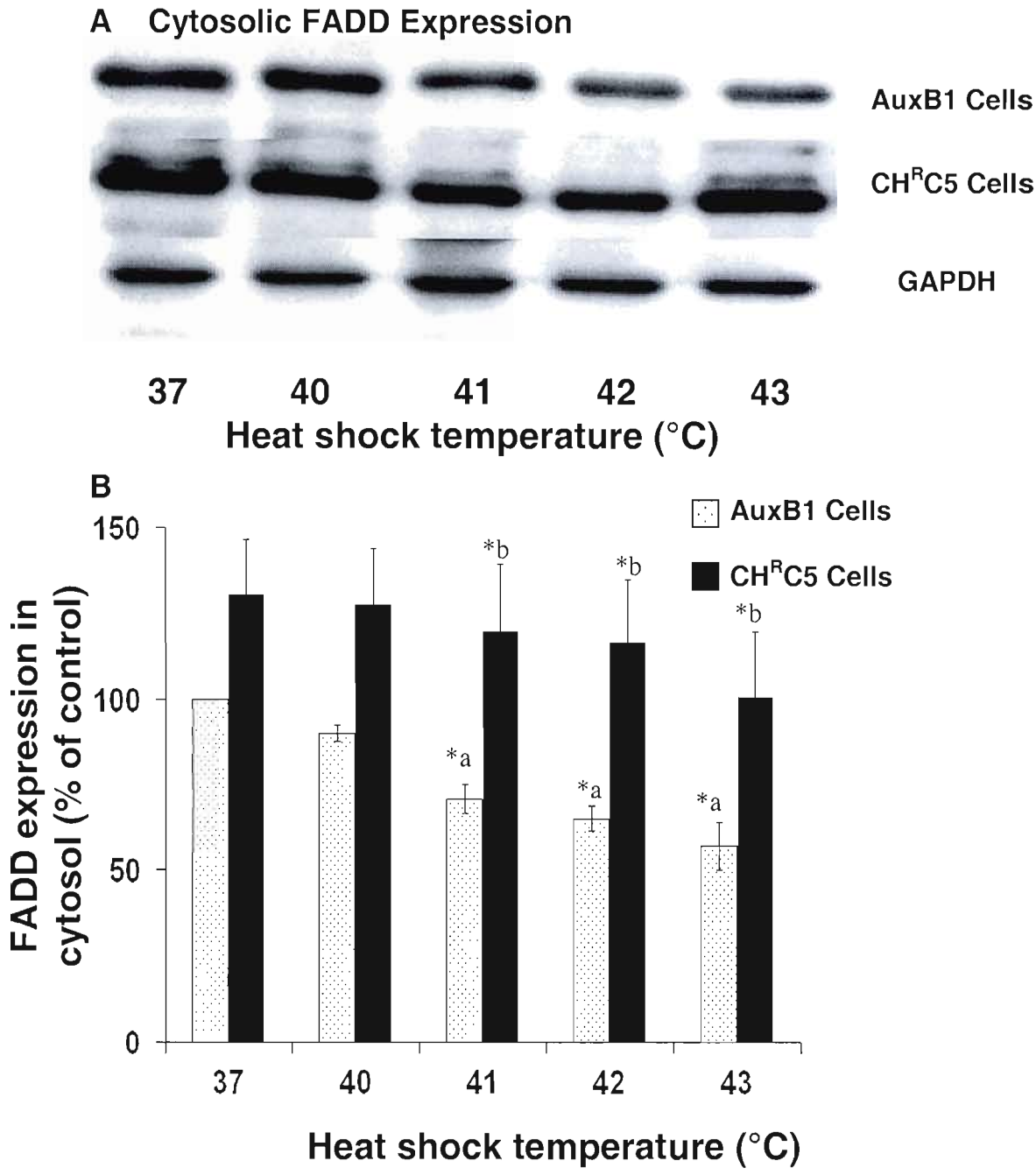

A Cytosolic FADD Expression
AuxB1 Cells
CH^RC5 Cells
GAPDH
37
40
41
42
43
Heat shock temperature (°C)
B
AuxB1 Cells
CH^RC5 Cells
FADD expression in cytosol (% of control)
150
100
50
0
*a
*b
37
40
41
42
43
Heat shock temperature (°C)

Figure 1, A-B

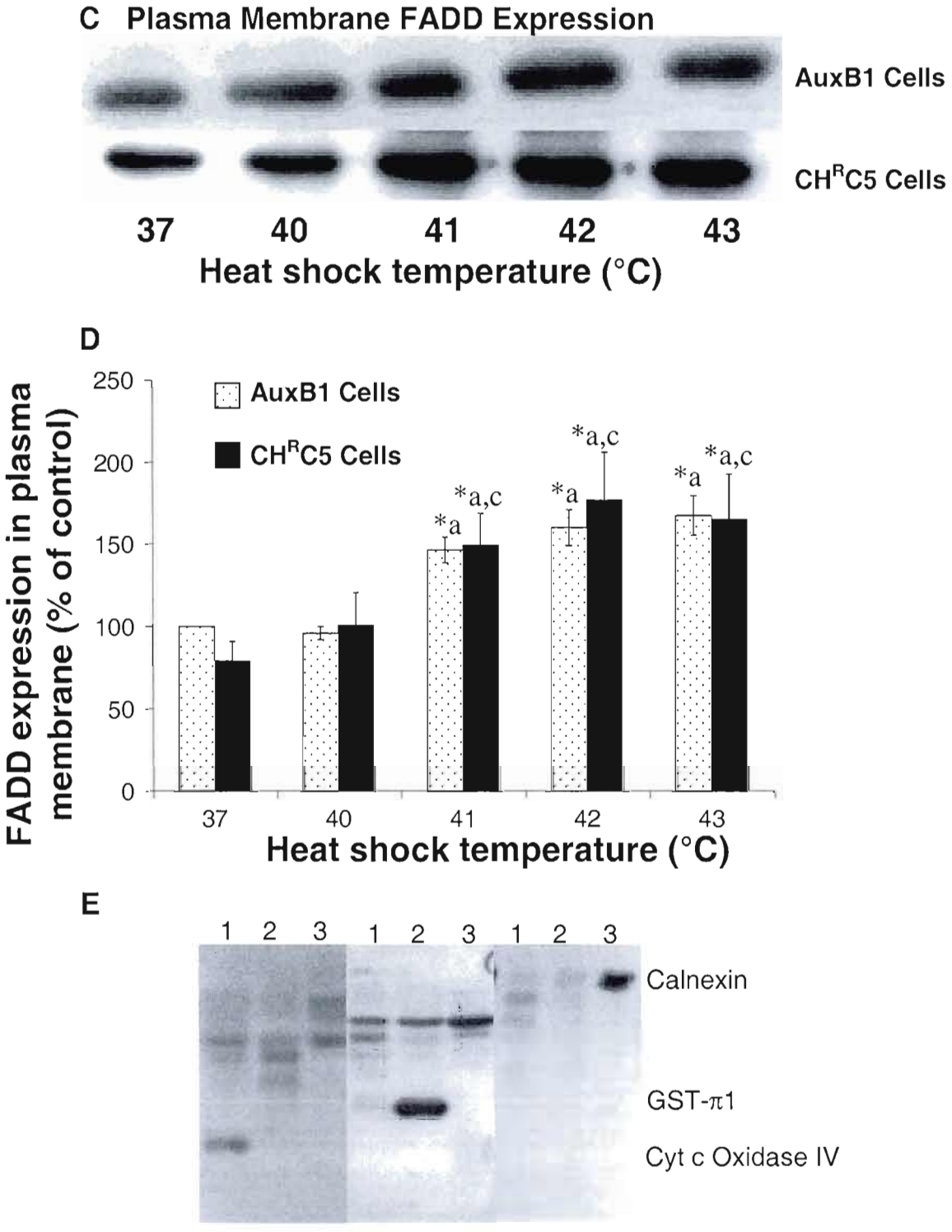
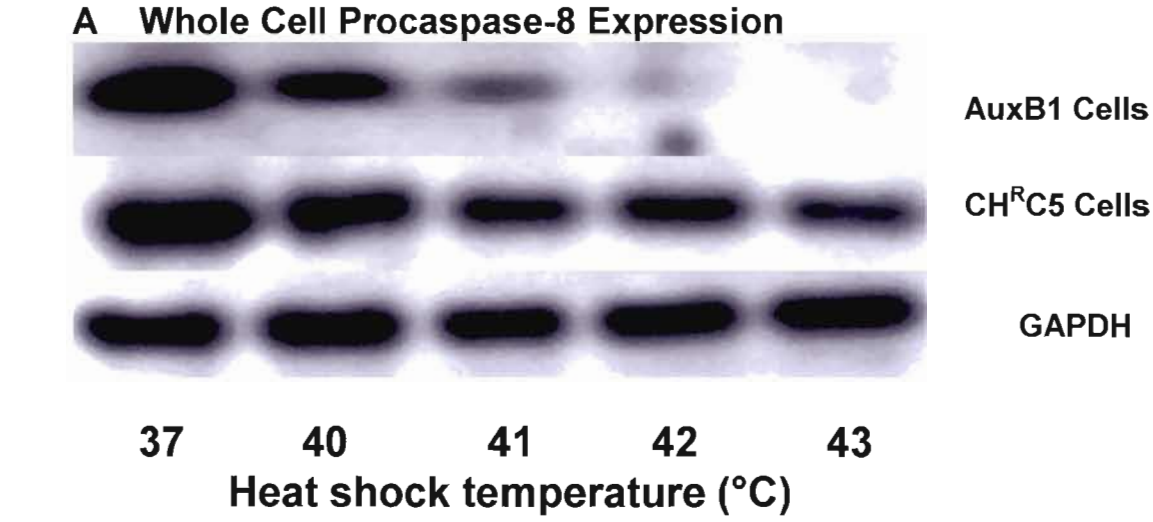

C Plasma Membrane FADD Expression
AuxB1 Cells
CH^RC5 Cells
37
40
41
42
43
Heat shock temperature (°C)
D
FADD expression in plasma membrane (% of control)
AuxB1 Cells
CH^RC5 Cells
250
200
150
100
50
0
*a
*a,c
37
40
41
42
43
Heat shock temperature (°C)
E
1 2 3 1 2 3 1 2 3
Calnexin
GST-π1
Cyt c Oxidase IV

**Figure 1, C-E**


A Whole Cell Procaspase-8 Expression
AuxB1 Cells
CH^RC5 Cells
GAPDH
37
40
41
42
43
Heat shock temperature (°C)

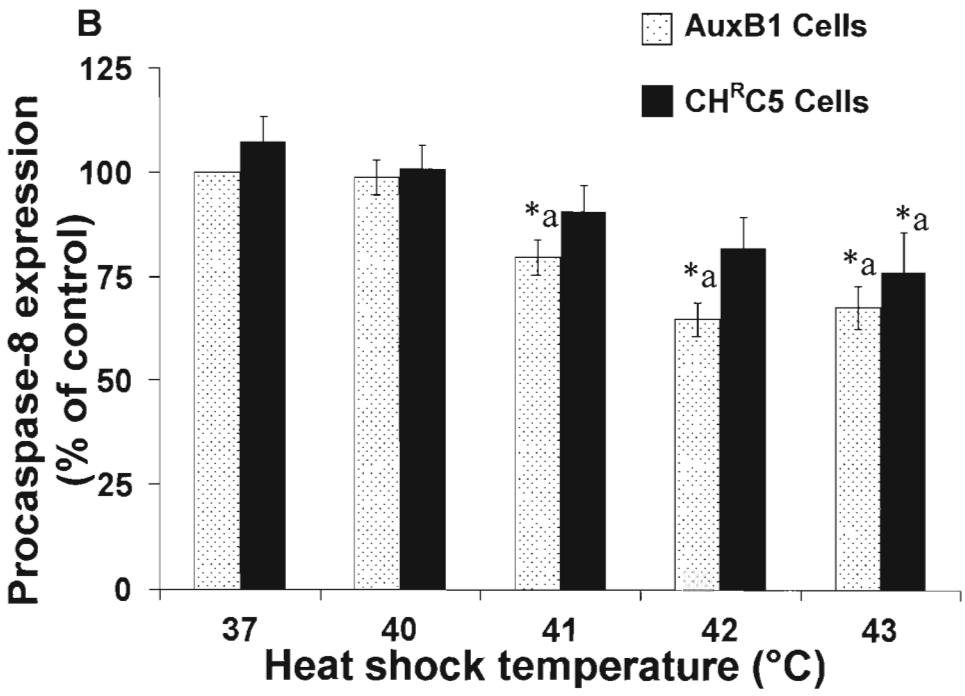

B
AuxB1 Cells
CH^RC5 Cells
Procaspase-8 expression (% of control)
125
100
75
50
25
0
*a
*a
*a
37
40
41
42
43
Heat shock temperature (°C)

Figure 2, A-B

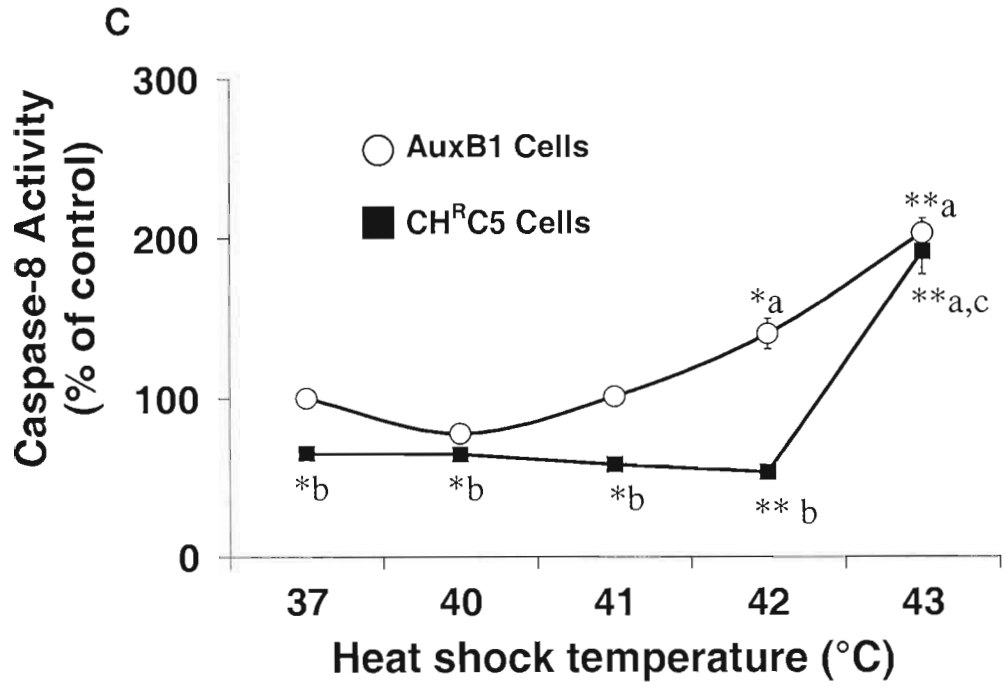

C
Caspase-8 Activity
(% of control)
300
200
100
0
AuxB1 Cells
CH^RC5 Cells
**a
*a
**a,c
*b
*b
*b
** b
37
40
41
42
43
Heat shock temperature (°C)

Figure 2, C

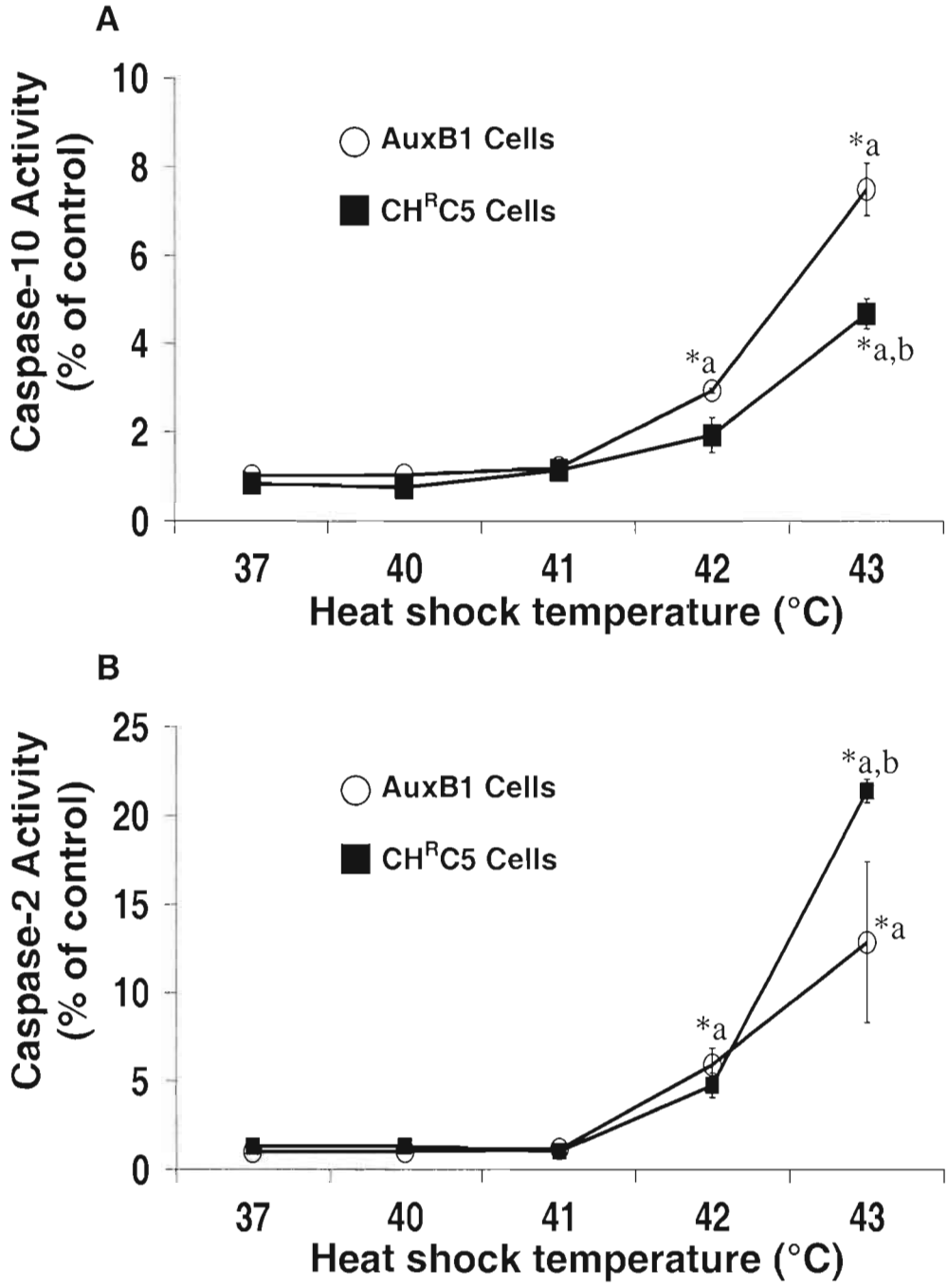

A
Caspase-10 Activity
(% of control)
10
8
6
4
2
0
AuxB1 Cells
CH^RC5 Cells
*a
*a
*a,b
37
40
41
42
43
Heat shock temperature (°C)
B
Caspase-2 Activity
(% of control)
25
20
15
10
5
0
AuxB1 Cells
CH^RC5 Cells
*a,b
*a
*a
37
40
41
42
43
Heat shock temperature (°C)

**Figure 3, A-B**

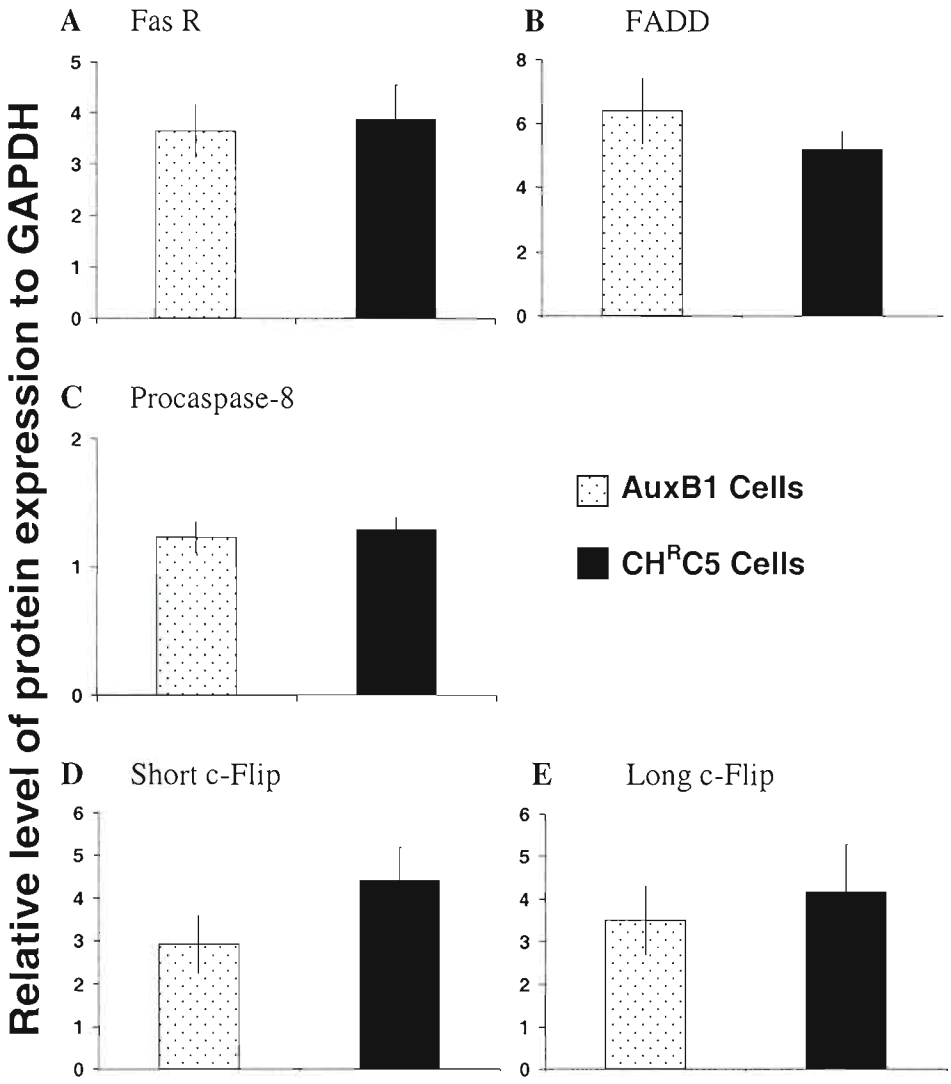

A Fas R
B FADD
C Procaspase-8
D Short c-Flip
E Long c-Flip
Relative level of protein expression to GAPDH
AuxB1 Cells
CH^RC5 Cells

**Figure 4, A-E**

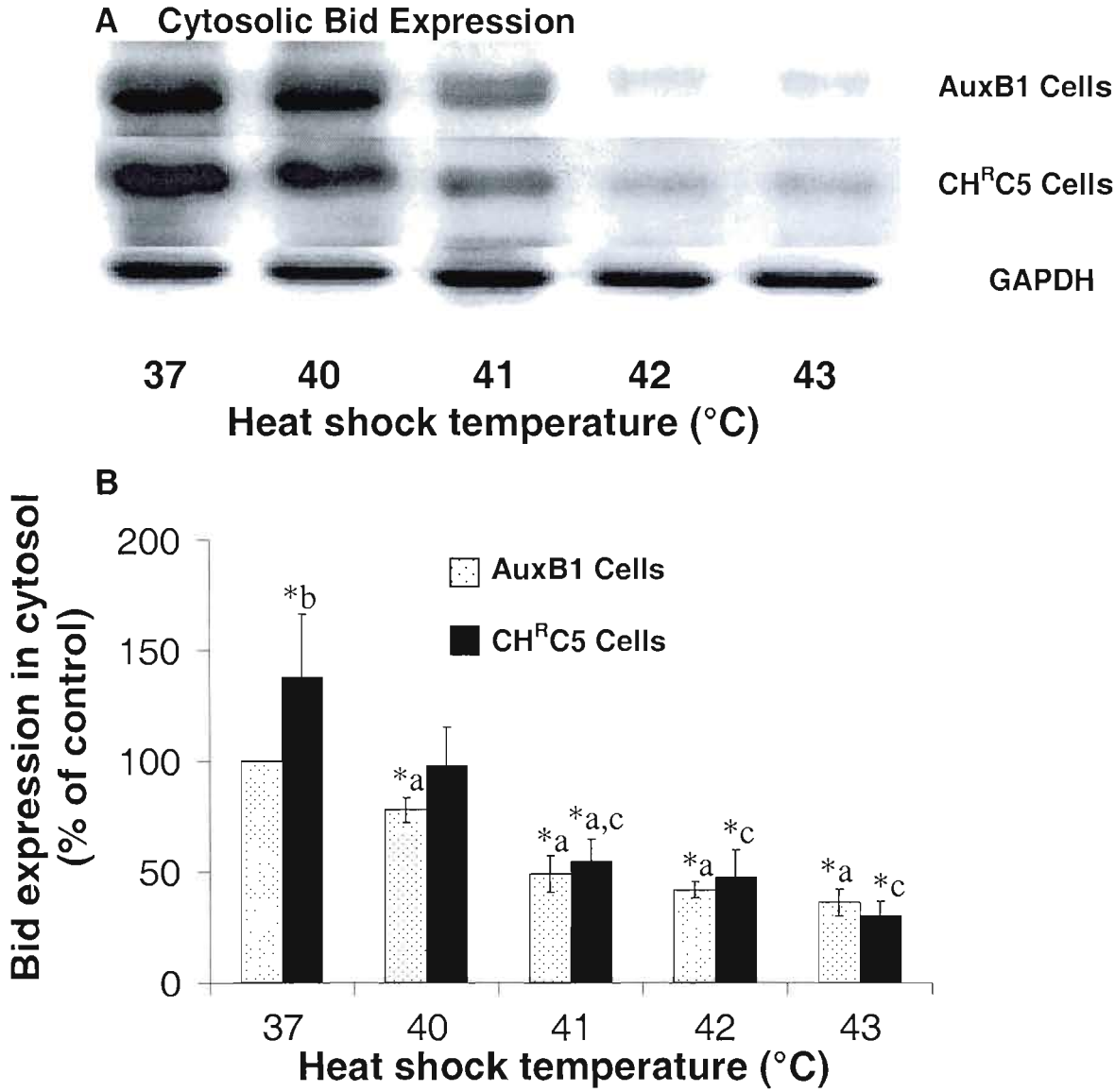
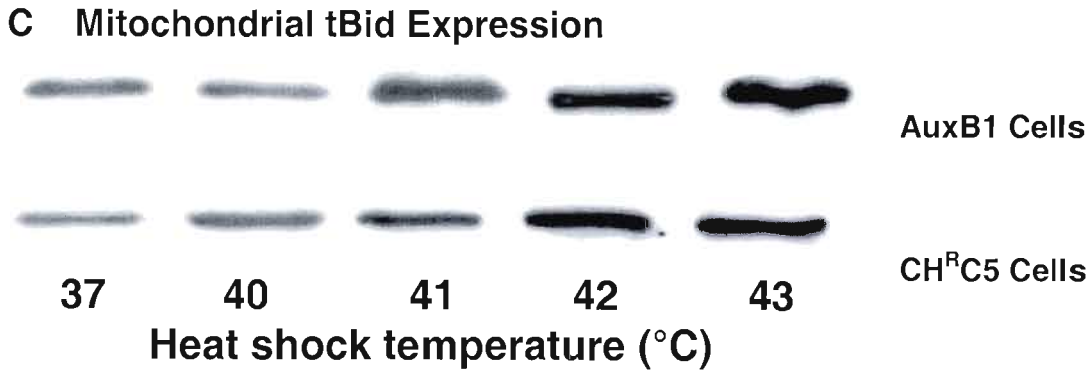

A Cytosolic Bid Expression
AuxB1 Cells
CH^RC5 Cells
GAPDH
37
40
41
42
43
Heat shock temperature (°C)
B
Bid expression in cytosol (% of control)
200
150
100
50
0
AuxB1 Cells
CH^RC5 Cells
*b
*a
*a
*a,c
*a
*c
*a
*c
37
40
41
42
43
Heat shock temperature (°C)

Figure 5, A-B


C Mitochondrial tBid Expression
AuxB1 Cells
CH^RC5 Cells
37
40
41
42
43
Heat shock temperature (°C)

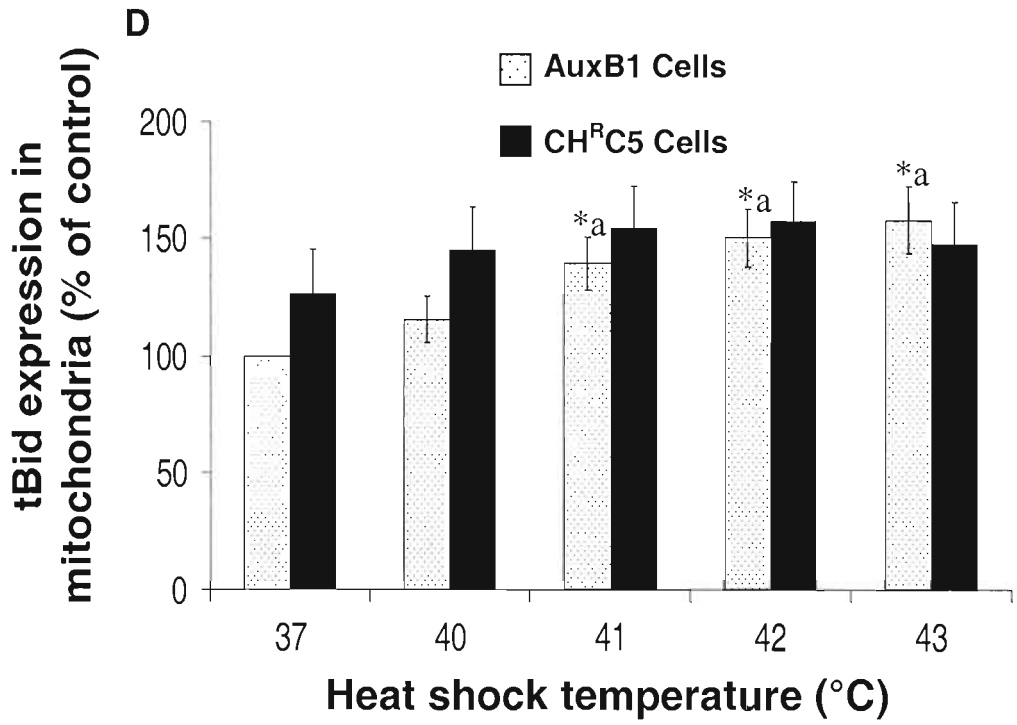
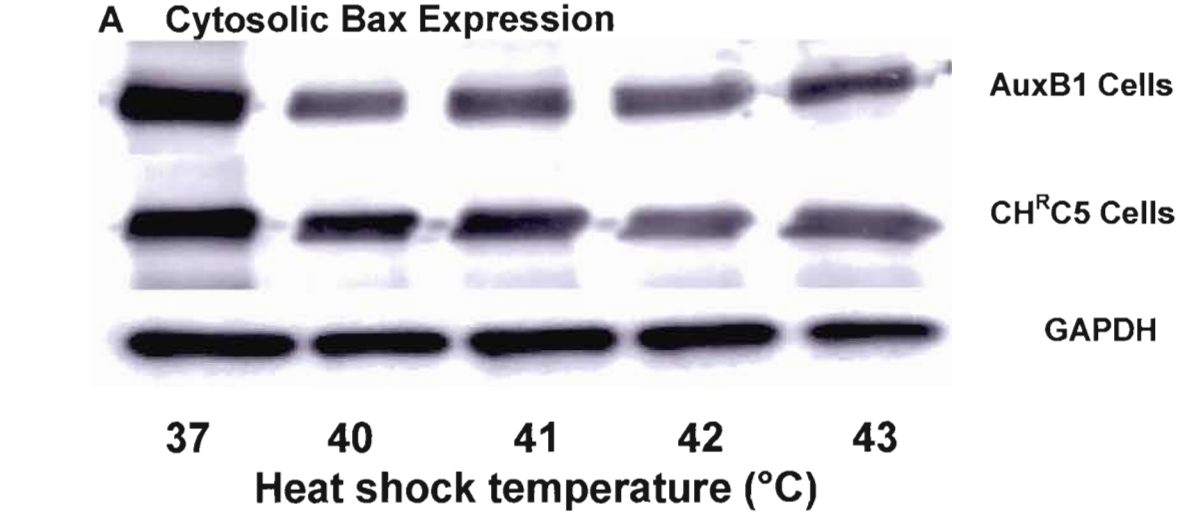

D
AuxB1 Cells
CH^RC5 Cells
tBid expression in mitochondria (% of control)
200
150
100
50
0
*a
*a
*a
37
40
41
42
43
Heat shock temperature (°C)

Figure 5, C-D


A Cytosolic Bax Expression
AuxB1 Cells
CH^RC5 Cells
GAPDH
37
40
41
42
43
Heat shock temperature (°C)

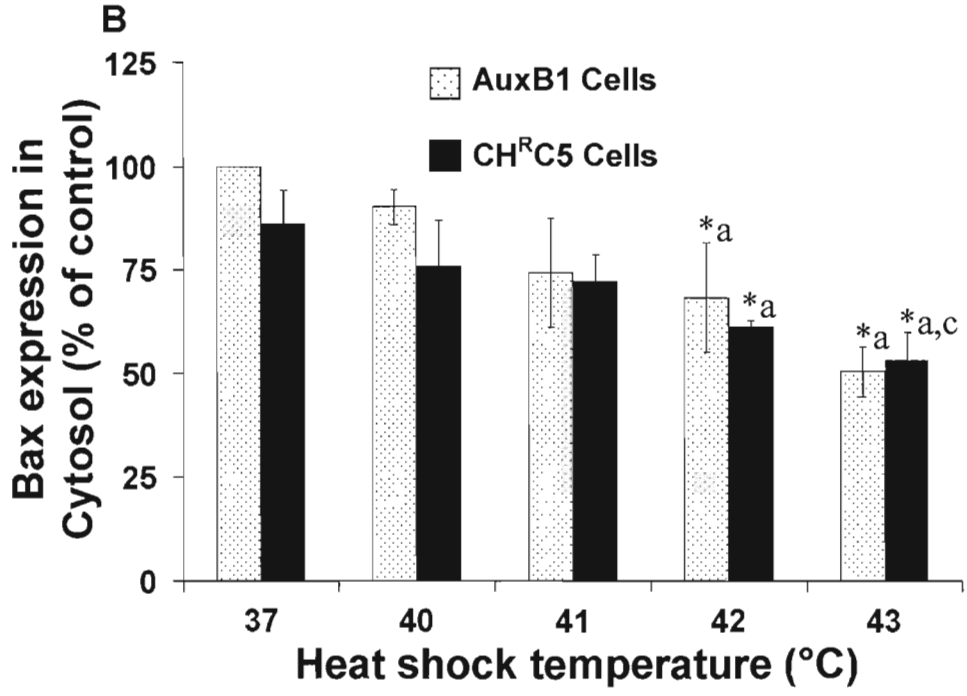
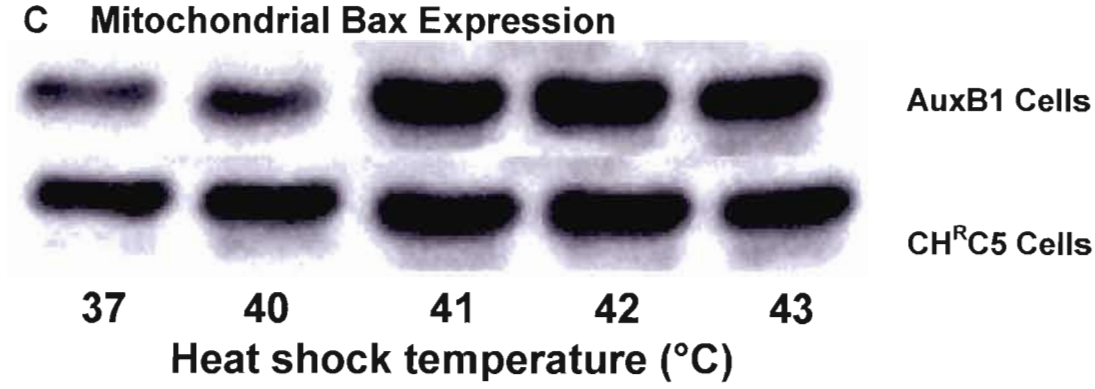

B
Bax expression in Cytosol (% of control)
125
100
75
50
25
0
AuxB1 Cells
CH^RC5 Cells
*a
*a
*a
*a,c
37
40
41
42
43
Heat shock temperature (°C)

**Figure 6, A-B**


C Mitochondrial Bax Expression
AuxB1 Cells
CH^RC5 Cells
37
40
41
42
43
Heat shock temperature (°C)

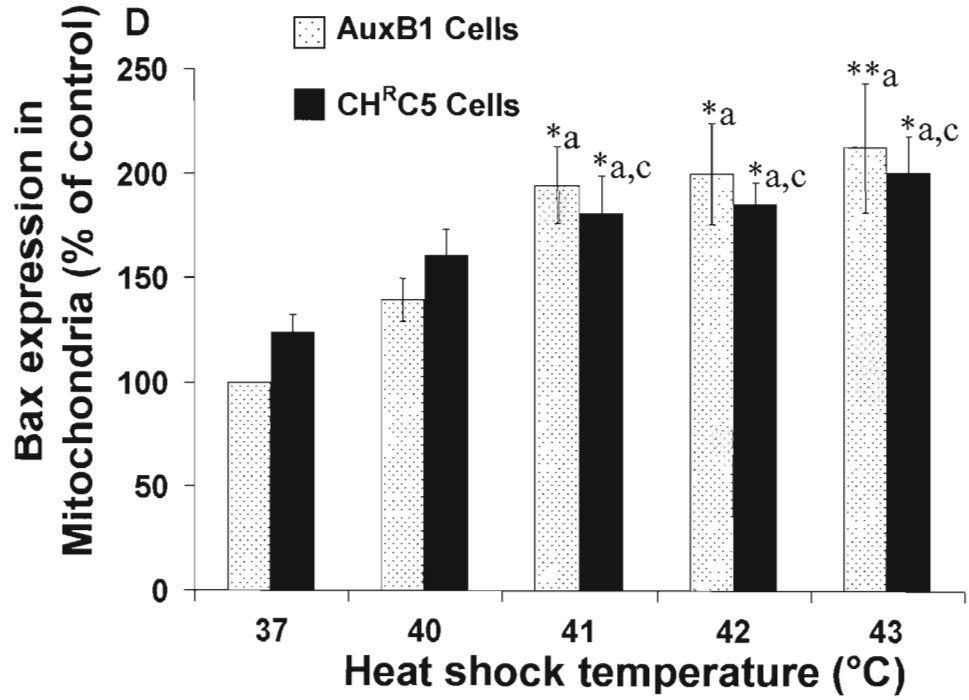

D
AuxB1 Cells
CH^RC5 Cells
Bax expression in Mitochondria (% of control)
250
200
150
100
50
0
*a
*a,c
*a
*a,c
**a
*a,c
37
40
41
42
43
Heat shock temperature (°C)

Figure 6, C-D

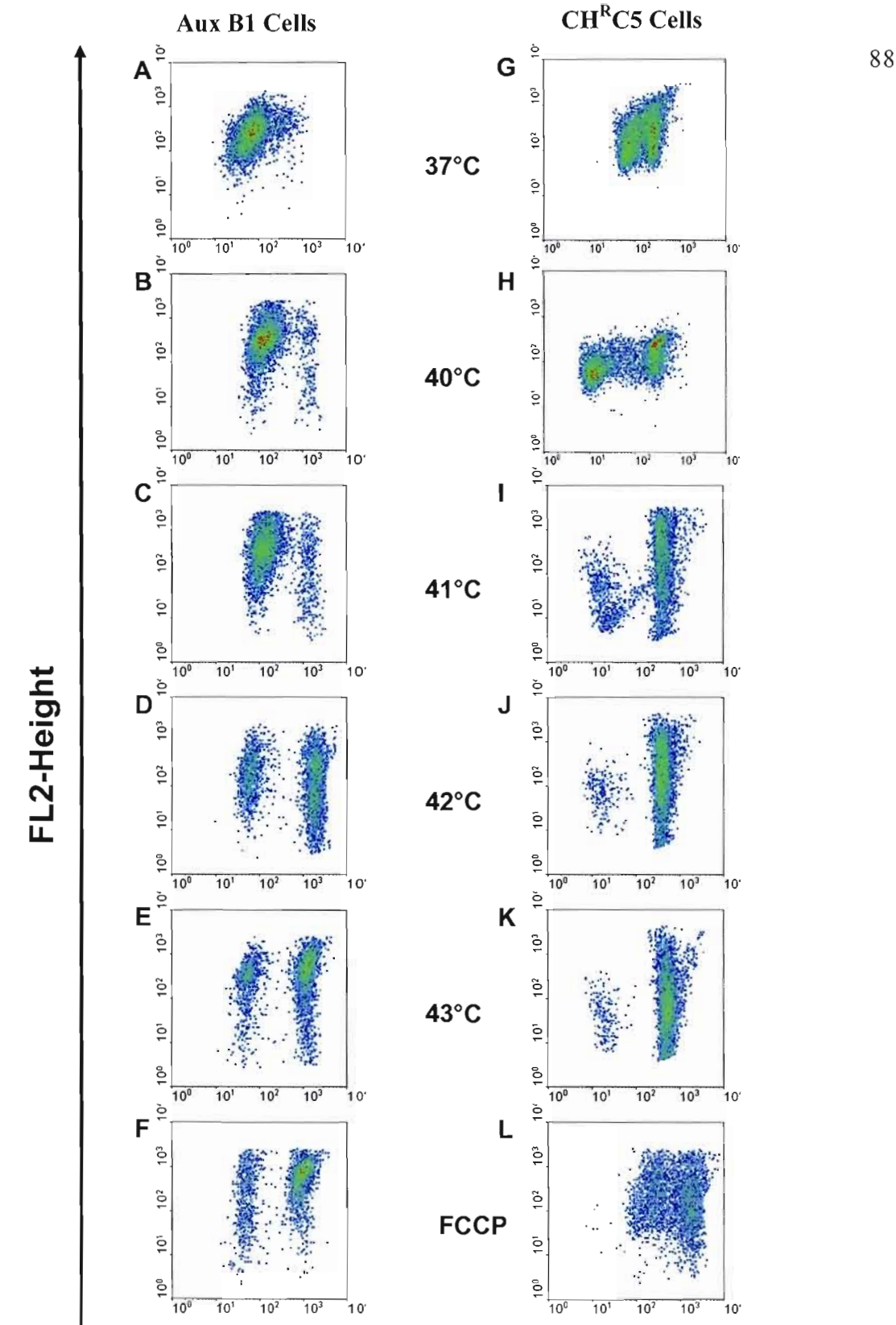

Aux B1 Cells
CH^RC5 Cells
A
B
C
D
E
F
G
H
I
J
K
L
37°C
40°C
41°C
42°C
43°C
FCCP
FL2-Height
FL1-Height

Figure 7, A-L

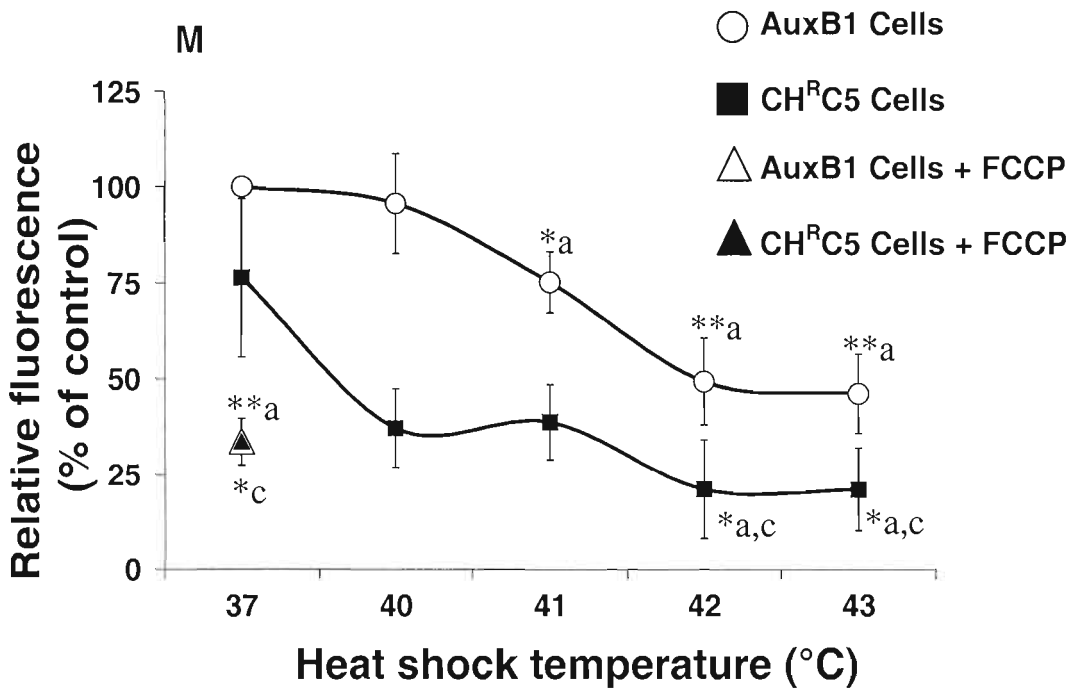
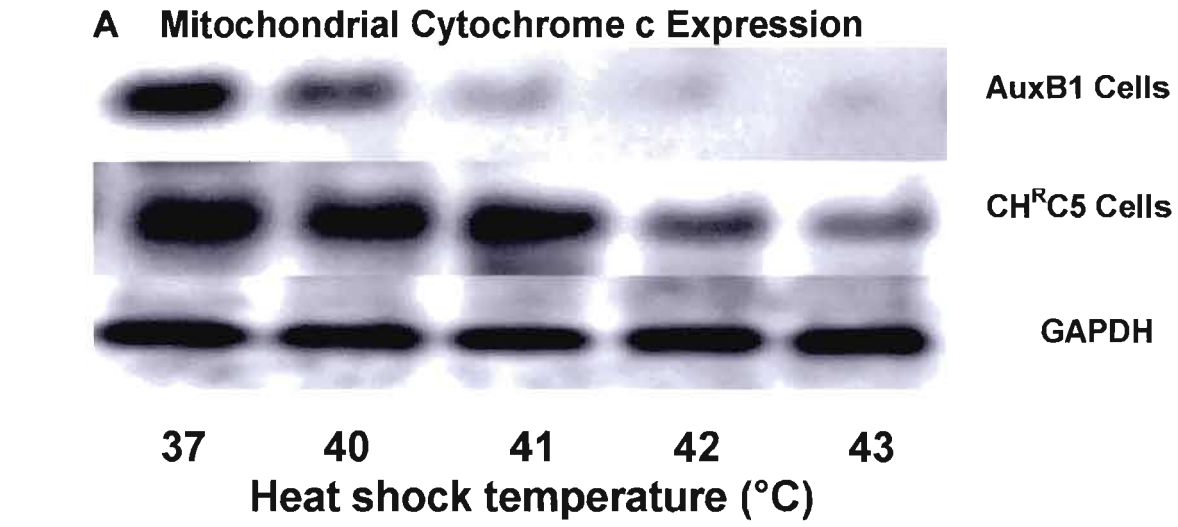

M
AuxB1 Cells
CH^RC5 Cells
AuxB1 Cells + FCCP
CH^RC5 Cells + FCCP
Relative fluorescence (% of control)
125
100
75
50
25
0
37
40
41
42
43
Heat shock temperature (°C)
**a
*c
*a
**a
**a
*a,c
*a,c

**Figure 7, M**


A Mitochondrial Cytochrome c Expression
AuxB1 Cells
CH^RC5 Cells
GAPDH
37
40
41
42
43
Heat shock temperature (°C)

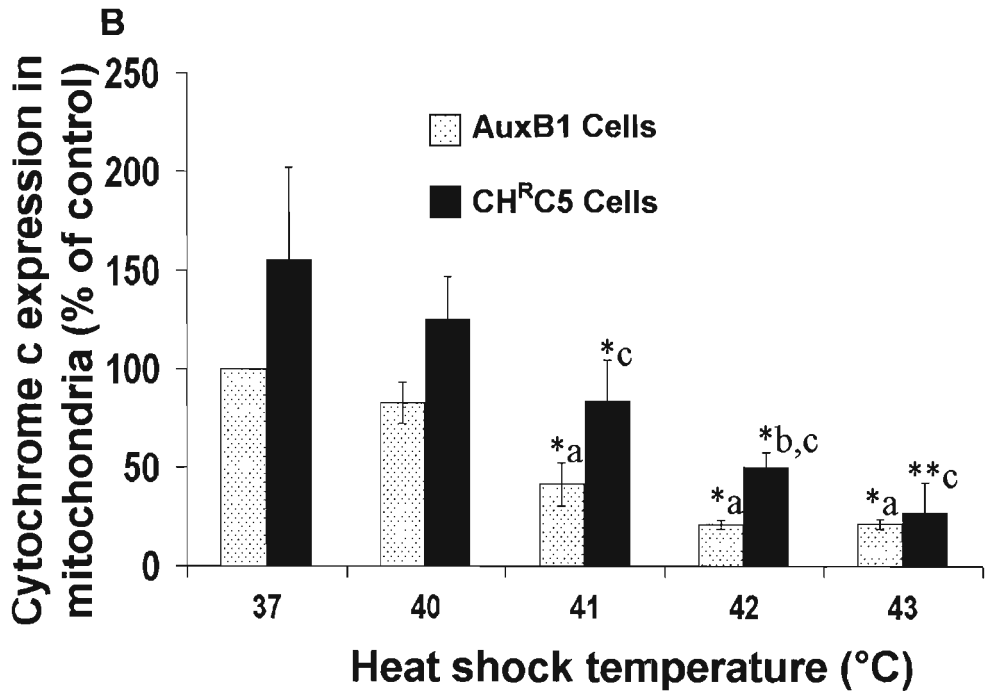
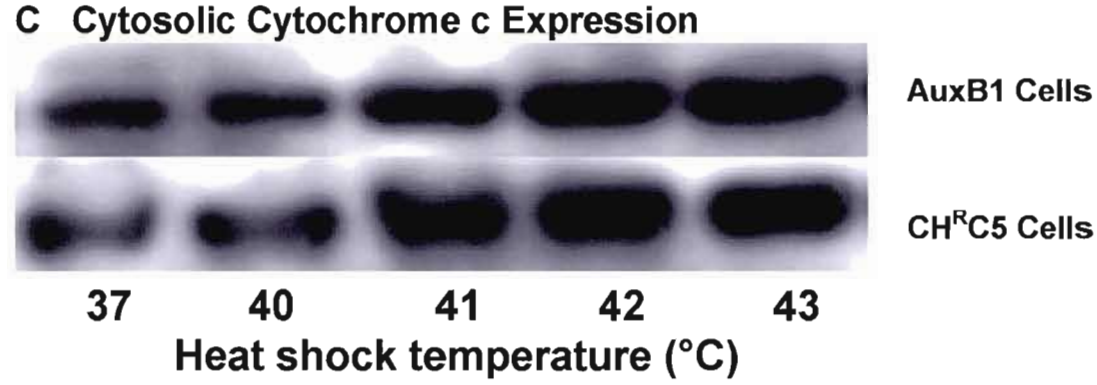

B
Cytochrome c expression in mitochondria (% of control)
250
200
150
100
50
0
AuxB1 Cells
CH^RC5 Cells
*a
*c
*a
*b,c
*a
**c
37
40
41
42
43
Heat shock temperature (°C)

Figure 8, A-B


C Cytosolic Cytochrome c Expression
AuxB1 Cells
CH^RC5 Cells
37
40
41
42
43
Heat shock temperature (°C)

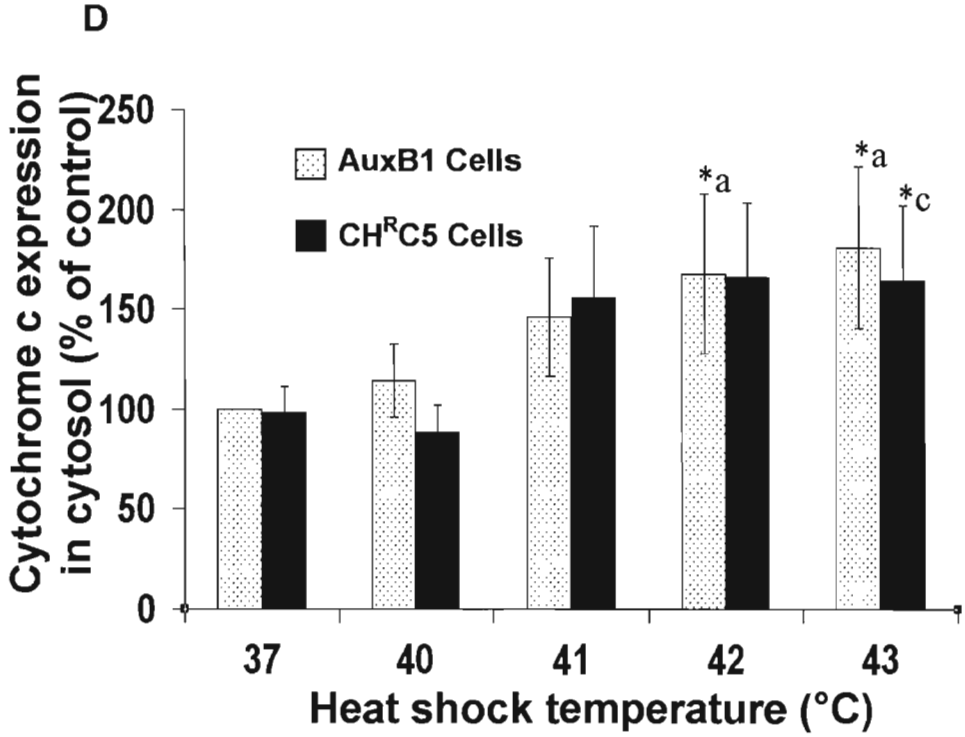

D
Cytochrome c expression in cytosol (% of control)
250
200
150
100
50
0
AuxB1 Cells
CH^RC5 Cells
*a
*a
*c
37
40
41
42
43
Heat shock temperature (°C)

Figure 8, C-D

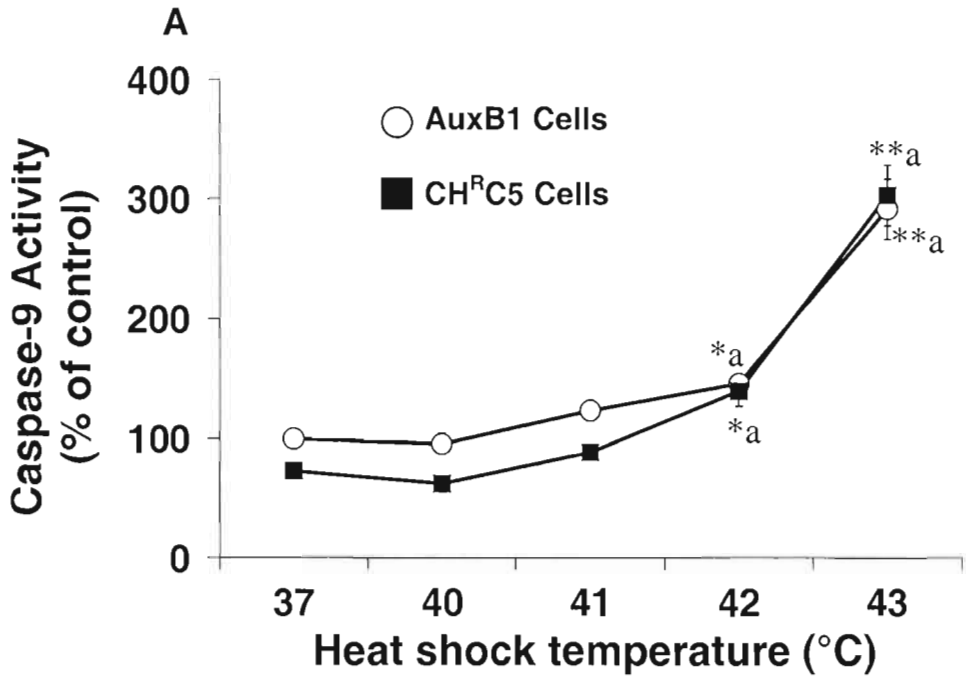

A
400
300
200
100
0
Caspase-9 Activity
(% of control)
AuxB1 Cells
CH^RC5 Cells
**a
**a
*a
*a
37
40
41
42
43
Heat shock temperature (°C)

Figure 9, A

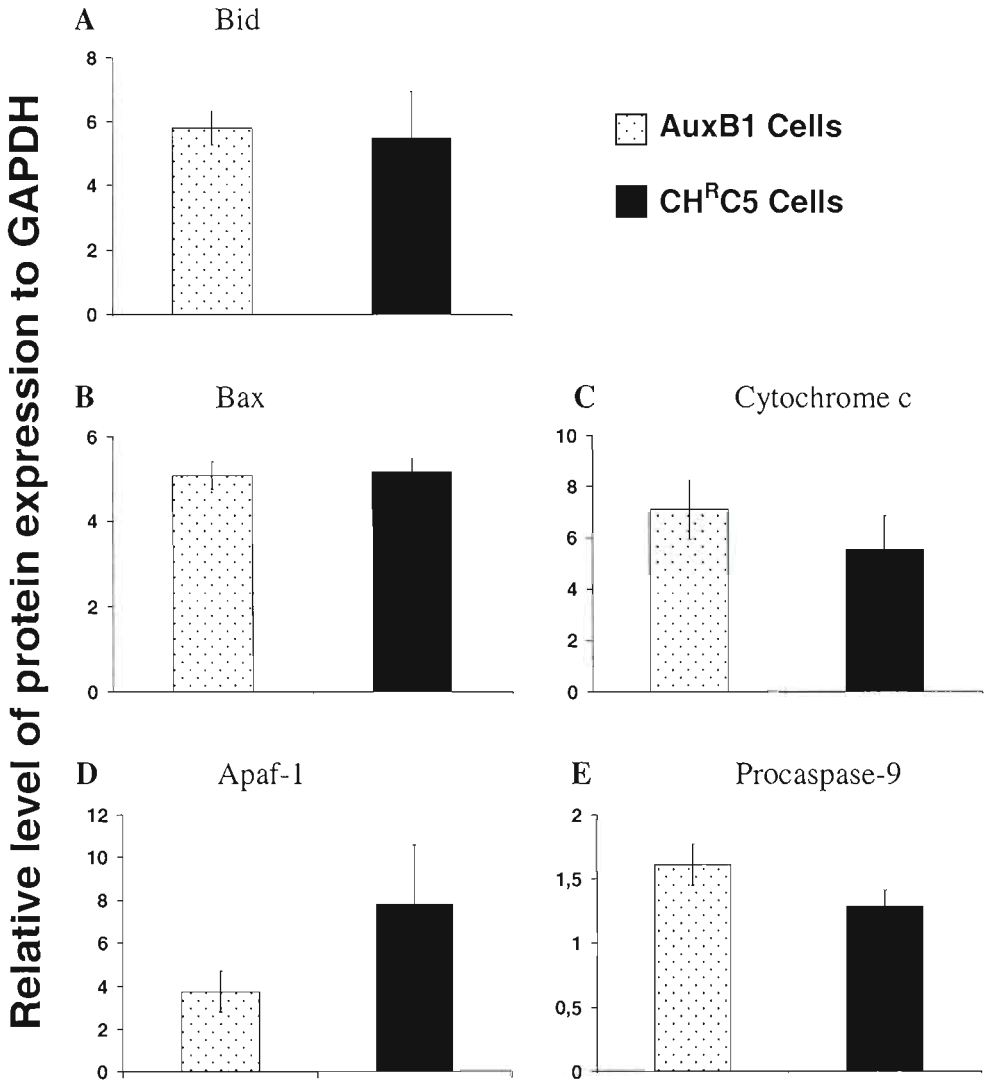

Relative level of protein expression to GAPDH
A
Bid
B
Bax
C
Cytochrome c
D
Apaf-1
E
Procaspase-9
AuxB1 Cells
CH^RC5 Cells

**Figure 10, A-E**

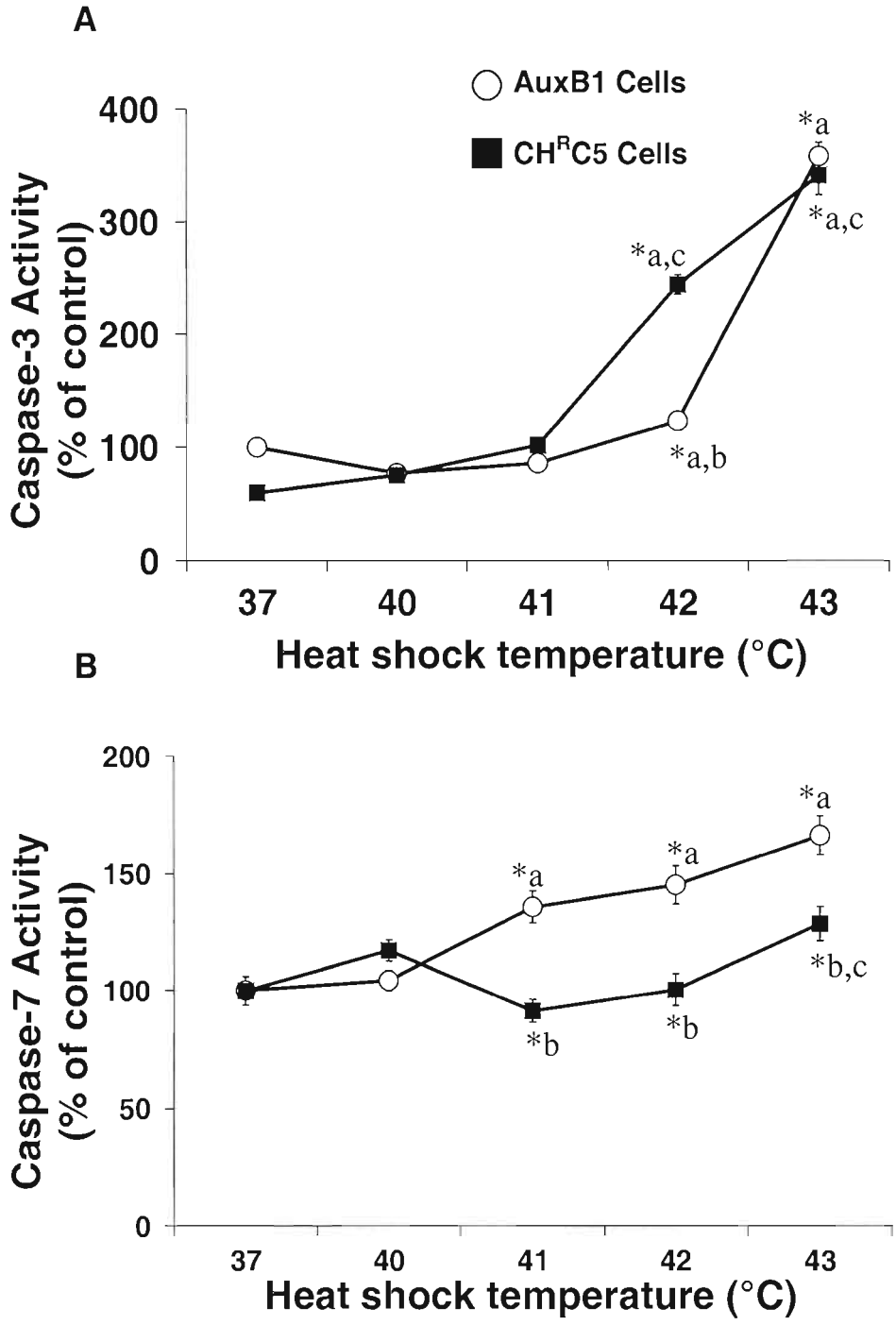
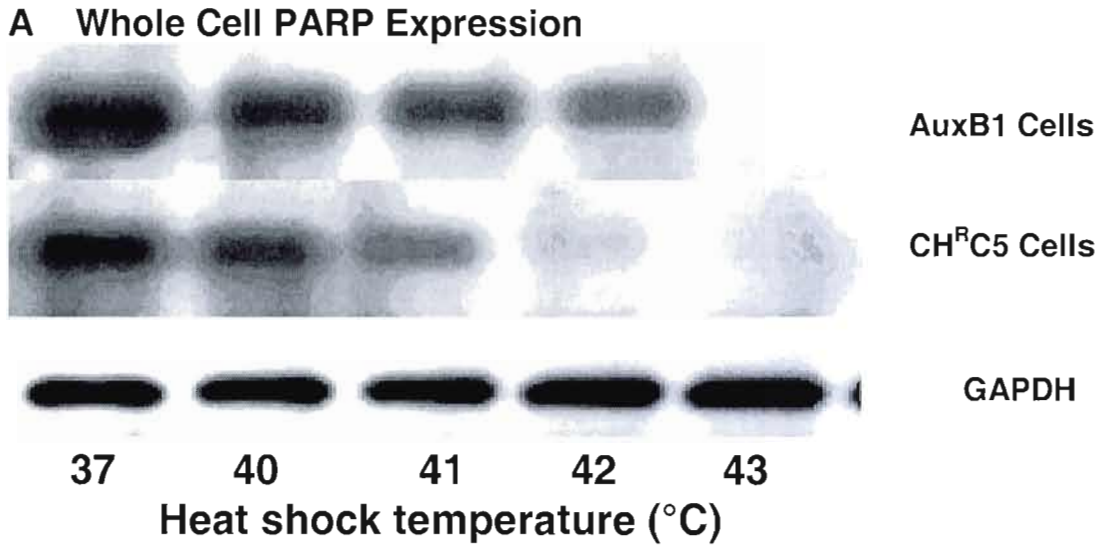

A
AuxB1 Cells
CH^RC5 Cells
Caspase-3 Activity (% of control)
400
300
200
100
0
*a
*a,c
*a,c
*a,b
37
40
41
42
43
Heat shock temperature (°C)
B
Caspase-7 Activity (% of control)
200
150
100
50
0
*a
*a
*a
*b
*b
*b,c
37
40
41
42
43
Heat shock temperature (°C)

Figure 11, A-B


A Whole Cell PARP Expression
AuxB1 Cells
CH^RC5 Cells
GAPDH
37
40
41
42
43
Heat shock temperature (°C)

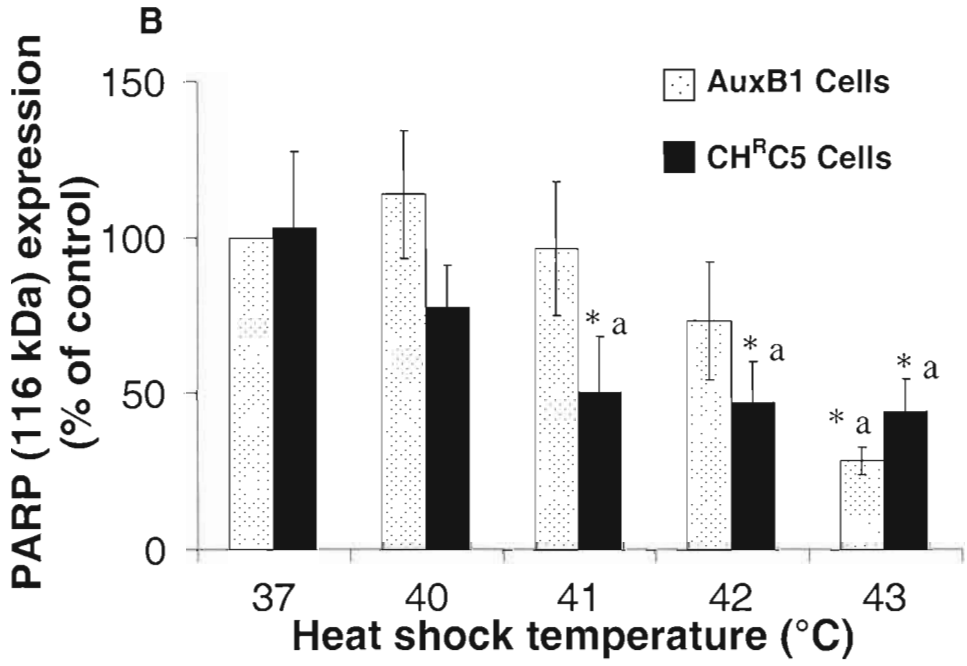

B
PARP (116 kDa) expression (% of control)
150
100
50
0
AuxB1 Cells
CH^RC5 Cells
* a
37
40
41
42
43
Heat shock temperature (°C)

Figure 12, A-B

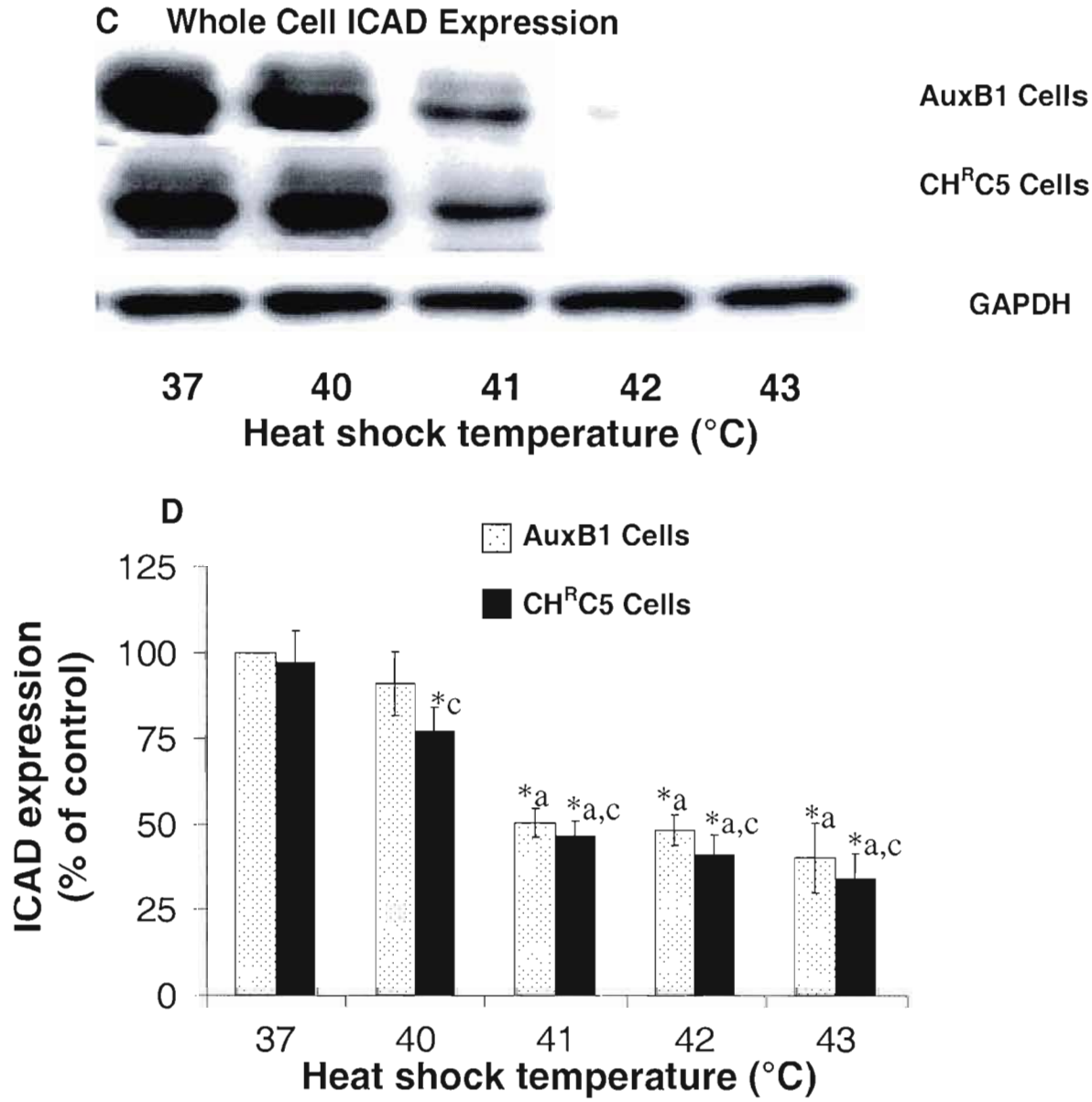

C Whole Cell ICAD Expression
AuxB1 Cells
CH^RC5 Cells
GAPDH
37
40
41
42
43
Heat shock temperature (°C)
D
AuxB1 Cells
CH^RC5 Cells
ICAD expression (% of control)
125
100
75
50
25
0
*c
*a
*a,c
*a
*a,c
*a
*a,c
37
40
41
42
43
Heat shock temperature (°C)

**Figure 12, C-D**

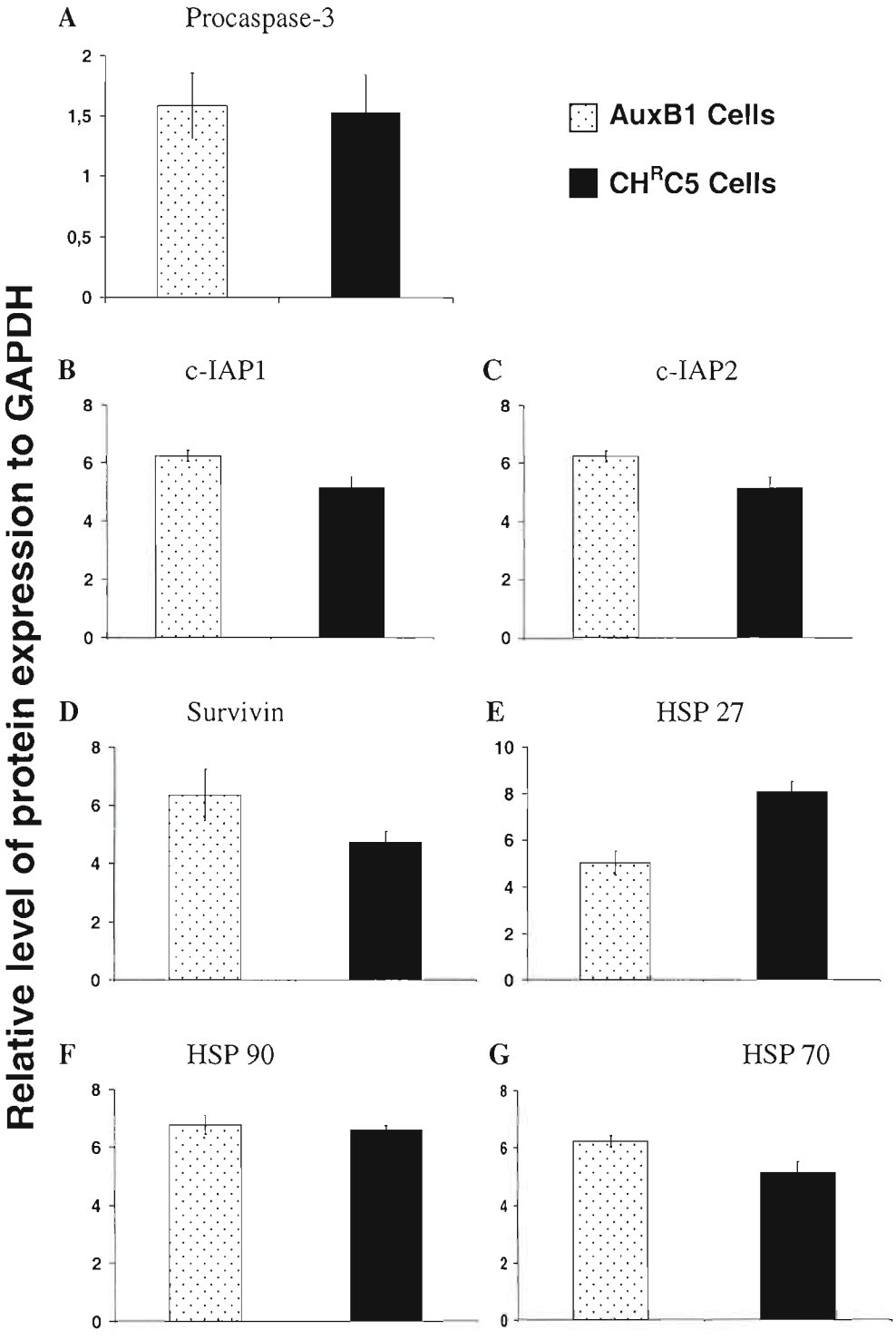

A
Procaspase-3
2
1,5
1
0,5
0
AuxB1 Cells
CH^RC5 Cells
Relative level of protein expression to GAPDH
B
c-IAP1
C
c-IAP2
D
Survivin
E
HSP 27
10
F
HSP 90
G
HSP 70

**Figure 13, A-G**

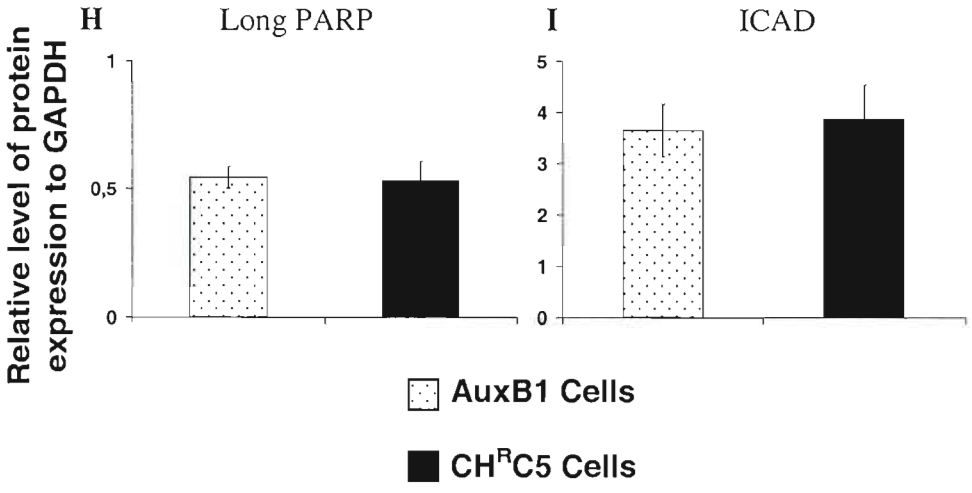

H
Long PARP
I
ICAD
Relative level of protein expression to GAPDH
1
0,5
0
5
4
3
2
1
0
AuxB1 Cells
CH^RC5 Cells

**Figure 13, H-I**

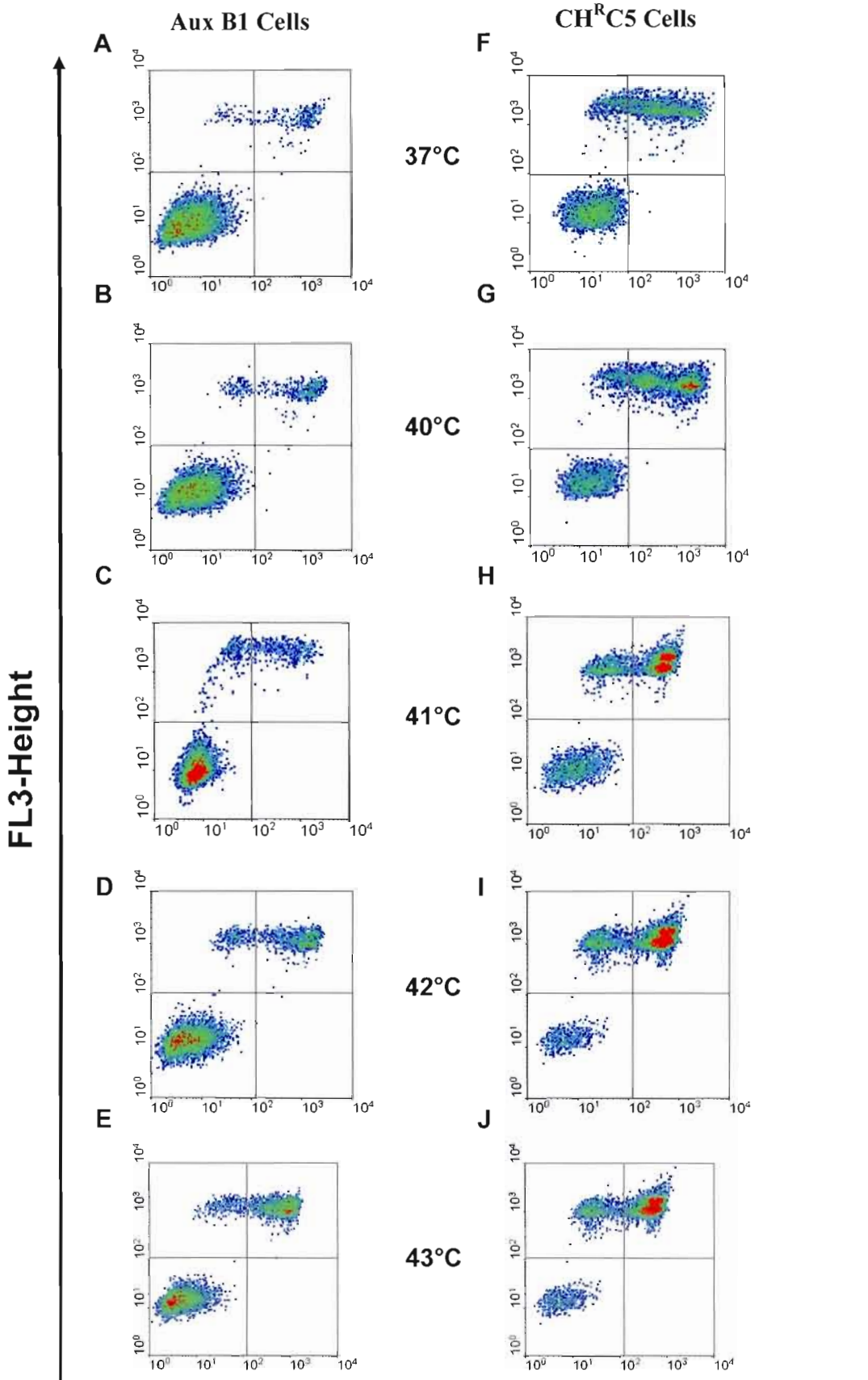

Aux B1 Cells
CH^RC5 Cells
A
B
C
D
E
F
G
H
I
J
37°C
40°C
41°C
42°C
43°C
FL3-Height
FL1-Height

Figure 14, A-J

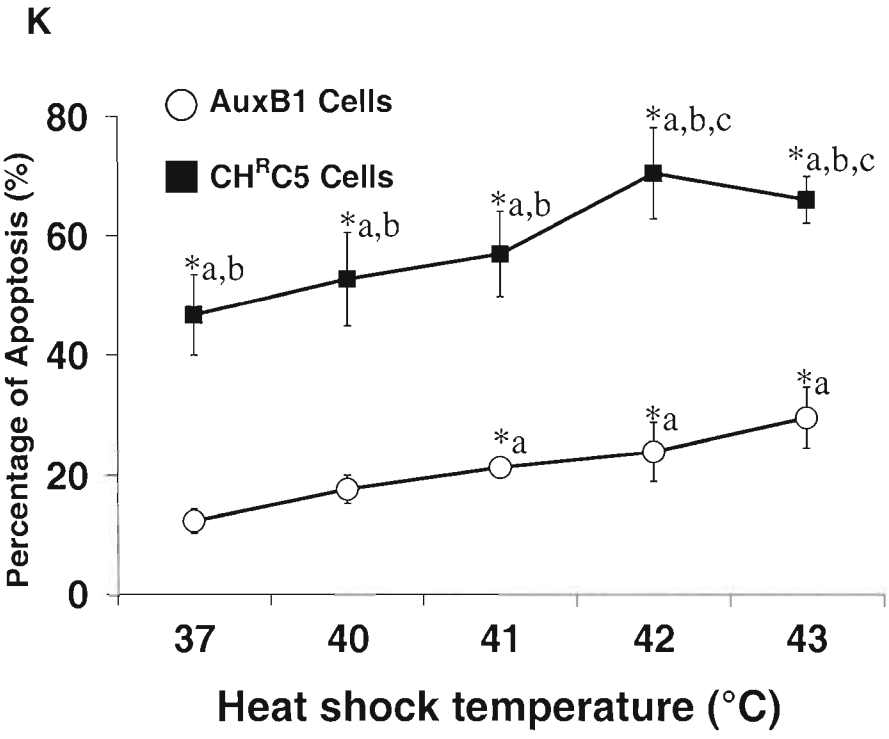

K
AuxB1 Cells
CH^RC5 Cells
Percentage of Apoptosis (%)
0
20
40
60
80
*a,b
*a,b
*a,b
*a,b,c
*a,b,c
*a
*a
*a
37
40
41
42
43
Heat shock temperature (°C)

Figure 14, K

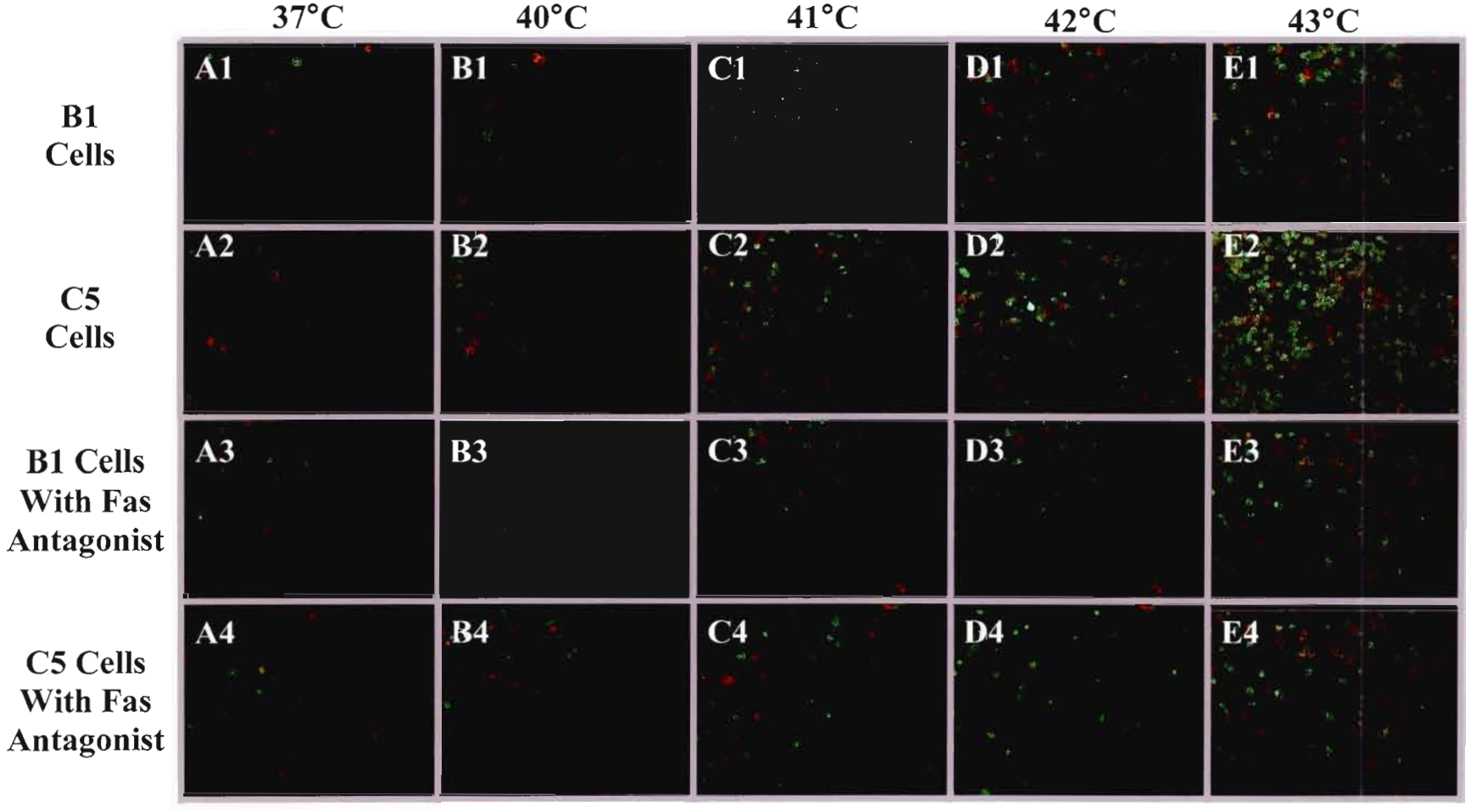

37°C
40°C
41°C
42°C
43°C
B1 Cells
C5 Cells
B1 Cells With Fas Antagonist
C5 Cells With Fas Antagonist
A1
B1
C1
D1
E1
A2
B2
C2
D2
E2
A3
B3
C3
D3
E3
A4
B4
C4
D4
E4

**Figure 15, A-E**

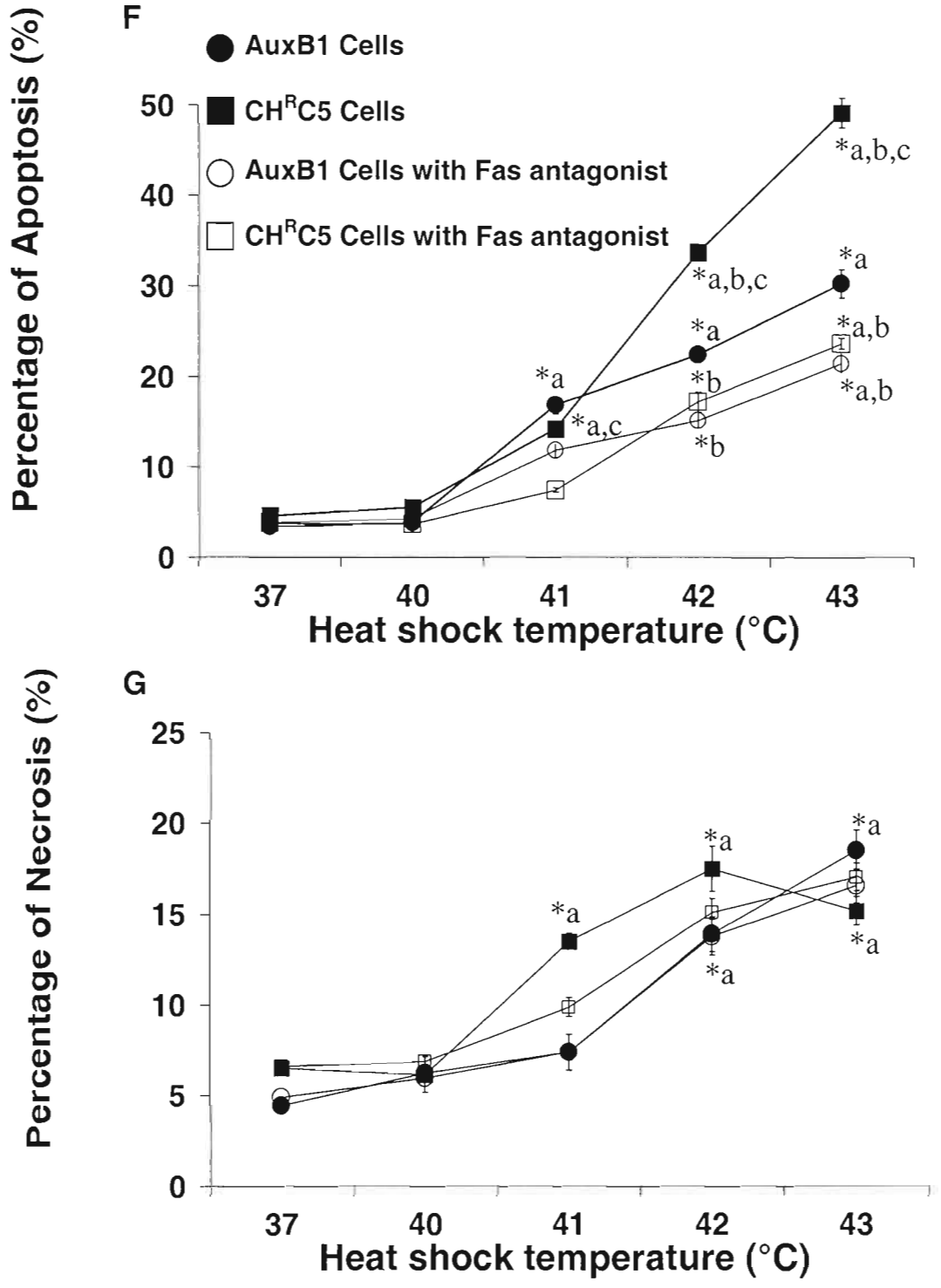

F
AuxB1 Cells
CH^RC5 Cells
AuxB1 Cells with Fas antagonist
CH^RC5 Cells with Fas antagonist
Percentage of Apoptosis (%)
50
40
30
20
10
0
*a,b,c
*a,b,c
*a
*a
*a,b
*a,b
*a
*b
*b
*a,c
37
40
41
42
43
Heat shock temperature (°C)
G
Percentage of Necrosis (%)
25
20
15
10
5
0
*a
*a
*a
*a
*a
37
40
41
42
43
Heat shock temperature (°C)

**Figure 15, F-G**

## FIGURE LEGENDS

**Figure 1:**

**Heat shock induced translocation of FADD from the cytoplasm to plasma membrane.** Drug-sensitive AuxB1 cells (▨) and MDR $CH^RC5$ cells (■) were exposed to 1 h heat shock at the indicated temperatures (40-43°C) relative to controls at 37°C. Levels of expression of FADD (30 kDa) protein were expressed relative to untreated control AuxB1 cells (37°C) designated as 100%. Purity of subcellular fractionations was confirmed using antibodies against cytochrome oxidase (1), $GST\pi_1$ (2), and calnexin (3), specific to mitochondrial, cytoplasmic, and microsomal fractions, respectively (E). Each blot is representative of at least three independent experiments. The data represent means ± SEM of at least three independent experiments and significance is represented by $p < 0.05$ (*). (a) significant difference for $CH^RC5$ and heated AuxB1 cells from experiment control (AuxB1 cells at 37°C). (b) significant difference between AuxB1 and $CH^RC5$ cells at the specific treatment temperature. (c) significant difference for heated $CH^RC5$ from same cell line control ($CH^RC5$ cells at 37°C).

**Figure 2:**

**Heat shock induced activation of initiator caspase-8.** Cells were exposed to 1 h heat shock at the indicated temperatures (40-43°C). Levels of total cellular expression of procaspase-8 (55 kDa) protein (A-B) in AuxB1 cells (▨) and MDR $CH^RC5$ cells (■) are expressed relative to untreated AuxB1 control cells (37°C). Each blot is representative of at least three independent experiments. Levels of Vmax for caspase-8 activity in AuxB1 cells (○) and MDR $CH^RC5$ cells (■) are expressed relative to untreated AuxB1 control cells (37°C), designated as 1.0 (C). The data represent means ± SEM of at least three independent experiments and significance is represented by $p < 0.05$ (*) and $p < 0.001$ (**). (a) significant difference for $CH^RC5$

and heated AuxB1 cells from experiment control (AuxB1 cells at 37°C). (b) significant difference between AuxB1 and $CH^RC5$ cells at the specific treatment temperature. (c) significant difference for heated $CH^RC5$ from same cell line control ($CH^RC5$ cells at 37°C). When not shown, error bars lie within the symbol.

**Figure 3:**

**Heat shock induced activation of initiator caspases-10 and -2.** Levels of caspase-10 (A) and caspase-2 (B) activity in AuxB1 (○) and $CH^RC5$ cells (■) were determined by an enzyme activity assay using caspase-10 and -2 substrates. Both caspase activity levels in $CH^RC5$ and AuxB1 cells were expressed relative to untreated AuxB1 (37°C). The data represent means ± SEM of at least three independent experiments and significance is represented by $p < 0.05$ (*). (a) significant difference for $CH^RC5$ and heated AuxB1 cells from experiment control (AuxB1 cells at 37°C). (b) significant difference between AuxB1 and $CH^RC5$ cells at the specific treatment temperature. When not shown, error bars lie within the symbol.

**Figure 4:**

**Comparison of endogenous expression of extrinsic apoptotic pathway protein levels between drug-sensitive AuxB1 and MDR $CH^RC5$ cells.** Confluent drug-sensitive AuxB1 cells (▨) and MDR $CH^RC5$ cells (■) were harvested and the total cellular levels of protein expression of Fas receptor (A), FADD (B), Procaspase-8 (C), c-$FLIP_S$ (D), c-$FLIP_L$ (E) were measured by Western blot (not shown). The quantitative data are shown for blots and represent means ± SEM of at least three independent experiments.

**Figure 5:**

**Heat shock induced cleavage of pro-apoptotic Bid in the cytoplasm and translocation of truncated-Bid to the mitochondria.** Drug-sensitive AuxB1 cells (▨) and MDR $CH^RC5$ cells (■) were exposed to 1 h heat shock at the indicated temperatures (40-43°C). Levels of expression of Bid and tBid proteins were determined by Western blot. Expression of Bid was measured in the cytosol (A, B) and expression of tBid in the mitochondria (C, D). Bid (21 kDa) and tBid (14 kDa) levels in $CH^RC5$ and AuxB1 cells were expressed relative to the untreated AuxB1 control (37°C). Each blot is representative of at least three independent experiments. The data represent means ± SEM of at least three independent experiments and significance is represented by $p < 0.05$ (*). (a) significant difference for $CH^RC5$ and heated AuxB1 cells from experiment control (AuxB1 cells at 37°C). (c) significant difference for heated $CH^RC5$ from same cell line control ($CH^RC5$ cells at 37°C).

**Figure 6:**

**Heat shock induced translocation of pro-apoptotic Bax from the cytoplasm to mitochondria.** Drug-sensitive AuxB1 cells (▨) and MDR $CH^RC5$ cells (■) were exposed to 1 h heat shock at the indicated temperatures (40-43°C). Levels of expression of Bax protein were determined by Western blot. Expression of Bax was measured in the cytosol (A, B) and in the mitochondria (C, D). Bax (21 kDa) levels in $CH^RC5$ and AuxB1 cells were expressed relative to untreated AuxB1 control cells (37°C). Each blot is representative of at least three independent experiments. The data represent means ± SEM of at least three independent experiments and significance is represented by $p < 0.05$ (*) and $p < 0.001$ (**). (a) significant difference for $CH^RC5$ and heated AuxB1 cells from experiment control (AuxB1 cells at 37°C). (c) significant difference for heated $CH^RC5$ from same cell line control ($CH^RC5$ cells at 37°C).

**Figure 7:**

**Mitochondrial membrane potential in response to heat shock.** Mitochondrial membrane potential was measured by flow cytometry using JC-1 staining. Drug-sensitive AuxB1 cells (A-E) and MDR $CH^RC5$ cells (G-K) were exposed to 1 h heat shock at the indicated temperatures (40-43°C) with FCCP as positive control (F and L, respectively) as represented by the FACS Scan dot plots. The relative fluorescence for mitochondrial membrane potential for AuxB1 (○) and $CH^RC5$ cells (■) (M) was expressed relative to untreated AuxB1 control cells (37°C). The data represent means ± SEM of at least three independent experiments and significance is represented by $p < 0.05$ (*) and $p < 0.001$ (**). (a) significant difference for $CH^RC5$ and heated AuxB1 cells from experiment control (AuxB1 cells at 37°C). (c) significant difference for heated $CH^RC5$ from same cell line control ($CH^RC5$ cells at 37°C).

**Figure 8:**

**Heat shock induced release of cytochrome *c* from the mitochondria into cytoplasm.** Drug-sensitive AuxB1 cells (▨) and MDR $CH^RC5$ cells (■) were exposed to 1 h heat shock at the indicated temperatures (40-43°C). Levels of expression of cytochrome *c* protein were determined by Western blot. Expression of cytochrome *c* was measured in the mitochondria (A, B) and in the cytosol (C, D). Cytochrome *c* (14 kDa) levels in $CH^RC5$ and AuxB1 cells were expressed relative to the untreated AuxB1 control (37°C). Each blot is representative of at least three independent experiments. The data represent means ± SEM of at least three independent experiments and significance is represented by $p < 0.05$ (*) and $p < 0.001$ (**). (a) significant difference for $CH^RC5$ and heated AuxB1 cells from experiment control (AuxB1 cells at 37°C). (b) significant difference between AuxB1 and $CH^RC5$ cells at the specific treatment temperature. (c) significant difference for heated $CH^RC5$ from same cell line control ($CH^RC5$ cells at 37°C).

**Figure 9:**

**Heat shock induced activation of initiator caspase-9.** CHO cells were exposed to 1 h heat shock at the indicated temperatures (40-43°C). Levels of caspase-9 activity in AuxB1 (○) and MDR $CH^RC5$ cells (■) were determined by an enzyme activity assay using caspase-9 substrate, Ac-LEHD-AFC. The data represent means ± SEM of at least three independent experiments and significance is represented by $p < 0.05$ (*) and $p < 0.001$ (**). (a) significant difference for $CH^RC5$ and heated AuxB1 cells from experiment control (AuxB1 cells at 37°C). When not shown, error bars lie within the symbol.

**Figure 10:**

**Comparison of endogenous expression of intrinsic apoptotic pathway protein levels between drug-sensitive AuxB1 and MDR $CH^RC5$ cells.** Confluent drug-sensitive AuxB1 cells (▨) and MDR $CH^RC5$ cells (■) were harvested and the total cellular levels of protein expression of Bid (A), Bax (B), Cytochrome *c* (C), Apaf-1 (D), Procaspase-9 (E) were measured by Western blot (not shown). The quantitative data are shown for blots and represent means ± SEM of at least three independent experiments.

**Figure 11:**

**Heat shock induced activation of effector caspases-3 and -7.** CHO cells were exposed to 2 h heat shock at the indicated temperatures (40-43°C). Levels of caspase activity in AuxB1 (○) and $CH^RC5$ cells (■) were determined by an enzyme activity assay using caspase-3 substrate, Ac-Asp-Glu-Val-Asp-AMC (A) and caspase-7 substrate, MCA-VDQVDGWK(DNP)-$NH_2$ (B). The caspase activity levels in both cell lines were expressed relative to untreated AuxB1 (37°C). The data represent means ± SEM of at least three independent experiments and significance is represented by $p < 0.05$ (*). (a) significant difference for $CH^RC5$ and heated AuxB1

cells from experiment control (AuxB1 cells at 37°C). (b) significant difference between AuxB1 and $CH^RC5$ cells at the specific treatment temperature. (c) significant difference for heated $CH^RC5$ from same cell line control ($CH^RC5$ cells at 37°C). When not shown, error bars lie within the symbol.

**Figure 12:**

**Heat shock induced cleavage of caspase-3 substrates PARP and ICAD.** CHO cells were exposed to 2 h heat shock at the indicated temperatures (40-43°C). Levels of expression of PARP and ICAD proteins in AuxB1 cells (▨) and $CH^RC5$ cells (■) were determined by Western blot. Expression of PARP (116 kDa) (A-B) and ICAD (47 kDa) (C-D) protein levels in $CH^RC5$ and AuxB1 cells was measured in whole cell lysates and expressed relative to the untreated AuxB1 control (37°C). Each blot is representative of at least three independent experiments. The data represent means ± SEM of at least three independent experiments and significance is represented by $p < 0.05$ (*). (a) significant difference for $CH^RC5$ and heated AuxB1 cells from experiment control (AuxB1 cells at 37°C). (c) significant difference for heated $CH^RC5$ from same cell line control ($CH^RC5$ cells at 37°C).

**Figure 13:**

**Comparison of endogenous expression of apoptotic execution protein levels between drug-sensitive AuxB1 and MDR $CH^RC5$ cells.** Confluent drug-sensitive AuxB1 cells (▨) and MDR $CH^RC5$ cells (■) were harvested and the total cellular levels of protein expression of Procaspase-3 (A), c-IAP1 (B), c-IAP2 (C), Survivin (D), HSP27 (E), HSP90 (F), HSP70 (G), Long PARP (H) and ICAD (I) were measured by Western blot (not shown). The quantitative data are shown for blots and represent means ± SEM of at least three independent experiments.

**Figure 14:**

**Heat shock induced apoptosis in AuxB1 and $CH^RC5$ cells via flow cytometry.** Apoptosis and necrosis levels were measured by flow cytometry using Annexin V-FITC staining (FL1-Channel) and PI uptake (FL3-Channel). Drug-sensitive AuxB1 cells (A-E) and MDR $CH^RC5$ cells (F-J) were exposed to 1 h heat shock at the indicated temperatures (40-43°C). Upper right quadrant, late apoptosis/early necrosis; lower right quadrant, early apoptosis; upper left quadrant necrosis. Percentages of apoptosis (K) in AuxB1 (○) and $CH^RC5$ cells (■) are expressed relative to the untreated AuxB1 control (37°C). The data represent means ± SEM of at least three independent experiments and significance is represented by $p < 0.05$ (*). (a) significant difference for $CH^RC5$ and heated AuxB1 cells from experiment control (AuxB1 cells at 37°C). (b) significant difference between AuxB1 and $CH^RC5$ cells at the specific treatment temperature. (c) significant difference for heated $CH^RC5$ from same cell line control ($CH^RC5$ cells at 37°C).

**Figure 15:**

**Heat shock induced apoptosis in AuxB1 and $CH^RC5$ cells via fluorescence microscopy.** Apoptosis and necrosis levels were measured by fluorescence microscopy using Hoechst 33258 and PI staining. Drug-sensitive AuxB1 cells (A1,3-E1,3) and MDR $CH^RC5$ cells (A2,4-E2,4) were exposed to 2 h heat shock at the indicated temperatures (40-43°C). Percentages of apoptosis (F) and necrosis (G) in AuxB1 (●) and $CH^RC5$ cells (■) are expressed relative to the untreated AuxB1 control (37°C). A set of AuxB1 (○) and $CH^RC5$ cells (□) were treated with Fas/FasL antagonist in addition to heat. The data represent means ± SEM of at least three independent experiments and significance is represented by $p < 0.05$ (*). (a) significant difference for $CH^RC5$ and heated AuxB1 cells from experiment control (AuxB1 cells at 37°C). (b) significant difference between AuxB1 and $CH^RC5$ cells at

the specific treatment temperature. (c) significant difference for heated $CH^RC5$ from same cell line control ($CH^RC5$ cells at 37°C).

## CHAPTER 4: CONCLUSION

Cancer represents one of the most common causes of death in developed countries today. The battle against cancer is made difficult by the development of cellular mechanisms to evade death by contemporary treatments. Chemotherapy represents one such classical treatment of cancer. The development of MDR by tumour cells constitutes a major obstacle in the use of chemotherapy. Thus, understanding the molecular mechanisms of MDR and being able to once again render these cells sensitive to chemotherapeutic drugs symbolizes a great leap forward in this field and would maximize the success of patient treatment methods. Some strategies for sensitizing MDR cells to chemotherapeutic drugs already exist, involving chemosensitizers such as cyclosporin A and verapamil, nevertheless, their use is limited by their toxicity and deleterious pharmacokinetic and pharmacodynamic effects. However, the use of heat shock or hyperthermia is an MDR sensitizing approach that may represent a promising avenue of reversing the effects of MDR.

Research on combination therapy using heat shock with either radiation or chemotherapy has shown a high success rate in improvement of tumour response (Wust et al., 2002). Several recent clinical studies demonstrated positive responses of tumours with these types of combination treatments with higher patient survival rates (Jones et al., 2005; De Wit et al., 1999; van der Zee et al., 2000; Issels et al., 2001; Wendtner et al., 2001; Hindebrandt et al., 2004; Fraker, 2004).

Findings from several *in vitro* studies indicate that chemotherapy combined with either chemosensitizers or heat shock/hyperthermia can reverse the MDR phenotype mediated by MDR proteins such as P-GP and MRP1 (Bates and Mackillop, 1990; Averill and Larrivée, 1998; Dumontet et al., 1998; Averill and Su, 1999; Larrivée and Averill, 2000; Turcotte and Averill-Bates, 2001; Liu et al., 2001; Souslova and Averill-Bates, 2004). Yet, little is understood about the mechanisms by

which heat shock/hyperthermia render MDR cells sensitive to chemotherapeutic agents.

This study investigated the molecular mechanisms of heat shock induced cell death by apoptosis in CHO cells, assessing the differences in response to heat shock that occur between drug-sensitive AuxB1 and MDR $CH^RC5$ cells. Whereas many cellular models of MDR possess a combination of different mechanisms of resistance, to the best of our knowledge, the $CH^RC5$ cells confer their resistance to drugs solely by overexpressing the efflux transporter P-GP. This makes the $CH^RC5$ cells an excellent model to study the mechanisms of resistance rendered by P-GP. A comprehensive look into these heat shock induced differences that occur between drug-sensitive AuxB1 and MDR $CH^RC5$ cells can give insight into the maintenance of resistance to cancer treatments by tumour cells and could unveil potentially new treatments strategies. The study found that in fact differences in heat shock induced apoptosis do exist between AuxB1 and P-GP overexpressing $CH^RC5$ cells. These differences manifest themselves in variation of response of the two main apoptotic pathways: receptor-mediated and mitochondria mediated pathways of apoptosis. The results suggest that $CH^RC5$ cells confer resistance to heat shock induced apoptosis mainly via the receptor mediated pathway. It has also been shown that several key players involved in the mitochondria mediated pathway are found to be more abundant in $CH^RC5$ cells, possibly tipping the balance toward this pathway in response to heat. Overall, the $CH^RC5$ cells are not resistant to heat shock induced apoptosis. This suggests that heat shock may provide a useful part of the treatment for eliminating MDR tumour cells.

Nonetheless, when investigating the molecular mechanisms of heat shock induced apoptosis in MDR cells, other mechanisms of cell death, independent of caspases, must be considered. These alternative types of cell death do not occur under physiological conditions but may be induced by, for example, TNF-$\alpha$ or chemotherapeutic drugs (Jaattela, 2004). Whether caspase-dependent or

-independent, the various types of regulated cell death are all executed by active cellular processes that can be interrupted by interfering with intracellular signalling. This is the main difference between these regulated types of cell death and accidental necrosis (Leist and Jaaletta, 2001). Several models of caspase independent death programs have been proposed. These include autophagy, paraptosis, mitotic catastrophe and slow cell death. Autophagy is characterized by sequestration of bulk cytoplasm and organelles in double and multimembrane autophagic vesicles which are then delivered to and destroyed by the cell's lysosomal system (Schweichel and Merker, 1973; Clarke, 1990). Further, cells that undergo excessive autophagy trigger their death without the activation of caspases (Gozuacik and Kimchi, 2004). Paraptosis is characterized by cytoplasmic vacuolation that begins with progressive swelling of the mitochondria and endoplasmic reticulum (ER). Typically, it does not involve caspase activation or any other morphological characteristics associated with apoptosis (Sperandio et al., 2000). It is thought to be mediated by MAPKs and can be triggered by the TNF receptor family member, TAJ/TROY and the insulin-like growth factor I receptor (Sperandio et al., 2004; Wang et al., 2004). Another death pathway is mitotic catastrophe, which is triggered by mitotic failure caused by defective cell cycle checkpoints and the development of aneuploid cells that eventually die. It can be triggered by agents that stabilize or destabilize microtubules and/or cause DNA damage. Cells that die by this pathway do so either during or close to metaphase in a p53-independent manner, or in a partially p53 dependent manner after failed mitosis by activation of a polyploidy checkpoint (Roninson et al., 2001). It is not yet fully understood whether mitotic catastrophe represents a completely caspase-independent cell death pathway since it has been reported to be accompanied by mitochondrial membrane permeabilization and caspase activation (Castedo et al., 2004). However, it has been argued that it is fundamentally different from apoptosis since caspase inhibition and Bcl-2 overexpression fail to prevent mitotic catastrophe (Lock and Stribinskiene, 1996; Roninson, 2001). Finally, slow

cell death has been described to represent a delayed type of regulated cell death, which occurs when caspases are inhibited or absent (Blagosklonny, 2000).

Yet despite the numerous types of regulated cell death, making exclusive definitions is difficult and would not be accurate as there is much crosstalk and many shared signalling pathways between the various death programs. The mitochondria, lysosomes and ER are the main players involved in the different types of regulated cell death in a variety of ways. Firstly, the involvement of the mitochondria in apoptotic cell death is through the release of toxic proteins from the mitochondrial intermembrane space upon the permeabilization of the outer membrane. This is tightly controlled by the Bcl-2 protein family members, which includes both pro- and anti-apoptotic proteins. Proteins which are released from the mitochondria include cytochrome *c*, endonuclease G and AIF. Cytochrome c is mostly associated with forming the apoptosome, which serves to activate caspase-9 and subsequently effector caspases. However, endonuclease G is a protease, which is able to induce caspase-independent DNA fragmentation, most likely through cooperation with caspase-dependent exonucleases and DNase I to produce internucleosomal DNA fragments under physiological conditions (Li et al., 2001; Widlak et al., 2001). Apoptosis inducing factor (AIF) is normally found in the mitochondrial intermembrane space and performs an oxydoreductase function (Miramar et al., 2001). Upon being released into the cytoplasm it translocates to the nucleus where it is involved in triggering peripheral chromatin condensation and high molecular weight (50 kb) DNA loss (Susin et al., 1999; Loeffler et al., 2001; Yu et al., 2002). The apoptogenic effects of AIF have been shown to be alleviated by HSP70 (Ravagnan et al., 2001). It has also been shown that the lysosomal protease cathepsin D is able to trigger AIF release independently of caspases (Bidere et al., 2003). AIF release can also be triggered by excessive calcium influx, which leads to overactivation of poly (ADP-ribose) polymerase-1 (Yu et al., 2002). Further, it has been suggested that AIF can act as a precautionary defence in causing death in cancer cells with flawed caspase activation (Joseph et al., 2002; Liao and Dickson, 2003).

Secondly, the lysosomal involvement in cell death has just recently become better defined and accepted as an organized set of events determining cell survival or death. The participation of lysosomal proteases has been observed in cell death induced by oxidative stress, TNF-α, bile-salt and chemotherapeutic drugs (Zhao et al., 2001; Foghsgaard et al., 2001; Guicciardi et al., 2000; Roberts et al., 1997; Johansson et al., 2003). The magnitude of lysosomal permeabilization and the amount of proteolytic enzymes released into the cytoplasm are the main factors in determining cell death type (Li et al., 2000). Several mechanisms have been proposed for the translocation of lysosomal proteases into the cytoplasm. The accumulation of sphingosine in lysosomes has been shown to facilitate the release of lysosomal enzymes into the cytosol (Kogedal et al., 2001). Generation of reactive oxygen species (ROS) can also induce lysosomal leakage as can the translocation of pro-apoptotic Bcl-2 proteins to lysosomes, where they induce pore formation and membrane permeabilization (Roberg and Ollinger, 1998; Roberg et al., 1999; Zhoa et al., 2000). HSP 70 has recently been found to promote cell survival by inhibiting lysosomal membrane permeabilization (Nylandsted et al., 2004). The most common lysosomal proteases cathepsin B and D are involved in carrying out cell death in both caspase-dependent and -independent manners. Cathepsin B is able to act as an effector protease downstream of caspases in some cells as well as to execute cell death independently of apoptotic machinery in cancer cells (Foghsgaard et al., 2001; Broker et al., 2004). Cathepsin D has been shown to activate Bax leading to selective release of AIF resulting in cell death (Bidere et al., 2003). The cleavage and translocation of pro-apoptotic Bid has also been shown in response to lysosomal disruption (Cirman et al., 2004). Thus lysosomal proteases seem to promote cell death via mitochondrial dysfunction and the subsequent release of mitochondrial proteins. Additionally, it has been reported that lysosomal proteases can cleave caspases thus suggesting that lysosomal permeabilization occurs early in apoptosis (Schotte et al., 1998; Vancompernolle et al., 1998).

Finally, the ER is crucial in sensing cellular stress and restoring cellular homeostasis by withholding protein synthesis and metabolism (Travers et al., 2000). Very severe damage to the ER results in cell death by the unfolded protein response, the release of calcium into cytoplasm and/or the induction of mitochondrial membrane permeabilization. The unfolded protein response (UPR) represents the signalling that occurs between the ER and the nucleus in response to ER stress (Schroder and Kaufman, 2005). During the UPR, accumulated proteins either undergo correct refolding or if unsuccessfully refolded are degraded by the ubiquitin-proteosome pathway. Two signalling pathways respond to ER unfolded protein overload: the UPR pathway, which triggers the expression of ER chaperones such as GRP78/Bip (Schroder and Kaufman, 2005) and the activation of nuclear factor-kappa B (NF-κB) (Szczesna-Skorupa et al., 2004). The UPR occurs through three main mechanisms: reduced translation of misfolded protein (Koumenis et al., 2002), enhanced translation of ER chaperones like GRP78 and GRP94 (Schroder and Kaufman, 2005) and ER-associated degradation that degrades misfolded proteins within the ER, which finish in the cytoplasm with the 26S proteosome (Ahner and Brodsky, 2004). Three membrane receptors, PERK, IRE1 and ATF6, are involved in triggering the UPR to ER stress signalling (Sargsyan et al., 2004). Damaged cells whose unfolded proteins exceed the threshold undergo cell death mediated by ATF4 and ATF6, as well as activation of the JNK/AP-1/Gadd153-signaling pathway. Gadd 153 suppresses activation of Bcl-2 and NF-κB. UPR cell survival or death is regulated by the balance of GRP78 and Gadd153 expression, which is co-regulated by NF-κB in accordance with the magnitude of ER stress (Kim et al., 2006).

Moreover, intracellular $Ca^{2+}$ influx caused by ER stress results in the activation of calpains (Guroff, 1964). Calpains are cytosolic proteases, which act downstream of caspase activation and are in turn inhibited by calpastatin (Wood and Newcomb, 1999; Wood et al., 1998). Calpastatin is inactivated by calpain- or caspase-mediated cleavage (Wang, 2000). The release of $Ca^{2+}$ into the cytosol leads to the activation of caspase-12, which normally resides on the cytosolic face of the

ER, and the subsequent activation of downstream caspases. This is thought to occur via translocation of Bim to the ER (Morishima, 2004). ER damage can also induce permeabilization of the mitochondrial outer membrane and the subsequent release of pro-apoptotic proteins contained within, inducing the apoptotic pathway (Jimbo, 2003). Bcl-2 family proteins and $Ca^{2+}$ shifts in the cytosol are believed to be involved in the signalling between the ER and mitochondria (Annis et al., 2004; Mattson and Chan, 2003).

Undertaking this study, which investigated the molecular mechanisms of heat shock induced apoptosis in drug sensitive AuxB1 and MDR $CH^{R}C5$ cells, aside from giving important insights, more importantly prompts more questions. The study has shown differences between the two CHO cell types in heat shock induced apoptosis. The $CH^{R}C5$ cells, which overexpress P-GP, appear to confer resistance to heat shock via the receptor mediated pathway of apoptosis and push the equilibrium toward the mitochondria mediated pathway in response to heat shock. These findings are important in understanding induction of cell death in MDR cells using heat shock as a sensitizer to cancer treatments such as chemotherapy. If the mechanisms of the response of MDR cells to heat shock can be fully elucidated, development of more tailored and successful treatments could be possible. This study is a cornerstone first glance into the molecular mechanisms involved in heat shock induced cell death in MDR $CH^{R}C5$ cells which overexpress P-GP. Nevertheless, more work lies ahead in defining the signalling pathways involved and further, formulating conclusions that may be applied in design of cancer treatment in the future.

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