UNIVERSITÉ DU QUÉBEC À MONTRÉAL

INDUCTION DE L'APOPTOSE PAR L'HYPERTHERMIE ET L'ADRIAMYCINE CHEZ LES CELLULES HeLa AVEC SUREXPRESSION DE LA MRP1

MÉMOIRE
PRÉSENTÉ
COMME EXIGENCE PARTIELLE
DE LA MAÎTRISE EN CHIMIE
(CONCENTRATION BIOCHIMIE)

PAR

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Mai 2006

ACKNOWLEDGEMENTS

A journey is easier when you travel together. Interdependence is certainly more valuable than independence. This thesis is the result of two and half years of work whereby I have been accompanied and supported by many people. It is a pleasant aspect that I have now the opportunity to express my gratitude for all of them.

I am deeply indebted to my research director Prof. Dr. Averill whose help, stimulating suggestions and encouragement helped me in all the time of research and for the thesis. I would also like to thank my colleagues André Tanel, Tatiana Souslova, Qixiang Ke, Anissa Chérif, Zhenghui Wang and Oliver Didur who helped me throughout my study by inspiring conversations, by their valuable suggestions and by the beautiful moments spent together. I would like to give my special thanks to my friend and colleague Ahmed Bettaieb for moral support and for always being there for me and for our many discussions and providing me brotherly advice and tips that helped me a lot in staying on the right track. I am also thankful to my friend and colleague Paulina Wrzal for helping me correcting English and offering suggestions for improvement.

I am grateful to my mother for giving me her auspicious blessing and positive support. I am also thankful to my brother Vishal and sisters Yamini, Ragini, Damini and Bhavini for rendering me the sense and the value of sisterhood. I am glad to be one of them. I also want to give many thanks to my mother in law for understanding and helping me through difficult times.

The necessary financial support for the development of the work presented in this thesis was provided by the Cancer Research Society (DAB), Natural Sciences and Engineering Research Council of Canada (DAB) through research grants to Dr. Diana Averill. Funding was also obtained from Université du Québec à Montréal in the form of internal scholarships: PAFARC, Rosendahl-Lapointe and Mario Plouffe.

DEDICATION

I am honoured to dedicate this thesis to the three most important men of my life, first my father, Shree Balvant Bhai Barot, whose blessing gives me courage and confidence in my self. I feel a deep sense of gratitude to my father who formed part of my vision of life and taught me the good things that really matter in life. The happy memory of my father still provides a persistent inspiration for my journey in this life. Secondly, my husband, Hitesh whose love and patience enabled me to complete this work, for putting up with me during this most stressful year and his continual presence, moral support and faith in me which helped me to continue my study. Finally I am so glad to devote this thesis to my son Maharshi. One of the best experiences that my husband and I lived through in this period was the birth of our son Maharshi, who provided an additional and joyful dimension to our life mission.

TABLE OF CONTENTS

HBV	Hepatitis-B
HCV	Hepatitis-C
HeLaMRP	HeLa cells with overexpressed MRP1 protein with the phenotype of multidrug resistance to chemotherapy
HPV	Human papilloma virus
HSPs	Heat shock proteins
HT	Hyperthermia
HTLV-I	Human T cell leukemia virus type I
IAP	Inhibitors of apoptosis proteins
L-BSO	L-buthionine sulfoximine
LRP	Protein associated with resistance in lung cancer
LTC_4	Cysteinyl leukotriene C_4
MDR	Multidrug resistant
MRP	Multidrug resistance protein
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate-buffered saline
P-gp	P-glycoprotein
RNA	Ribonucleic acid
SDS-PAGE	Polyacrylamide gel electrophoresis
SEM	Standard error of the mean
Smac	Second mitochondrial activator of caspases
TNF	Tumor necrosis factor
TNFR1	Receptor of TNF
TRADD	TNF receptor 1-associated protein
γ-GCS	γ-glutamylcysteine synthetase

LIST OF ABBREVIATIONS

ABC	ATP-Binding cassette
ADR	Adriamycin
AIF	Apoptosis inducing factor
Apaf-1	Apoptotic protease activating factor-1
ATP	Adenosine triphosphate
AuxB1	Epithelial cells of ovaries of Chinese hamster
BH domains	Bcl-2 homology domains
Bid and tBid	(truncated) BH3-Interacting death domain agonist
Caspase	Cysteinyl aspartate-specific protease
CAT	Catalase
CHO	Chinese hamster ovary
CH^RC_5	AuxB1 cells with overexpressed P-glycoprotein with a phenotype of multidrug resistance to chemotherapy
DD	Death Domain
Diablo	Direct IAP binding protein with low pI
DISC	Death-inducing signaling complex
DMEM	Dulbecco modified eagle's medium
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
EBV	Epstein-Barr Virus
Endo-G	Endonuclease G
FADD	Fas-associated with death domain
FBS	Fetal bovine serum
GPx	Glutathione peroxidase
GS	Glutathione synthetase
GSH	Reduced glutathione
GST	Glutathione S-transferase

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RESUME

Introduction: L'apoptose est un mécanisme étroitement contrôlé qui répond à des besoins particuliers de l'organisme et permet une élimination physiologique des cellules excessives ou endommagées. Elle est donc nécessaire au développement et au maintien du bon fonctionnement de tout organisme vivant puisqu'elle joue un rôle important dans l'embryogenèse, dans les changements morphologiques, dans l'homéostasie cellulaire, dans l'atrophie et la réparation des tissus et dans la régression des tumeurs. Cette forme de mort cellulaire fait intervenir une famille de sérines protéases connues sous le nom de caspases. Leur activation survient après stimulation des cellules par différents facteurs comme des signaux physico-chimiques (UV, rayons gamma), la privation en facteurs de croissance ou une grande variété des molécules chimiques comme les polluants environnementaux et les médicaments anti-cancéreux. Parmi ces derniers on cite l'Adriamycine qui est un médicament anti-tumoral efficace, cependant son utilisation entraîne malheureusement de sévères altérations du fonctionnement cardiaque. Ceci limite par conséquent les doses pouvant être données sans danger aux patients cancéreux. D'autre part l'utilisation clinique de ce médicament semble être également limitée par la résistance que développent certaines formes de tumeurs à la chimiothérapie. Pour surmonter ces deux limites les chercheurs ont essayé de développer certaines stratégies thérapeutiques comme l'hyperthermie, visant à sensibiliser la tumeur aux faibles concentrations des médicaments.

Objectifs : La présente étude permettra d'étudier le mécanisme de mort cellulaire induite par l'Adriamycine et l'hyperthermie. Les deux principaux piliers de ce projet sont : (1) Déterminer si l'Adriamycine ou l'hyperthermie utilisées séparément peuvent déclencher l'apoptose chez des cellules tumorales du col utérin humain (HeLa) et chez la même lignée exprimant la protéine de résistance aux médicaments MDR (HeLa MRP). (2) Déterminer si l'hyperthermie pourrait sensibiliser les cellules à l'action de l'Adriamycine.

Résultats : L'Adriamycine ou l'hyperthermie utilisés séparément ou combiné avait comme effet une activation de la caspase initiatrice (9) et de la caspase effectrice (3) ainsi qu'un clivage de l'inhibiteur du facteur de fragmentation d'ADN activé par les caspases (ICAD). D'un autre coté l'hyperthermie a causé l'accumulation intracellulaire de l'Adriamycine, la diminution du potentiel membranaire mitochondriale due à une translocation du Bax vers la mitochondrie suivi d'un relarguage du cytochrome c vers le cytosol.

Conclusion : L'hyperthermie seule pourrait induire l'apoptose et pourrait servir comme une stratégie utile pour augmenter l'effet pro-apoptotique de l'Adriamycine chez des cellules HeLa parentales ou résistantes aux médicaments (HeLa MRP).

Mots clefs : Hyperthermie, Résistance pléiotropique, MRP1, Apoptose, Adriamycine

ABSTRACT

Abstract

Introduction: Apoptosis is a mechanism of cell death, which provides a physiological elimination of excess or damaged cells. It follows a characteristic program of events, including activation of the caspase cascade. A wide variety of toxic compounds (environmental pollutants, drugs) can induce cell death by apoptosis. Adriamycin is a widely used anticancer drug, however, its mechanisms of antitumour activity have been a long term matter of debate. In addition, the mechanisms of severe cardiac toxicity, which limit the usefulness of the drug, are not well understood. Multidrug resistance is a major obstacle in the successful use of cancer chemotherapy at clinical level. However, at present hyperthermia is used as a useful technique to overcome the problem of multidrug resistance. Purpose of using hyperthermia, is to increase the anticancer effect of Adriamcyin by sensitizing cell towards drug, at the same time decreasing the toxic effect of high dose of Adriamycin.

Objectives: The present study investigates the mechanism of cell death induced by Adriamycin and hyperthermia. The two main objectives of this project are: (1) to determine whether Adriamycin and hyperthermia alone can trigger apoptosis in human cervical adinocarcinoma cells over expressing MRP1 (HeLaMRP) relative to parental HeLa cells and (2) to determine whether hyperthermia can enhance the induction of apoptosis by Adriamycin.

Results: Treatment of cells with Adriamycin or hyperthermia alone or combined together resulted in activation of caspase-9 and caspase-3 as well as cleavage of inhibitor of caspase-activated DNase (ICAD), which leads to induction of apoptosis. Hyperthermia enhanced intracellular accumulation of Adriamycin, associated to a decrease in the mitochondrial membrane potential and translocation of pro-apoptotic proteins, Bax and cytochrome c between the cytoplasm and mitochondria of the cell.

Conclusion: Adriamycin is able to induce apoptosis by the mitochondrial pathway of apoptosis in both drug-sensitive and MRP1-overexpressing HeLa cells and hyperthermia alone is enable to induce apoptosis and could be a useful strategy to enhance the induction of apoptosis by Adriamycin in both drug-sensitive and MRP1-overexpressing HeLa cells.

Keywords: Hyperthermia, Multidrug resistance, MRP1, Apoptosis, Adriamycin

CHAPTER 1

INTRODUCTION

1.1 Cancer

1.1.1 Introduction and history of cancer

Cancer is a disease, which originates within a single cell. Body cells grow, divide and die in an orderly manner until the person becomes an adult. After that, cells in most parts of the body divide only to replace depleted or dying cells and to repair injury. Cancer develops when there is uncontrolled growth of cells in a part of the body. It is a mutation in the cells, which causes them to replicate continuously. All types of cancer start due to uncontrolled growth of abnormal cells. The discovery of "oncogenes", "tumor suppressor genes" and "DNA repair genes" during the 1980s and 1990s provided a clearer picture explaining the reason for uncontrollable growth of cancer cells.

The origin of the word "cancer" is credited to the Greek physician Hippocrates (460-370 B.C.). He found swollen blood vessels around the area of a malignant tumor which reminded him of crab claws leading to the coining of terms like *karkinos* and *karkinoma* (the Greek name for crab) to depict it. Later on, these terms evolved into carcinos or carcinoma. An Egyptian papyrus shows the oldest description of human cancer which was written between 3000-1500 BC. Galen, an ancient Roman, noted the crab like appearance of cancerous tumors and thought it to be caused by an excess of black bile. While the word cancer is frequently used to describe this disease, it is important to understand that there are over 200 different types of cancer and each has a specific name, treatment and probability of recovery.

1.1.2 Development of cancer

Damage to the DNA is a primary step of development of a tumor cell. Generally, the cell is able to repair the damaged DNA. In cancer cells, the damaged DNA is not repaired and leads to the formation of a tumor. Tumors can be either benign (non-cancerous) or malignant (cancerous). Benign tumors reside in one place

in the body and are usually not critical, while for malignant tumors, cells are able to invade the tissues around them and spread to other parts of the body. However, there are some cancers that do not form solid tumors (e.g. leukaemia).

1.1.3 Canadian 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 2004 an estimated 68,300 deaths were due to cancer and the number of new cases of cancer was an approximate 145,500. Breast cancer continued as the most common cancer in women and prostate cancer for men, whereas lung cancer remained the other leading common area of concern for both men and women. On the basis of current incidence rates, during their lifetimes 38 percent of Canadian women and 43 percent of men will develop cancer and on the basis of current death rates, 23 percent of women and 28 percent of men will die of cancer. According to the age and sex distribution of cancer, people who are at least 60 years old are most likely to suffer from cancer. For example, among women, 63 percent of new cases and 78 percent of cancer deaths occur among those who are at least 60 years old. In addition, it was also predicted that cancer would be the leading cause of premature death in Canada in the year of 2004 (www.cancer.ca).

1.1.4 Risk factors of cancer

Understanding the causative factors of cancer could contribute to prevention of this disease. There are several risk factors leading to development of cancer in humans and most of them are preventable. Tobacco smoke is the single

factor known to have caused the highest proportion of cancer and is known to cause 30% of all cancers. Research shows that tobacco consumption is related to cancer of the lung, mouth, larynx, esophagus, bladder, kidney and pancreas (Levitz *et al.,* 2004; Doll *et al.,* 1994). Several studies that measured the individual dietary fat intake of large groups of women showed that unhealthy diet is one of the main causes of breast cancer (Hardy, 2005; van Gils *et al.,* 2005). Environment in the work place is also a risk factor for certain forms of cancer. For instance, workers that have direct contact with carcinogenic agents (e.g. arsenic, aluminium, asbestos, chromium, cadmium, etc) in the workplace are at risk for developing certain forms of cancer (Wogan *et al.,* 2004; Heath, 1996). Viral infection (e.g. H-Pylori, HTLV-I, EBV, HBV, HCV and HPV) is also one of the recognized risk factors of cancer (Poland and Jacobson, 2004; Evans *et al.,* 1998). Furthermore, lack of exercise, excessive alcohol, environmental pollutants, UV rays and genetic susceptibility are also well known risk factors for cancer.

1.2 Treatment of cancer

Cancer treatment varies depending upon the nature of cancer and the phase of cancer. Moreover, treatment may vary depending on whether or not the objective of treatment is to cure the cancer, to prevent the cancer from dispersion, or to ease the symptoms caused by cancer. The three most common ways to treat cancer are surgery, radiation therapy and chemotherapy. More recent methods in cancer treatment include immunotherapy, gene therapy, enzymotherapy, molecular therapy, galvanotherapy (electrochemotherapy), photodynamic therapy, herbal therapy, nutritional therapy, and adjunctive therapies such as hyperthermia, oxygen therapies (including ozone), dimethyl sulfoxide (DMSO) therapy, live cell therapy, etc.

1.2.1 Surgery

Surgery is the branch of medical science that treats disease or injury by operative procedures. Surgery is useful to diagnose, to determine the stage and to treat cancer. There are several types of surgeries, depending on the stage of cancer (Fleming, 2001; Pollock and Morton, 2003) and they are often performed to accomplish more than one of the above mentioned objectives. Preventive (prophylactic) surgery is used to remove body tissue that is not malignant but that is likely to become malignant such as polyps in colon. Diagnostic surgery helps to analyse whether the sample is cancerous or not e.g. biopsy, staging surgery helps determine the extent of disease. Curative surgery is the removal of a tumor when it appears to be confined to one area. There are several other classes of surgery that are commonly used for cancer treatment such as debulking (cytoreductive) surgery, palliative surgery, supportive surgery and restorative (reconstructive) surgery.

1.2.2 Radiation therapy

Radiation therapy is the use of high-energy radiation from X-rays, gamma rays, neutrons and other sources to kill cancer cells and shrink tumors. Radiation therapy is a highly targeted and effective way to destroy cancer cells and is often used after surgery (Perez and Brady, 1998). This reduces the risk of recurrence. Radiation therapy is relatively easy to endure and the side effects are restricted to the area being treated (Hof and Debus, 2005) and it is also efficient to reduce pain due to the tumor (Yorozu *et al.*, 2003).

1.2.3 Chemotherapy

Chemotherapy is the use of chemical agents (anti-cancer or cytotoxic drugs) which interact with cancer cells to eliminate or control the growth of cancer (Undevia

et al., 2005; Trotice, 1997). It is a mainstay in the treatment of malignancies. Cancer chemotherapy may consist of single drugs or combinations of drugs and can be administered intravenously, injected into a body cavity, or delivered orally (Burke *et al.,* 1996). A major advantage of chemotherapy over radiation and surgery is its ability to treat widespread or metastatic cancer, rather than physically removing a tumor or a part of it. Hence chemotherapy is considered a systemic treatment. More than half of all people diagnosed with cancer receive chemotherapy.

Listed below are several major categories of chemotherapeutic agents based on their chemical structures. Alkylating agents such as nitrogen mustards and ethylamines are the most commonly used in chemotherapy. Secondly, plant (vinca) alkaloids are antitumor 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 (Earhart, 1999), which are widely used to treat advanced ovarian and breast cancers (Khayat *et al.,* 2000; Lamb and Wiseman, 1998). Finally, antimetabolites, nitrosoureas and anti-tumor antibiotics are among the well known categories of chemotherapeutic agents.

Anti-tumor 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-tumor antibiotics such as Adriamycin, dactinomycin, bleomycin and mithramycin are used to treat a variety of cancers.

1.3 Adriamycin

1.3.1 An overview

Anthracycline antibiotics (Review: Gianni *et al.,* 2003) such as Adriamycin are among the most important anti-tumor drugs available (Weiss, 1992). They have been in clinical practice since the 1960s. Adriamycin is a water soluble anticancer agent that was first isolated from *Streptomyces peucetius* variety *casius* (Arcamone *et al.,* 1969). Doxorubicin is the common trade name of Adriamycin. This drug has been used to treat a broad range of malignancies including tumors arising in breast, bile ducts, endometrial tissue, liver, soft tissue sarcomas, as well as various disseminated neoplasms, namely leukemia, bone marrow sarcoma, carcinomas of the thyroid and bladder and others (Gewirtz, 1999).

1.3.2 Structure of Adriamycin

Adriamycin possesses an anthracycline chromophore containing four fused rings and a positively charged amino sugar (Figure 1). Adriamycin shares structural similarity with another anthracycline molecule named daunorubicin (DNR). It differs only by the presence of a hydroxyl group (-OH) at the 14-position (Taatjes *et al.,* 1997). Adriamycin consists of a hydroxylated tetracycline quinone attached to a sugar residue via a glycoside bond (Wallace, 2003). As shown in the figure, the planar ring system has electronic resonance in the first and third rings with a conjugated quinone structure in the second ring. The secondary hydroxyl group on the 3rd ring and tertiary group on the 4th ring seem to be key participants in hydrogen bonding interactions that stabilize the drug-DNA complex (Gao and Wang, 1991; Pohle *et al.,* 1990).

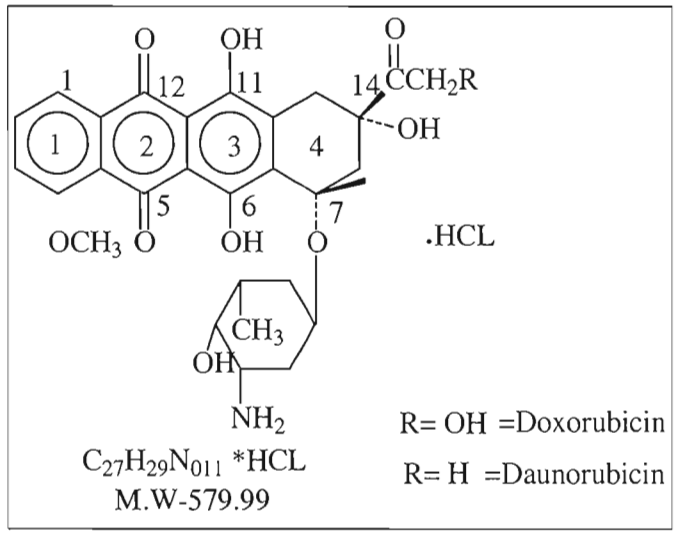

Figure 1.1: Schematic representation of Adriamycin and Daunorubucin.
(Figure adapted from Frederick *et al.*, 1990)

The quinone portion of the anthracycline ring is lipophilic, however the saturated end of the ring system contains abundant hydroxyl groups adjacent to the amino sugar, producing a hydrophilic center. Thus the actual molecule is amphoteric displaying both acidic and basic properties. The well conjugated structure of Adriamycin is responsible for the red-orange color and fluorescent nature which is a useful tool for its chemical, biological and pharmacological studies.

1.3.3 Mechanisms of action of Adriamycin

Elucidation of the mechanisms by which anticancer agents induce apoptosis is necessary in order to understand the basis of drug resistance as well as for optimization of therapy. The exact mechanism of action of Adriamycin is unknown. Several studies suggest that Adriamycin has the propensity to display multiple cellular effects, which contribute to its antineoplastic properties (Sharples *et al.*, 2000; Mayers *et al.*, 1998). Adriamycin intercalates into the DNA double helix by inserting itself into the strands of genetic material (DNA) inside the cell and binding

them together. This prevents the cell from replicating its genetic material (Halliwell and Gutteridge, 1999; Mayers *et al.,* 1998). It also appears to interfere with an enzyme called topoisomerase II that is involved in DNA replication (Hurley, 2002; Jung and Reszka, 2001; Gewirtz, 1999). Finally, it can also form free radicals which are molecules capable of damaging cells (Chandra *et al.,* 2000; Gewirtz, 1999; Bachur *et al.,* 1977). Thus, these different actions of Adriamycin impede many cellular functions (Sharples *et al.,* 2000).

1.3.3.1 Interactions with DNA

Recent interest in the use of the DNA-Adriamycin complex as an approach to improve the therapeutic effectiveness and to reduce toxicity of Adriamycin for cancer chemotherapy requires an in-depth understanding of the physicochemical and biochemical properties of such complexes. Although the interaction of Adriamycin with other cellular targets may play a role in the selective cytotoxicity of this drug, binding to DNA is generally believed to be essential for its activity. The primary mode of action of Adriamycin is believed to be its reversible binding to nuclear DNA which causes inhibition of both replication and transcription processes (Neidle *et al.,* 1997; Zunino *et al.,* 1977, 1975) and subsequently leads to cell death. Numerous biochemical studies including evidence from NMR spectroscopic and X-ray crystallographic studies have shown that Adriamycin intercalates into the β-form of the DNA double stranded helix with guanine-cytosine d(CpG) site-specific interactions (Chaires *et al.,* 1990). These findings were similar to the work of Manfait and co-workers (Manfait *et al.,* 1982). They analyzed the Raman and resonance Raman spectra of the DNA-Adriamycin complex in aqueous solution. They reported that the chromophore of Adriamycin is intercalated in the GC sequences and the substituents on the rings give hydrogen-bonding interactions with the DNA base pairs above and below the intercalation site. It was also observed that the phenolic groups

of the chromophore were involved in the drug-DNA intercalation, in addition to pi-pi, hydroxyl and amino group interactions (Manfait *et al.,* 1982).

The following events are prerequisite for intercalative interactions between Adriamycin and DNA to take place. First, the DNA must undergo a conformational transition to form the intercalation site. For this event the DNA base pairs separate 3.4 Å leading to the formation of a cavity for the incoming Adriamycin-chromophore to insert. This is accomplished by localized unwinding of the contiguous base pairs at the intercalation site and increasing of the distance between the phosphate groups on the sugar phosphate backbone on both strands. This results in reduction of the localized charge density and facilitates the release of condensed counter ions such as Na^+. The next event that occurs involves the transfer of the drug from aqueous solution to the intercalation site and finally, it's insertion into the DNA duplex (Manning, 1978; Record *et al.,* 1978). The non-covalent interactions between the ligand and the base pairs associated with the DNA binding site are driven by several forces including hydrophobic effects, reduction of columbic repulsion as a result of the polyelectrolyte effect, van der Waals interactions, pi-stacking interactions and hydrogen bonding (Record and Spolar, 1990).

1.3.3.2 Generation of reactive oxygen species

Adriamycin is also known to be involved in oxidation/reduction reactions. A number of NADPH-dependent cellular reductases such as mitochondrial NADPH dehydrogenase (Muraoka and Miura, 2003), xanthine dehydrogenase (Yee and Pritsos, 1997) and endothelial nitric oxide synthase (Vasquez-Vivar *et al.,* 1997) are able to reduce Adriamycin to a semiquinone free radical. Under aerobic conditions, the semiquinone is oxidized by molecular oxygen back to the parent compound (Adriamycin) and the reaction produces superoxide radical anion ($O_2^{\cdot-}$) (Figure 2). The formation of $O_2^{\cdot-}$ is the beginning of a cascade that generates highly reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and the hydroxyl radical

($OH^{\cdot}$). For instance, superoxide can react with itself leading to the formation of hydrogen peroxide (Figure 2) (Minotti *et al.*, 1999; Bounias *et al.*, 1997; Michalska *et al.*, 1996). Hydrogen peroxide can react with either the Adriamycin semiquinone free radical or it will undergo reductive cleavage to hydroxyl radicals ($OH^{\cdot}$) (Bates and Winterbourn, 1982). All of these highly reactive free radical species are widely invoked as the primary mechanism underlying many of the toxicities observed with Adriamycin and related anthraquinones (Tsang *et al.*, 2003; Nakagawa *et al.*, 2002). For example, the generation of this semiquinone free radical of Adriamycin has been shown to result in the cleavage of DNA (Lown *et al.*, 1977). Adriamycin is also responsible for oxidative modification of nucleic acids, lipids and proteins (Monti *et al.*, 1995; Keizer *et al.*, 1990; Piccinini *et al.*, 1990).

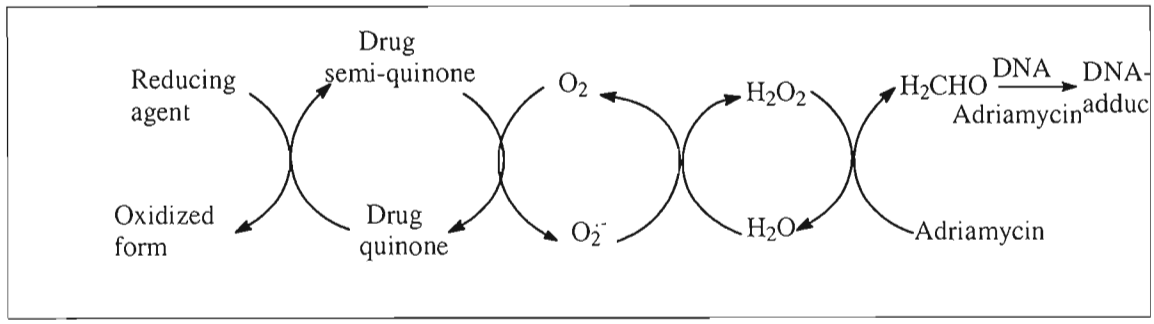

Figure 1.2: The steps from drug reduction to DNA-drug adduct formation. This process involves a cascade of reactions starting with drug reduction and catalytic production of reactive oxygen species followed by oxidative formation of formaldehyde and ending with drug-DNA alkylation mediated by formaldehyde (Adapted from Taatjes et al., 1997).

1.3.3.3 Inhibition of topoisomerase II

The interaction of Adriamycin with topoisomerase II to form DNA-cleavable complexes also appears to be an eminent mechanism of Adriamycin cytocidal activity. Over a decade ago, topoisomerase II was identified to be a primary cellular target for many of these DNA binding agents. Topoisomerase II is a nuclear enzyme which, in the presence of Adriamycin, causes extensive fragmentation of DNA by catalyzing the interconversion of topological isomers of DNA, thereby playing a key role in DNA metabolism (Toonen and Hande, 2001; Hengstler *et al.*, 1999).

Adriamycin has been shown to exert antineoplastic activity through the formation of a ternary complex between the ligand, DNA and enzyme.

1.3.3.4 Metal-ion chelation

Metal-ion chelation is also one of the cytotoxic characteristics of Adriamycin. As previously mentioned, the anthracycline chromophore contains a hydroxyl quinone, which is a well-described iron chelating structure. Adriamycin is involved in the chelation of metal ions such as Cu^{+2}, Fe^{+2} and Fe^{+3} (Kalyanaraman *et al.,* 2002). For instance, in the case of iron, Adriamycin tends to form the Adriamycin-Fe-DNA complex which catalyses the transfer of electrons from glutathione to oxygen resulting in the formation of active oxygen species, which leads to cleavage of DNA.

1.3.3.5 Membrane effects

Further action for Adriamycin can be demonstrated at the cell membrane level. This drug can bind to cell membrane lipids and affect a variety of functions (Solem *et al.,* 1994; Solem and Wallace, 1993). Many studies have highlighted the fact that Adriamycin disrupts mitochondrial function by inducing the mitochondrial permeability transition (Ling *et al.,* 1993). This mechanism consists of metamorphosis in properties of the inner mitochondrial membrane transforming it from a restrictive barrier into passive permeation (Wallace, 2003). In conclusion, cytotoxicity and/or antiproliferative activity of Adriamycin may result as a consequence of any of the above mentioned mechanisms and/or those not yet identified.

1.3.4 Side effects of Adriamycin

Regardless of all the advantages, this drug has various side effects namely: diarrhea, facial flushing, red coloration of urine, anemia, leucopenia, stomatitis,

immunosuppression, mucositis, oesophagitis, nausea and vomiting. However, the main limiting factor in its usage as an antitumoral agent is chronic or acute cardiotoxicity (Pagnini *et al.*, 2000; Minow *et al.*, 1977).

Many applications have explained the selective cardiotoxicity of Adriamycin (Olson and Mushlin, 1990). However, the pathogenesis of the Adriamycin-induced cardiomyopathy is not well understood. Cardiac toxicity occurs after prolonged administration of Adriamycin, eventually leading to congestive heart failure. It has been suggested that Adriamycin undergoes redox cycling on mitochondrial complex I and liberates highly reactive oxygen free radicals (Doroshow, 1983). These highly reactive oxygen species have been widely implicated as a primary cause for Adriamycin-induced cardiac toxicity (Xu *et al.*, 2001; Lee *et al.*, 1991).

1.4 Multidrug resistance

1.4.1 An overview

Resistance to multiple chemotherapeutic agents is considered a major cause of chemotherapy failure (Linn and Giaccone, 1995). Drug resistance can be classified into two categories: 1) intrinsic drug resistance (e.g. renal and prostate cancers) where cells already have a relatively resistant phenotype to anticancer agents without drug-selection; 2) acquired drug resistance (Yoshiyama *et al.*, 2004; Selby, 1984), characterised by the attainment of a resistant phenotype after being exposed to cytotoxic agents. Acquired drug resistance in persistent tumors is the most critical and perilous occurrence for patients treated by chemotherapy. Furthermore, once drug resistance is developed, cancer cells often acquire cross-resistance to a variety of chemically and functionally unrelated compounds. This phenomenon is known as multidrug resistance (MDR) (Seiji Naito *et al.*, 1999).

MDR was first described in 1970 during cell culture by Biedler and Riehm. They found cellular resistance to actinomycin D in Chinese hamster cells *in vitro* (Biedler and Riehm, 1970). Later on, they discovered that this cell line also shows

resistance to vinca alkaloids, epipodophyllotoxines, anthracyclines, dactinomycin and taxol. These drugs had different cellular targets and mechanisms of action. It was later discovered that increased active drug efflux out of the MDR cells resulted in reduced accumulation of drugs (Dano, 1973). In 1986, the multidrug resistance gene mdr1 was cloned and P-glycoprotein (P-gp) recognized as the first molecule to explain the MDR phenomenon (Roninson *et al.,* 1986; Chen *et al.,* 1986, Gros *et al.,* 1986)

The mechanisms mainly studied for investigation of MDR with known clinical significance are namely: 1) activation of transmembrane proteins, effluxing different chemical substances from the cells; 2) activation of enzymes of the glutathione detoxification system; 3) alterations of genes and proteins involved in the control of apoptosis (Review: Stavrovskaya, 2000).

1.4.2 Multidrug resistance proteins

A variety of ATP binding cassette (ABC) transporters, localized in the cell membrane, cause the MDR phenomenon by extruding a variety of chemotherapeutic agents from tumor cells. These transporters play a key role in drug availability, metabolism and toxicity. The four foremost groups of ABC transporters which are involved in MDR are namely: the classical P-glycoprotein (P-gp) (MDR1), the multidrug resistance associated proteins (MRP1, MRP2 and probably MRP3, MRP4 and MRP5) (ABCB1), the ABCG2 protein and ABC half-transporters. All these proteins have been known to act as catalysts for ATP-dependent active transportation of anticancer agents (Bodo *et al.,* 2003). The following text briefly summarises the most important MDR related proteins.

1.4.2.1 P-glycoprotein

1.4.2.1.1 An overview

P-gp (Sakaeda *et al.,* 2004) is a 170 kDa membrane-bound protein and a product of the mdr1 gene. It was first characterised in MDR Chinese hamster ovary cells by Ling and co-workers (Kartner *et al.,* 1983; Ling and Thompson, 1974). It has been implicated as a primary cause of MDR in tumors (Georges *et al.*, 1990). It is generally found in the gut, gonads, kidneys, biliary system, brain and other organs. P-gp is found not only in cancer cells but also in normal tissue cells, such as hepatocytes, renal proximal tubular cells, and epithelial cells. It serves as an ATP-dependent efflux pump by actively transporting chemicals and anti-tumor agents out of cells thereby reducing their cytosolic concentration. This leads to decreased toxicity in cells (Gottesman and Pastan, 1993; Doige *et al.*, 1992). Apart from its drug efflux function, P-gp has many proposed physiological functions. Expression of P-gp in CD34+ stem cells and specific peripheral blood subsets raises the possibility that P-gp could play a role in haemopoietic development and immune cell functions (Lucia *et al.,* 1995; Drach *et al.,* 1992). Specifically, P-gp has been shown to be involved in the release of certain cytokines from T lymphocytes (Frank *et al.,* 2001; Drach *et al.,* 1998; Raghu *et al.,* 1996).

1.4.2.1.2 Structure and mechanisms

P-gp is composed of 1280 amino acids. It has two homologous halves, each containing six hydrophobic transmembrane segments and a nucleotide sequence with both NH_2 and COOH terminals of the protein located in the cytoplasm (Gottesman and Pastan, 1993). This molecule has two ATP-binding domains indicating that the function of P-gp is energy-dependent (Chen *et al.,* 1986). In cancer cells, P-gp is associated with the MDR phenotype, mediating resistance to anthracyclines, vinca alkaloids, colchicines, epipodophyllotoxins and paclitaxel (Avendano and Menendez, 2002). Despite numerous studies, it is still difficult to decipher the mechanism by which P-gp confers cellular resistance to cytotoxic attack by structurally unrelated

drugs. One of the most popular hypotheses proposes that the drug binds to a specific site of P-gp within the lipid bilayer of the cell plasma membrane. Then, by means of the energy of ATP hydrolysis, the drug molecule is transported out of the cell. Chemotherapeutic drugs were shown to be bound better to the membranes of drug resistant cells than to those of drug sensitive cells.

1.4.2.1.3 Modulators of P-glycoprotein

P-gp activity is controlled by a variety of endogenous and environmental stimuli which induce stress responses including heat shock, irradiation, genotoxic stress, inflammation, growth factors and cytotoxic agents (Sukhai, 2000). P-gp mediated MDR can be reversed by various chemo sensitizers or reversing agents such as Ca^{2+} channel blockers (e.g. verapamil), immuno-suppressants (e.g. cyclosporine A), antiarrhythmic drugs (e.g. quinidine) and many other lipophilic compounds (Gottesman and Pastan, 1993). All of these diverse compounds are hydrophobic and may bind to the P-gp molecule directly and impede its function in a competitive manner (Safa *et al.,* 1989; Naito and Tsuruo, 1989; Nogae *et al.,* 1989). P-gp mediated MDR to Adriamycin can be altered by a combination of hyperthermia with cyclosporine A and verapamil (Averill and Larrivée, 1998; Averill and Su, 1999). This combination is also useful in overcoming melphalan resistance by increasing intracellular drug accumulation in multidrug-resistant cells $CH^{R}C5$ cells (CH(R)C5) (Larrivée and Averill, 1999).

1.4.2.2 Multidrug resistance associated protein (MRP1)

1.4.2.2.1 An overview

Multidrug resistance protein 1 (MRP1) is a member of the C branch of the super family of the ATP-binding cassette (ABCC1) transporter proteins. MRP1 was originally cloned from an Adriamycin selected MDR human lung cancer cell line H69 (Grant *et. al.,* 1994; Cole *et al.,* 1992). MRP1 is a human 190 kDa protein,

encoded by the mrp1 gene, which is located on chromosome 16 (Loe *et al.,* 1996; Zaman *et al.,* 1993). The over expression of MRP1 is sufficient to confer MDR to structurally diverse natural products and cytotoxic drugs, and to mediate their efflux in an ATP dependent manner (Payen and Gao, 2003; Zaman, *et al.,* 1994; Cole *et al.,* 1992). MRP1 is present in almost all cells of the human body. MRP1 is not only expressed at the cell membrane, but is also located in the cytoplasm, endoplasmic reticulum, or golgi apparatus in some cells (e.g. HL/ADR, etc) (Almquist, 1995; Marquardt, 1992). It has been detected in tumors with many different cellular origins (Leonard, 2003). MRP1 is also able to protect normal tissues from the effects of toxic substances. The ubiquitous presence of MRP1 in many cells, together with the fact that this ABC protein can export unconjugated bilirubin (UCB) from the cell, suggests that this transporter functions throughout the organism to protect cells against accumulation of toxic levels of UCB.

The clinical cancers exhibiting MRP1 expression include hematological (Burger *et al.,* 1994a, b; Versantvoort *et al.,* 1994), lung (Savaraj *et al.,* 1994), acute lymphoblastic leukemia relapses and chronic myeloid leukemia (Hirose *et al.,* 2003; Beck *et al.,* 1994). Moreover, MRP1 is found to be over expressed in many non-P-gp expressing MDR cell lines (Slovak *et al.,* 1993; Cole *et al.,* 1992).

1.4.2.2.2 Structure of MRP1

The core structure of MRP1 shows similarity to other ABC transporters (Figure 3). It has two membrane-spanning domains (MSDs), each followed by a nucleotide-binding domain (NBD) (Leslie *et al.,* 2001).

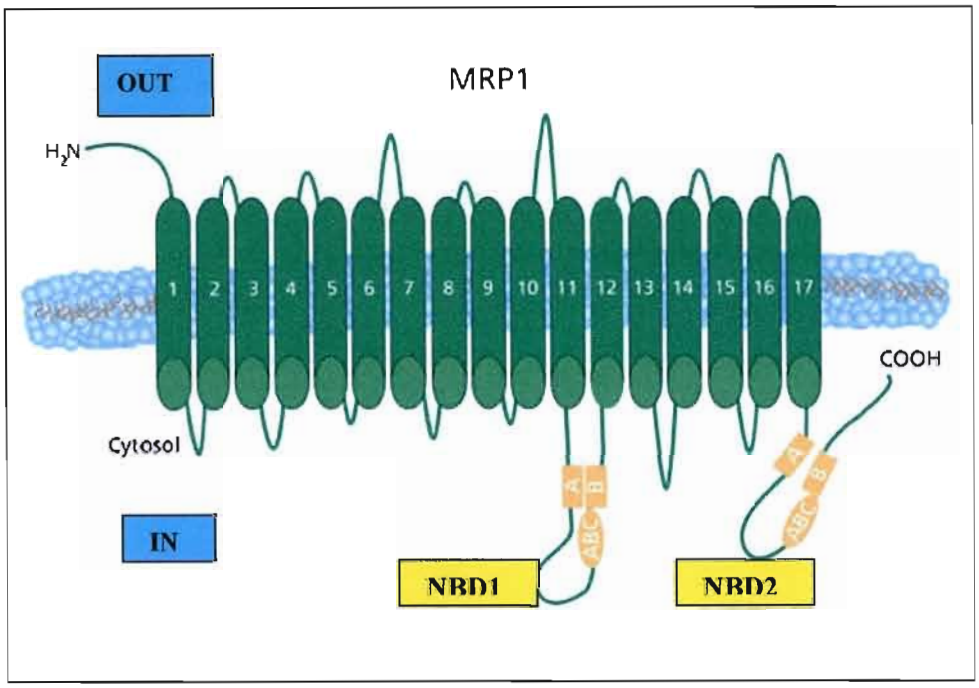

Figure 1.3: Structure of Multidrug Resistance Protein 1 (MRP1): MRP1 is thought to encode for 17 putative transmembrane domains (TMs). The rectangular bars represent the TM domains of MRP1. The nucleotide binding domains are indicated as NBD1 and NBD2. The extracellular (OUT) and intracellular (IN) sides of the membrane are also indicated. (Figure: adapted from Borst *et al.*, 2000)

A major part of this protein is composed of five transmembrane helices (TMD0) and a small cytoplasmic loop of about 80 amino acids (L0) (Bakos *et al.*, 1998, 1996; Gao *et al.*, 1998, 1996). This intracellular loop (L0) plays a vital role in transport activity of the MRP1 and MRP2 proteins (Fernandez *et al.*, 2002). However, unlike P-gp, MRP1 contains an additional third NH_2 proximal membrane-spanning domain with approximately 280 amino acids (Hipfner *et al.*, 1999).

1.4.2.2.3 Mechanisms of MRP1

The MDR phenotype conferred by MRP1 is similar, but not identical, to that conferred by P-gp. The exact mechanism of MRP1 mediated transport of cytotoxic compounds is not very clear. However, several studies indicate that MRP1 can

mediate efflux of several conjugated compounds by co-transport with GSH, or in a GSH-stimulated fashion by acting as a glutathione-S-conjugate export pump (GS-X pump) (Figure 4) (Renes *et al.*, 1999; Loe *et al.*, 1998). MRP1 mediated transport is ATP dependent, as for P-gp (Muller *et al.*, 1994). Several unconjugated hydrophobic drugs such as vinca alkaloids (e.g. vincristine) are transported by MRP1 in a glutathione dependent manner (Bagrij *et al.*, 2001; Renes *et al.*, 1999; Loe *et al.*, 1996). The exact mechanism of involvement of GSH in MRP1-mediated transport is not clear. One presumption is that glutathione S-transferases (GSTs) catalyze the conjugation of GSH to a number of electrophilic xenobiotics and form transportable complexes with cationic agents. As a result, the rate of drug detoxification increases (Black and Wolf, 1991).

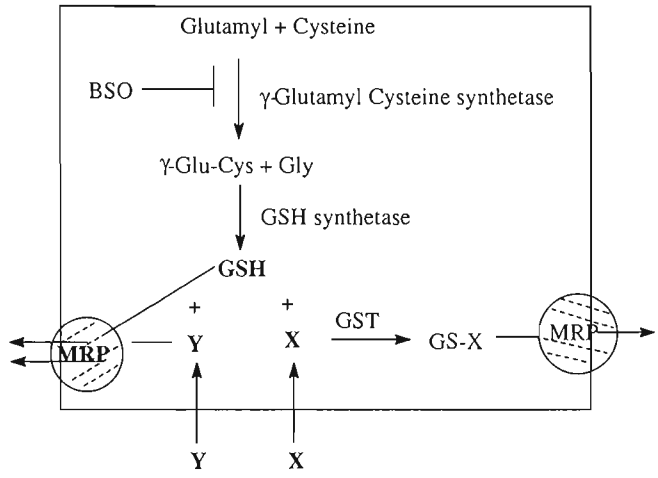

Figure 1.4: Interrelation between multidrug resistance-associated protein (MRP) and glutathione (GSH). Some drugs (X) can be conjugated to GSH by glutathione S-transferase (GST) and are then transported out of the cell by MRP. Other drugs (Y) are co-transported with GSH. In both cases, transportation of the drug depends on the continued synthesis of GSH, which can be blocked by buthionine sulfoximine (BSO) (Figure: adapted from Borst *et al.*, 2000).

Finally, MRP1 confers resistance to certain antimonial and arsenical oxyanions, a function which is not associated with P-gp (Stride *et al.*, 1997; Cole *et al.*, 1994). MRP1 also appears to be responsible for the preferential drug resistance to topoisomerase II inhibitors (Hendrikse *et al.*, 1999).

1.4.2.2.4 Substrates of MRP1

Unlike P-gp, MRP1 is a primary active transporter of many conjugated organic anions such as sulfate-, glucoronide- and GSH-conjugates. MRP1 is also the major high-affinity transporter of leukotriene C4, which is an important signaling molecule for the migration of dendritic cells from the epidermis to lymphatic vessels (Karwatsky *et al.,* 2005; Hipfner *et al.,* 1999; Keppler *et al.,* 1998). The structural elements that contribute to the affinity of a molecule for MRP1 are not clearly defined. Several findings suggest that the presence of positively charged arginine and lysine residues in the MSDs of MRP1 (Ito *et al.,* 2001; Seelig *et al.,* 2000) may facilitate transmembrane transport of charged substrates. GSH conjugates have at least two carboxylate residues, which contribute to recognition by MRP1. Several other substrates for MRP have been identified, such as oxidized GSH disulfide (GSSG) and steroid glucuronides (e.g. 17b-estradiol 17-(b-D glucuronide) (Homolya *et al.,* 2003; Loe *et al.,* 1996).

1.4.2.2.5 Modulators of MRP1

Reversal of MDR offers the hope of increasing the efficacy of conventional chemotherapy. Most MDR modulators act by either binding to membrane transport proteins (especially P-gp and MRP), thus inhibiting their drug-effluxing activity or by indirect mechanisms related to phosphorylation of the transport proteins or expression of the mdr1 and mrp1 genes. Expression of several MDR-associated genes can be affected by cytokines and immunological agents.

Derivatives of GSH are expected to be good inhibitors of MRP1. Addition of GSH to certain unconjugated xenobiotics, results in inhibition of MRP1 transport of organic anions out of membrane vesicles (Bagrij *et al.,* 2001; Loe *et al.,* 1996). For example, transport of LTC_4 is inadequately repressed by vincristine or verapamil alone, this effect is enhanced more than 20 fold in the presence of GSH. The

mechanism by which GSH enhances the inhibitory potency of certain compounds in MRP1-mediated conjugated organic anion transport appears to result from increasing their affinity for the protein. Some dietary flavonoids can also modulate the organic anion and GSH transport properties, ATPase activity, and drug resistance-conferring properties of MRP1. For example, in intact MRP1-overexpressing cells, quercetin reduced vincristine resistance from 8.9 to 2.2 fold. It was proposed that camptothecin (CPT) could be used for the reversal of the MRP1 phenotype at clinically achievable concentrations (Chauvier *et al.,* 2002). This study also established that mifepristone was a potent inhibitor of MRP1 in vincristine resistant cells (SGC7901/VCR) of MDR (Li *et al.,* 2004). A clinical study has shown that the taxane, tRA 98006 is a good MDR reversing agent (Brooks *et al.,* 2003). Inhibitors of activity GST of are also considered as potent modifiers of MRP1-mediated drug resistance. For example, BSO is one of the known inhibitors of γ-glutamyl-cysteine synthetase (Figure 4). BSO decreases intracellular level of glutathione and thereby overcomes resistance to many alkylating agents and reverses resistance to vincristine, rhodamine, doxorubicin and daunorubicin in MRP1 over expressing cell lines (Hui-Yun and Kang, 1998; Zaman *et al.,* 1994), whereas no effect was observed in MRP-negative parental cell lines.

1.4.2.3 The canalicular Multispecific Organic anion Transporter: cMOAT) (MRP2)

The canalicular membrane of the hepatocyte contains an ATP-dependent primary active drug transport system for organic anions, known as the canalicular Multispecific Organic Anion Transporter (cMOAT) (Smitherman *et al.,* 2004; Muller *et al.,* 1996; Ishikawa, 1992). cMOAT is encoded by a MRP1 homologue (Paulusma *et al.,* 1996; Ito *et al.,* 1996). It is one of the most extensively studied members of the MRP family and is mainly expressed in the liver. It shows 47.6% DNA sequence similarity with MRP1. It tends to be rate limiting for the hepatobiliary elimination of

drugs. cMOAT has similar substrates to MRP1, including drugs conjugated with glutathione, glucuronide and sulphate, and natural product anticancer drugs (e.g. anthracyclines, vinca alkaloids, methotrexate, etc). Most of the substrates for the cMOAT are bulky organic molecules with two separated negative charges (e.g. methotrexate (MTX) derivatives). It has been found that cMOAT related transport is associated with bilirubin glucuronide transport, with defects resulting in the Dubin-Johnson syndrome (Kobayashi *et al.,* 2004). Although its clinical significance in drug resistance remains to be determined, expression of cMOAT has been reported in human cancers such as breast, leukemia and ovary (Sparreboom *et al.,* 2003).

At the present time, there are many strategies such as use of pegylated chemotherapy (e.g. Pegylated liposomal doxorubicin), organ specific administration, intrathecal therapy, hyperoxygen, hyperthermia, etc, which are in use to reverse MDR, consequently increasing drug delivery in both cells and tissues (Wartenberg *et al.,* 2005; Hau *et al.,* 2004; Dong *et al.,* 1994). Among all of these approaches, hyperthermia is extensively used in the treatment of cancer (Terashima *et al.,* 2004; Van der Zee, 2002) and has potential as a MDR reversing technique, which could result in chemo sensitization of the tumor cells, denaturation of cell repair enzymes and induction of apoptosis (Souslova and Averill-Bates, 2004; Bates and Mackillop, 1986).

1.5 Hyperthermia

1.5.1 An overview

When cells are heated beyond their normal temperature (37°C), they can become more sensitive to therapeutic agents such as radiation and chemotherapy (Schlemmer *et al.,* 2004). The application of heat in a therapeutic setting is referred to as hyperthermia. Controlled use of hyperthermia can be used to combat disseminated cancers. The extensive amount of biological *in vitro* and *in vivo* experimental research on hyperthermia during the last decade has established it to be a valuable tool in cancer therapy (van der Zee, 2002; Law, 1982; Field and Bleehen, 1979). For

over two decades, different forms of hyperthermia have been used in the clinical treatment of cancer, thereby proving its effectiveness in combination with both radio- and chemotherapy (Hehr *et al.,* 2003; Tsuda *et al.,* 2003; Robins *et al.,* 1992).

1.5.2 History

The use of heat to treat disease is a primitive concept. Many ancient cultures, including the Egyptians, Greeks, Romans, Chinese, Indians and Japanese have used this concept for the treatment of various diseases (Coley, 1891). The field of modern hyperthermia was established in the late 19^{th} century when a number of physicians found the curative effects of hot mineral waters (Review: Herman *et al.,* 1982). Upon receiving heat treatment, they noted a regression of cancerous tumors for patients who had contracted fever inducing disease. In 1887, Dr. Julius Wagner-Jauregg began his study of the neurological effects of syphilis. On the basis of data collected from numerous reports, he observed a spontaneous remission and apparent cure, after a febrile (fever inducing) illness. In 1927, he was honoured by the Nobel Prize for this study (Wagner-Jauregg, 1887). Later that decade, Westermark reported the use of localized, non-fever produced heat treatments that resulted in the long-term remission of inoperable cancer of the cervix (Coley, 1891). Subsequently, some studies have been carried out highlighting the direct killing effect of heat upon various bacterial cultures (Thompson *et al.,* 1936). These results led to clinical trials using heat as a treatment for various diseases.

1.5.3 Types of induced hyperthermia

1.5.3.1 Extracorporeal whole body hyperthermia (EWBH)

EWBH offers a means of evenly elevating the temperature by extracorporeal circulation throughout the body in a controlled manner for a specified duration of time. It was developed to induce controlled, rapid and uniform heating of the body. In 1976, Leon Parks, a cardiothoracic surgeon, began a series of hyperthermic

treatments on patients who had failed to respond to any conventional treatments (Parks *et al.,* 1979). EWBH is also a useful treatment in patients with conventionally incurable malignant tumors. Several studies show that induction and maintenance of whole body hyperthermia is clinically possible (Lange *et al.,* 1983).

1.5.3.2 Whole body hyperthermia (WBH)

Externally induced whole body hyperthermia can be used to treat metastatic cancers that have proliferated throughout the body (Robins *et al.,* 1992). WBH heats the body from the outside in, using sources outside the body. As a result, body tissue is subjected to unevenly elevated temperatures. Whole body heating methods include saunas, hot air, microwaves and hydrotherapy (immersion in hot water) (Herman *et al.*, 1982). Pre-clinical and clinical studies have attributed a number of favorable effects to WBH including potentiation of the tumoricidal effects of specific cytotoxic agents as well as stimulation of different features of the immune system (Hildebrandt *et al.,* 2004a,b; Hegewisch-Becker *et al.,* 2003). In addition, the combined use of WBH and interleukin-2 resulted in enhancement of the anti-tumor response to sarcoma 45 in rats (Potapnev, 2004). Hence, it can be deduced that WBH could be able to contribute to overcoming drug resistance, as well as to increasing the response to re-treatment with cisplatin or carboplatin, even after multiple prior chemotherapies (Ohno *et al.,* 1991). Several studies have shown excellent response rates with the utilization of WBH and chemotherapy for ovarian cancer (Westermann *et al.,* 2001), as well as for other types of cancer, such as sarcoma (Wiedemann *et al.,* 1996; Cronau *et al.,* 1992). Irrespective of these benefits, there are a few drawbacks. For instance, several clinical observations indicate that diarrhea, nausea and vomiting are commonly observed after WBH (Kapp *et al.,* 2000). It was also observed that WBH could cause more serious side effects, including cardiac and vascular disorders, although these effects are uncommon (van der Zee *et al.,* 2002; Wust *et al.,* 2002; Kapp *et al.,* 2000).

1.5.3.3 Regional hyperthermia (RHT)

RHT is a method used for the treatment of isolated areas of the body, such as the liver, pelvis, stomach or limbs (Schlemmer *et al.,* 2004; Petrovich *et al.,* 1989). The principle of RHT is to heat intrinsic large tumors. Intraperitoneal hyperthermia is a form of regional hyperthermia that introduces heated solutions to the abdominal cavity via catheters. Magnets and devices that produce high energy such as arrays of antennas are placed over the region to be heated. In another approach named perfusion, blood is removed, heated and then pumped into the region that is to be heated internally. RHT has allowed the use of hyperthermia in conjunction with other modalities of antineoplastic therapy (Sticca, 2003). It is one of the promising methods for the treatment of prostate carcinoma (Tilly *et al.,* 2005; Petrovich *et al.,* 1991). Despite advances in this technology of heating, the non-homogeneous character of the treatment region can often affect the uniformity of the heat dispersion in the treated area. This means that is difficult to obtain a uniform regional rise in the temperature that is reproducible.

1.5.3.4 Local hyperthermia (LH)

LH entails elevating the temperature of superficial or subcutaneous tumors while sparing surrounding normal tissue, using either external or interstitial heating modalities. The area can be heated externally with high-frequency waves aimed at a tumor from a device outside the body. To achieve internal heating, one of several types of sterile probes may be used, including thin heated wires, hollow tubes filled with warm water, implanted microwave antennae, radio-frequency electrodes and ultrasound. LH has been successfully employed in the treatment of a wide range of tumors, particularly solid tumors (Karner *et al.,* 2004). The literature highlights that after giving systemic chemotherapy for prostate cancer to patients, LH could be carried out safely and effectively (Sherar *et al.,* 2003). Apart from of all these

benefits, treatments of blood diseases such as leukemia and certain tumor locations within the body, such as lung cancer, were difficult using LH.

1.5.4 Hyperthermia and combination therapy

Hyperthermia allied with radiotherapy or chemotherapy is a promising method for cancer treatment (Wust *et al.,* 2002; Robins *et al.,* 1992). There is considerable medical evidence demonstrating remarkable improvement in response rates when hyperthermia is used in combination with radiation therapy or chemotherapy (van der Zee *et al.,* 2002).

1.5.4.1 Hyperthermia and radiation therapy

The synergistic interactions between heat and radiation have been widely studied. The extent of synergism between heat and radiation depends on the temperature applied, the time interval between heat and radiation, and the treatment sequence (Dahl, 1988). An important mechanism for this interactive therapy is that hyperthermia interferes with the repair of radiation-induced DNA damage, probably due to an effect on cellular proteins (Kampinga *et al.,* 2001). In both experimental animal tumors and clinical treatment of human cancers, hyperthermia has been proven to increase the response of malignant tumors to radiation therapy. *In vivo* studies demonstrated that the effect of radiotherapy can be enhanced by a factor of 1.2 to 5 when combined with hyperthermia (Marino *et al.,* 1992). It has been shown that this combination therapy has the following benefits namely: a decrease in the radiation dose by 15% to 25%, a decrease in the side effects of X-Ray treatment and an increase in the effectiveness of the treatment of superficial and deep-seated tumors (Haim and Bicher, 2002).

The literature confirms that the combination of hyperthermia and radiation, with or without chemotherapy, might be a good treatment option for locally advanced

inoperable breast cancer (Li *et al.,* 2004). It is also an effective treatment for palliation of local symptoms, showing a tendency to achieve local control of large, ulcerative advanced breast lesions especially when such treatment is followed by salvage surgery (Iemwananonthachai *et al.,* 2003). Moreover, hyperthermia causes an increase in tumor blood flow, which results in an improvement in tissue oxygenation, thereby provisionally increasing their radio-sensitivity (Rau *et al.,* 2000; Song *et al.,* 1997). Overall, an important point is that hyperthermia is the most potent radio-sensitizer known to date.

1.5.4.2 Hyperthermia and chemotherapy

Analogous to thermal radio-sensitization, hyperthermia also enhances the cytotoxicity of various antineoplastic agents. Several types of interactions of heat with chemotherapeutic drugs have been investigated (Urano *et al.,* 1999; Hahn, 1982) such as supra additive (alkylating agents, platinum compounds) (Kubota *et al.,* 1993), threshold behavior (doxorubicin) and independent effects (fluorouracil, taxanes, vinca alkaloids). Hyperthermia increases the cytotoxicity of a wide variety of chemotherapeutic agents, including Adriamycin, melphalan, BCNU, bleomycin and cisplatin, both in vitro and in vivo (Honess, 1998; Raaphorst *et al.,* 1996; Orlandi *et al.,* 1995; Bates and Mackillop, 1990, 1986; Dahl, 1994; Herman *et al.,* 1988; Bates *et al.,* 1985).

Scientific evidence indicates that hyperthermia combined with chemotherapeutic drugs is a useful strategy to combat the MDR phenotype mediated by P-gp (Larrivée and Averill, 2000, 1999; Averill and Su, 1999; Averill and Larrivée, 1998; Bates and Mackillop, 1990). Melphalan resistance can be modulated by hyperthermia combined with ethacrynic acid in a P-gp overexpressing cell line (Turcotte and Averill-Bates, 2001). Hyperthermia is also useful in reversal of resistance to methotrexate in CHO cells (Herman *et al.,* 1981). Forty two degrees

hyperthermia could also be useful as a sensitizer in cisplatin resistant tumor cells (Raaphorst *et al.,* 1996).

The mechanisms by which heat enhances drug toxicity are likely to vary for different drugs. Available data suggests that optimal thermal chemo-sensitization occurs with synchronous application for most drugs, although there are some exceptions (e.g. oxacephasporines, cyclophosphamide and ifosfamide) (Urano *et al.,* 1999; Issels *et al.,* 1990). Recent data suggests that hyperthermia administrated with appropriate scheduling caused a modest increase in etoposide-induced apoptosis in both drug sensitive parental cell line (e.g. HeLa) and MDR cells with overexpression of MRP1 (Souslova and Averill-Bates, 2004). Scheduling was also required for the modification of etoposide (VP-16)-induced cell killing by hyperthermia in a radioresistant human melanoma (Sk-Mel-3) and a human normal (AG1522) cell line (Ng *et al.,* 1996).

Several phase II studies on hyperthermia in combination with pre- and/or postoperative chemotherapy in high-risk sarcomas have demonstrated quite impressive 5-year overall survival rates (Issels *et al.,* 2001; Wendtner *et al.,* 2001). Simultaneous combination of cisplatin and hyperthermia in cervical cancer, recurring following irradiation, resulted in a 50% response rate, which was expected to be 15% without hyperthermia (De Wit *et al.,* 1999; Rietbroek *et al,* 1997).

1.5.5 Molecular effects of hyperthermia

Different cell types vary widely in their intrinsic sensitivities to heat. There is no consistent discrepancy in heat sensitivity between tumor and normal cells, as well as between MDR cells and their drug sensitive counterparts. For example, hyperthermia is equally toxic to both drug sensitive CHO cells (AuxB1) and their multidrug resistant cell line (CH^RC_5) overexpressing P-gp (Bates and Mackillop, 1986). Similar results were obtained for human cervical adenocarcinoma cells (HeLa) and their MDR counterpart overexpressing MRP1 (Souslova and Averill-Bates,

2004). However, there appears to be a distinction in sensitivity among rodent and human cells. At temperatures between 41°C and 42°C, human tumor cells are less heat sensitive than rodent cells, and a potential therapeutic advantage can be achieved with prolonged heating at these non-lethal temperatures, though the reason for this difference is not known (Armour *et al.,* 1993). The sensitivity of cells to heat also varies with phase of the cell cycle, where cells in S phase and mitosis are the most heat sensitive (Yuguchi *et al.,* 2002)

The nature of the critical lesions that lead to cell death following heat treatment remains unknown. Several explanations could be that elevated temperature results in activation of cell metabolism which causes increased oxidative stress (Lord-Fontaine and Averill-Bates,1999, 2002) and acidosis of the tumor tissue (Vujaskovic et al., 2000; Bicher et al., 1980). Hyperthermia causes disturbances in the microcirculation of cancer tissue (Bogovic et al., 2001) resulting in an inhibition of the DNA repair mechanisms (Li et al., 1998; Osman, 1993) and induces apoptosis (Sakaguchi et al., 1995). Furthermore, it was shown that hyperthermia can cause a disruption of integrin-mediated actin cytoskeleton assembly and, possibly, of other integrin-mediated signaling pathways. These effects were shown to be influenced by The specific amplitude and exposure duration, as well as cell type. For example, exposure of mouse epithelial cells to elevated temperatures changed the organization of keratin filaments and actin filaments but had no effect on microtubules (Shyy et al., 1989). Similar results were observed in 9L cells where heat shock caused collapse of microfilaments and intermediate filaments but had only slight effects on microtubules (Wang et al., 1998). In contrast, microtubules were disrupted by heat shock in Chinese hamster ovary cells (Lin et al., 1982) and mouse 3T3 cells (Parrish et al., 1996).

Hyperthermia treatment modulates the activity of cytokines (Katschinski *et al.,* 1999; Neville and Sauder, 1988) and increases the antigenicity of tumor cells by the production of heat shock proteins (HSP) and activation of natural killer cells (Roigas *et al.,* 1998; Multhoff, 1997). Hyperthermia inactivates cellular antioxidant

defences against hydrogen peroxide (H_2O_2) (Lord-Fontaine and Averill-Bates, 2002; Averill-Bates and Przybytkowski, 1994). Hyperthermia can act by altering the transport functions of the plasma membrane. For example, in CHO cells, viscosity of the membrane decreased due to increased temperature. This resulted in the elevation of the activity of the sodium-potassium pump (Bates *et al.,* 1985). During hyperthermia, membrane permeability is changed to several compounds, including Adriamycin (Bates and Mackillop, 1987a), polyamines (Gerner *et al.,* 1980) and certain ions such as K^+ (Bates and Mackillop, 1987b).

Expression of HSPs is often correlated with the development and loss of thermotolerance (Hayashi *et al.,* 2001; Li and Werb, 1982; Landry *et al.,* 1982a, b). Expression of other genes modulated by heat requires further investigation such as the multiple drug resistance genes (Stein *et al.,* 1999).

Furthermore, cells exposed to acidic pH during heating have been found to be more sensitive to heat treatment (Song *et al.,* 1993). Exposure of cells to heat in a nutrient-deprived environment can also sensitize them to heat treatment. This effect appears to correlate with changes in the cellular ATP levels (Gerweck, 1988). These more specific temperature-dependent pathways in cells suggest new applications of hyperthermia such as heat controlled gene therapy or heat enhanced immunotherapy by vaccination.

1.6 Apoptosis

1.6.1 Introduction

Apoptosis is considered to be a distinct form of eukaryotic cell death, morphologically as well as biochemically, that occurs under a variety of physiological and pathological conditions (Arends and Whyllie, 1991). It is a continuous physiologic process of regulated non-inflammatory cell death (Hengartner, 2000). Apoptosis was first discovered by Carl Vogt in 1842 (Vogt *et al.,* 1842). The word "Apoptosis" comes from the ancient Greek origin, meaning 'falling

off of petals from a flower' or 'leaves from a tree in autumn'. The name "Apoptosis" was first introduced by John Kerr in 1972. Apoptosis is one of the most active fields of biomedical research. The importance of this research was recognized when Dr. Horvitz was honored with a Nobel Prize on October 7th 2002 for discovering and characterizing the genes controlling apoptosis in the nematode *Caenorhabditis elegans.*

Classically, cell death is believed to occur by one of two mechanisms, apoptosis or necrosis (Kerr and Harmon, 1974). These two pathways of cell death differ by several criteria. In necrosis, cell death occurs due to injurious agents leading to membrane swelling followed by the leakage of the cell contents resulting in inflammation of adjoining tissues as well as wide spread damage (Trump *et al.,* 1981). Apoptosis is a result of cells committing suicide and has three distinct phases; 1) shrinkage and fragmentation of cells and their nuclei; 2) condensation of chromatin; and 3) extensive degradation of chromosomal DNA (Wyllie and Kerr, 1980; Wyllie, 1980).

Apoptosis plays an important role in many biological events including the immune system, embryonic development (Barres *et al.,* 1992a,b), metamorphosis (Steller *et al.,* 1994; Ishizuya-oka and Shimozawa, 1992), hormone-induced tissue atrophy, chemical-induced cell death as well as tissue homeostasis (Arends and Whyllie, 1991). An imbalance between cell death and survival may result in premature cell death and uncontrolled proliferation (Evan and Vousden, 2001). In addition, apoptosis also plays a vital role in the pathogenesis of human disease (Fadeel *et al.,* 1999; Thatte and Dahanukar, 1997). For instance, uncontrolled apoptosis is implicated in various human diseases such as Alzheimer's, Parkinson's, carcinogenesis, intimal hyperplasia, leukemia, lymphoma, etc (Brunner *et al.,* 2003; Pritchard and Watson, 1996; Thompson, 1995).

1.6.2 Apoptotic pathways

The three principal mechanisms by which a cell can execute apoptosis are; 1) the Intrinsic pathway or mitochondrial pathway where apoptosis occurs by internal signals, arising within the cell (Adrain and Martin, 2001); 2) the Extrinsic or death receptor pathway, where apoptosis is triggered by external signals (Locksley *et al.,* 2001) and 3) the Endoplasmic reticulum pathway (Rao *et al,* 2004; Li *et al.,* 2001).

1.6.2.1 Extrinsic or death receptor pathway

Receptor mediated pathway is a major pathway for the induction of apoptosis (figure 1.5). The extrinsic pathway begins outside the cell, when conditions in the extra-cellular environment determine that a cell should undergo apoptosis. Up to date, there are six known members of the death receptors (DR) family including Fas/CD95/APO-1 and Tumor Necrosis Factor-α receptor (TNFα). These two members of the death receptor family play a key role in a variety of immunological, inflammatory and pathological conditions including initiation of apoptosis (Rudin *et al.,* 1997). Both of them belong to the TNF-R family and contain a cytosolic death domain (DD). They are located in the plasma membrane of the cell that is to undergo apoptosis and are activated by extra cellular ligands. Cytotoxic T lymphocytes express Fas ligands that activate cells bearing Fas receptors, thereby inducing apoptosis. The Fas receptor is generally found in epithelial tissues, tumors and hematopoietic tissues. It is activated by binding of Fas ligand (Fas-L) to cell membranes, undergoes trimerisation and recruits intracellular molecules known as Fas associated death domain (FADD) (Strasser *et al.,* 2000; Ashkenazi *et al.,* 1999). The extremity of FADD contains two death effector domains (DEDs) that recruit procaspase-8 (Salvesen and Dixit, 1999). The assembled complex of the cytoplasmic region of Fas, the adapter protein FADD and procaspase 8 is known as the Death-Inducing Signaling Complex (DISC). Once caspase-8 is activated, it can activate and

cleave downstream effector caspases such as caspase-3 and-7 (Budihardjo *et al.*, 1999). This results in cleavage of their specific substrates leading to apoptosis.

Fas-mediated apoptosis also involves Bid. In type-II cells the amount of DISC produced is low. This results in the activation of small amounts of caspase-8, which cleaves the cytosolic substrate Bid. This proteolytically modified Bid leads to induction of conformational changes in pro-apoptotic protein BAX that leads to permeabilization of the mitochondrial membrane and release of cytochrome c (Eskes *et al.*, 2000; Desagher *et al.*, 1999).

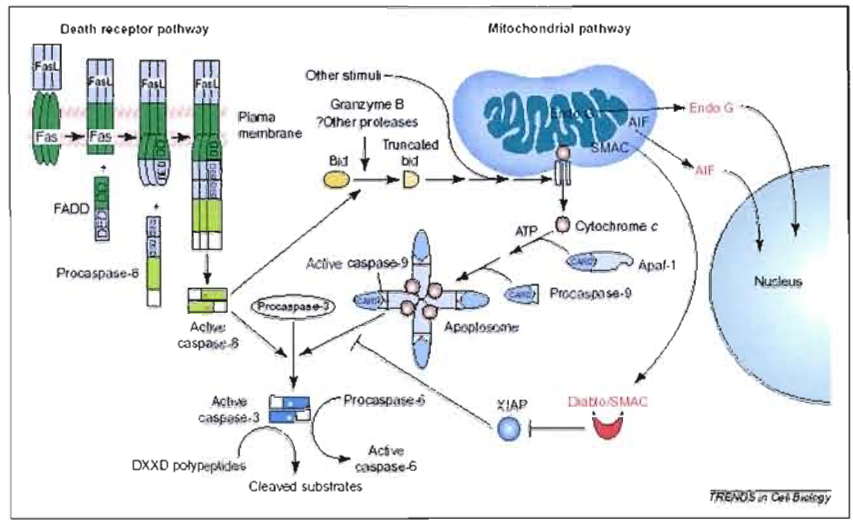

Figure 1.5: Extrinsic and intrinsic pathways of apoptosis. The death receptor pathway starts with ligation of death receptors such as Fas/CD95. This leads to the formation of the Death-Inducing Signaling Complex (DISC) and activation of caspase 8. Once activated, caspase-8 causes the cleavage of caspase 3 that eventually results in DNA fragmentation and apoptosis. In type II cells, the pro-apoptotic protein Bid can act to couple the death receptor pathway with the mitochondrial amplification loop. The intrinsic pathway can be stimulated by a variety of agents such as irradiation, chemotherapy drugs, reactive oxygen species, etc. Mitochondrial damage results in altered membrane permeability leading to the release of cytochrome c, that in turn binds to Apaf-1 and procaspase-9 to form a complex called the apoptosome. Activated caspase-9 then cleaves caspase-3 resulting in downstream events involved in cell death (Adapted from Kaufmann and Hengartner, 2001).

Cancer cells are also susceptible to the tumor necrosis factor receptor (TNF-R) mediated pathway. The TNF-R can induce apoptosis via different biochemical

pathways (Rudin *et al.,* 1997; Maclellan *et al.,* 1997). The most commonly described pathway of TNF-induced apoptosis is regulated by TNF-R associated death-domain (TRADD). It commences with the binding of TNF-related apoptosis-inducing ligand (TRAIL) to the transmembrane TNF-R and bind to TRADD through its death domain. The activated complex of TNF either recruits FADD, leading into signaling for apoptosis, or TRAF, leading to anti-apoptotic signaling (Ashkenazi, 2002; MacEwan, 2002). TRAF can suppress apoptosis by activating a protein known as nuclear factor kB (NF-kB), which acts as an inhibitor of apoptosis protein (IAP) that prevents the execution phase of apoptosis (Haimovitz *et al.,* 1994).

1.6.2.2 Mitochondrial or intrinsic pathway

A major pathway of apoptosis has been shown to be controlled at the mitochondrial level (Figure 1.5) (Susin *et al.,* 1999a; Zamzami *et al.,* 1996). This pathway can be activated by a variety of extra and intracellular stresses, including oxidative stress, γ and UV-radiation, removal of cytokines and treatment with different cytotoxic drugs such as Adriamycin, vincristine, vinblastine, etc (Kerr *et al.,* 1994). These cellular stresses result in the release of cytochrome c from the mitochondrial intermembrane to the cytosol, followed by binding of this cytosolic cytochrome c to the cofactor apoptotic protease activating factor-1 (Apaf-1). This binding leads to the formation of the apoptosome (Waterhouse *et al.,* 2002). The apoptosome is a ~1 MDa oligomeric complex, which in the presence of dATP/ATP self –oligomerizes to form an Apaf-1 multimer (Zou *et al.,* 1999) composed of cytochrome c, Apaf-1 and procaspase-9. This induces the auto activation of initiator caspase-9, which subsequently activates other effector caspases (e.g. caspase-3) and perpetrates the cell towards death. However, the precise mechanism of cytochrome c release is still unclear. Early data suggested that mitochondria played a very important role in this process. It was believed that release of cytochrome c was dependent on early loss of mitochondrial membrane potential and the opening of the

mitochondria permeability pores (Finkel *et al.,* 2001; Desagher *et al.,* 2000). Nevertheless, recent data suggest that both events are not essential for apoptotic cytochrome c release in all circumstances (Ly *et al.,* 2003; Waterhouse *et al.,* 2002).

It has been demonstrated that, upon activation of the intrinsic pathway, a range of pro-apoptotic molecules other then cytochrome c are released from mitochondria (Figure 5) (Van Loo *et al.,* 2002), such as Smac/Diablo, apoptosis inducing factor (AIF), Hsp60/10, endonuclease G (Endo G), etc (Li *et al.,* 2001; Du *et al.,* 2000; De laurenzi and Melino 2000; Susin *et al.,* 1999b; Xanthoudakis *et al.,* 1999; Samali *et al.,* 1999). Smac/Diablo promotes caspase activation by neutralizing the inhibitors of apoptosis proteins (IAP's), whereas AIF and Endo G (Jia *et al.,* 2003; Van loo *et al.,* 2002) lead to a new death pathway that could execute apoptosis-like cell death in the absence of caspases (Cregan *et al.,* 2002). Endonuclease G is a protein found in the inner mitochondrial membrane and like AIF it can also translocate from mitochondria to the nucleus during apoptosis and is capable of inducing DNA fragmentation independent of caspases (Li *et al.,* 2001).

1.6.2.2.1 Bcl-2 family of proteins

The mitochondrial pathway plays a critical role in the regulation of apoptosis as major mitochondrial dysfunction is likely to cause cell death. One of the principal regulators of the mitochondria-mediated pathway of apoptosis is the family of Bcl-2 proteins (Cory *et al.,* 2003; Gross *et al.,* 1999; Green and Reed, 1998). Bcl-2 was first identified in B cell lymphomas as an oncoprotein coded by a gene affected by translocations of chromosome 14 and 18. These proteins can be classified into two categories depending on their biological activity namely: Proapoptotic proteins that include Bax, Bak, Bik, Bad and Bid and anti-apoptotic proteins such as Bcl-2 and Bcl- X_L (Adams and Cory, 1998; review: Reed, 1997). Presently, at least 15 members of the Bcl-2 family are known which can have either a negative or a positive effect on the initiation of apoptosis (Shibue and Taniguchi, 2006; review). Despite their widely

opposing functions, all members of the Bcl-2 family have at least one copy of a BH motif (BH1-BH4). It was also observed that a subset of the pro-apoptotic members, including Bad, Bid and Bim contain only the BH3 domain (Coultas, 2003; Cory *et al.,* 2003). These BH domains play an important role in the ability of various family members of the Bcl-2 family to interact with each other (Keleker *et al.,* 1997; Cheng *et al.,* 1996). They can interact with each other via the BH domains and form either death- promoting or death inhibiting hetero-oligomeric complexes. However, the significance of these interactions has not been clearly defined (Cheng *et al.,* 1996). The relative levels of pro- and anti-apoptotic proteins determine a cell's susceptibility to apoptosis (Korsmeyer, 1995). The anti-apoptotic proteins inhibit apoptosis, however their site of action is not entirely understood. Proteins such as Bcl-2 can control apoptosis induced by various cytotoxic mechanisms and they appear to exert their effects by blocking the release of cytochrome c from mitochondria (Kluck *et al.,* 1997).

Pro-apoptotic proteins interact with anti-apoptotic proteins and halt their inhibition of apoptosis. It was also observed that pro-apoptotic members of the Bcl-2 family, including Bax, Bak, and Bid, might act directly by destabilizing the outer mitochondrial membrane and trigger mitochondrial release of apoptogenic cytochrome c and apoptosis-inducing factor into the cytoplasm (Basañez *et al.,* 1999).

1.6.2.3 Endoplasmic reticulum (ER) pathway

Recently, the regulation of cell death by endoplasmic reticulum (ER) stress has gained much interest (Momoi, 2004; Ferri and Kroemer, 2001). Three major apoptotic pathways are involved in ER stress-mediated cell death: 1) Caspase 12-dependent pathway (Nakagawa *et al.,* 2000); 2) ASK/JNK pathway, which leads to induction of cytochrome c release form mitochondria and caspase-9 activation (Tournier *et al.,* 2000) and 3) Bap31 and caspase-8 pathway, wherein caspase-8 will

be activated by its interaction with Bap31, resulting in cleavage of Bid and consequently cytochrome c-mediated cell death (Breckenridge *et al.,* 2002).

1.6.2.4 Caspases

The name caspase stands for aspartate-specific cysteinyl proteases. Caspases play an essential role in the process of apoptosis. They are involved in both initiation and execution of apoptosis (Philchenkov, 2004; Los, 1999; Earnshaw, 1999; Thornbery, 1998). They can be found in humans all the way down to insects, nematodes and hydra (Los, 2001; Budihardjo *et al.,* 1999; Cikala *et al.,* 1999; Earnshaw *et al.,* 1999). To date, at least 14 human caspases with different substrate specificity have been identified, although only two-thirds of these have been suggested to function in apoptosis (Siegel, 2006; review). Based on their function in apoptosis, caspases are categorized into two classes namely: initiator (caspase -8 and -9) and effector caspases (caspase -3, -6 and -7).

Initiator caspases are stimulated by a scaffold-mediated activation mechanism. This process involves assembly of a molecular platform in response to death stimuli and recruitment of procaspases. This results in the conformational change in the initiator procaspases leading to activation of the caspase. Afterwards, mature initiator caspases catalyse the processing of effector procaspases to their active enzymes, which in turn degrade specific substrates and activate further procaspases (Hengartner, 2000). Thus, the initiator caspases act as upstream activators of the effector caspases and effector caspases act as executioners by cleaving the proteins that actually induce apoptosis in the cell. Initiator caspases such as caspase-8 and -10 are directly linked to death inducing signaling complexes (DISCs), while caspase-9 is recruited to the apoptosome via its caspase activation and recruitment domain (CARD).

The caspases differ significantly in their cleavage specificity and thus have different proteins as substrates. A large number of caspase substrates have been

identified including the Bcl-2 family, cytoskeletal proteins: gelsoline, fodrin, actin, plectrin, cytokeratin; nuclear proteins: lamin, survival factor-focal adhesion kinase (FAK), p21-activated kinase (PAK), poly ADP-ribose polymerase (PARP), and inhibitor of caspase-activated DNase (ICAD) (Virag and Szabo, 2002; Enari *et al.*, 1998).

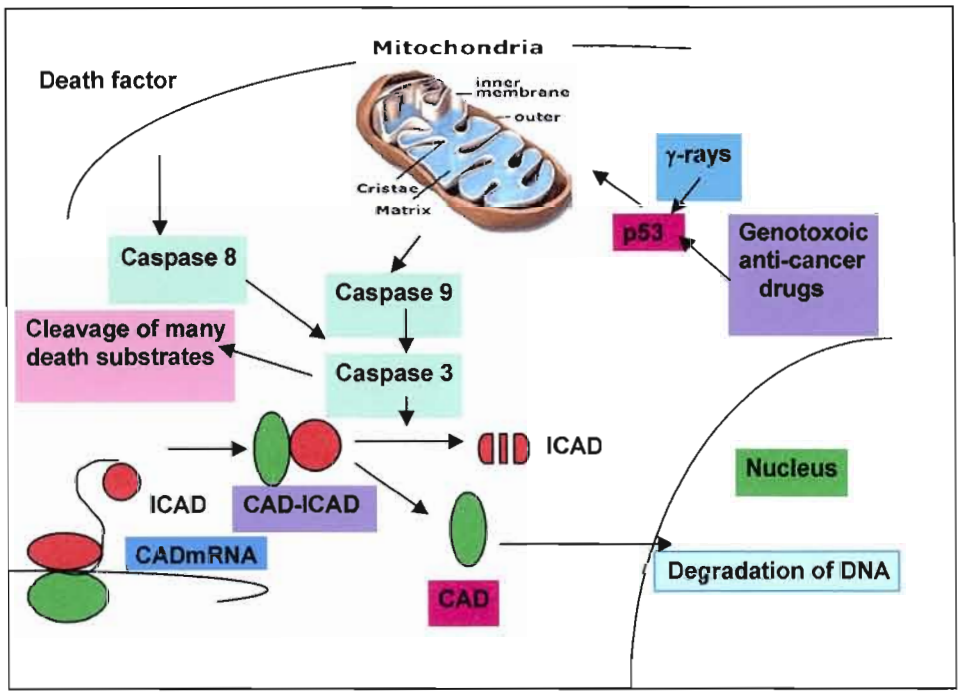

Figure1.6: A model for CAD-dependent DNA fragmentation during apoptosis. When CAD is synthesized on the ribosome, ICAD-L binds to its nascent chain and enhances its correct folding. After CAD synthesis is completed, ICAD-L remains complexed with CAD as DFF (DNA fragmentation factor) / (CAD/ICAD complex). When apoptotic stimuli activate caspase 3, this cleaves ICAD-L to release active CAD, which degrades chromosomal DNA. (Adapted from: Nagata, 2000)

ICAD is one of the important substrates of caspases (Hengartner, 2000). Caspases are responsible for the DNA fragmentation by cleaving the CAD-ICAD complex (Figure 6). CAD is a protein of 343 amino acids, which carries a nuclear-localization signal and produced as a complex with ICAD and exists as an inactive form. Treatment with caspase-3 can cleave this complex leading to the release of CAD allowing it to enter the nucleus and degrade chromosomal DNA (Enari *et al.*,

1998). ICAD seems to function as a chaperone for CAD during its synthesis, remaining complexed with CAD to inhibit its DNase activity; caspases activated by apoptotic stimuli then cleave ICAD, allowing CAD to enter the nucleus and degrade chromosomal DNA (Enari *et al.*, 1998).

1.7 Presentation of Project

1.7.1 Introduction

Hyperthermia is used today as a novel technique at the clinical level in cancer therapy. The potential of hyperthermia developed due to its ability to increase anticancer activity of radiation and of various anti-neoplastic agents and to decrease toxic side effects to normal tissue during cancer treatment. This allows targeting of chemotherapy with the use of regional heating. Resistance to multiple chemotherapeutic agents is considered a major cause of chemotherapy failure. Hyperthermia in combined use with chemotherapeutic agents could become a useful strategy to combat the multidrug resistance phenotype. Hyperthermia has been used increasingly, aimed to overcome drug resistance to chemotherapeutic agents in laboratory studies. However interactions between heat and individual drugs are not well understood. The mechanisms by which heat enhances drug toxicity are likely to vary for different drugs. Laboratory studies are essential to advance our perception at the cellular and molecular levels to ensure that hyperthermia can be combined successfully and optimally with chemotherapy in the cancer clinic.

1.7.2 Objectives of the project

The two main objectives of this project are first, to determine whether, hyperthermia can increase the cytotoxicity of the anticancer drug Adriamycin by triggering apoptosis in HeLa cells isolated from human cervical adenocarcinoma. The second objective is to determine whether hyperthermia can reverse resistance to

Adriamycin in HeLa cells with the overexpression of the multidrug resistance phenotype MRP1.

1.7.3 Choice of model

Two human cell lines from cervical adenocarcinoma were chosen as models for this project. The effect of hyperthermia on Adriamycin cytotoxicity was evaluated using cultured human adenocarcinoma cells (HeLa), which are drug sensitive and their drug resistant counterpart which, overexpresses multidrug resistance associated protein-MRP1 (HeLaMRP cells). HeLaMRP cells were a kind gift from Dr. Philippe Gros from McGill University, Montreal, QC (Kast and Gros, 1998). The HeLa cell is a well established *in vitro* cellular model which is widely used in laboratory research.

1.7.4 Experimental approach

The ability of hyperthermia (41°C to 45°C), alone or in combination with Adriamycin, to induce apoptosis was evaluated in HeLaMRP versus HeLa cells. Cellular uptake of Adriamycin was observed using Adriamycin fluorescence under UV illumination using a Zeiss microscope equipped with a mercury lamp. The UV illumination induced an orange fluorescence at sites of Adriamycin accumulation (Larrivée and Averill, 2000). Induction of apoptosis through the mitochondrial pathway was analyzed by subcelluler fractionation of Bax and cytochrome c, by measuring the translocation of Bax from the cytosol to mitochondria followed by the release of cytochrome c from mitochondria into the cytosol. The enzymatic activities of caspases 3, 8 and 9 were measured by a fluorimetric kinetic assay (Souslova and Averill-Bates, 2004). Chromatin condensation in the nucleus of both cell lines was evaluated using the fluorochrome Hoechst 33258 (apoptosis) by fluorescence microscopy and propidium iodide was used to observe the induction of the necrosis (Souslova and Averill-Bates, 2004). Cleavage of ICAD by caspase 3 was also observed using Western blotting (Tanel and Averill-Bates, 2005).

CHAPTER 2

EXPERIMENTAL RESULTS

2.1-Preface

This chapter includes a manuscript describing the experimental results of this project which I have carried out during the M.Sc program in the laboratory of Dr. Diana Averill. This manuscript will be submitted in the near future to the International Journal of Radiation Oncology, Biology and Physics and is entitled "Induction of apoptosis by heat shock and Adriamycin in a multidrug resistant human cell line". This manuscript was composed by myself, Kamini Barot and was revised by Dr Diana Averill. In this study, I have been engaged in the investigation of cell death mechanisms induced by the combined treatment of hyperthermia with Adriamycin and its effect on the human cervical adenocarcinoma cells with overexpression of multidrug resistance associated protein-MRP1.

2.2-Manuscript

INDUCTION OF APOPTOSIS BY HYPERTHERMIA AND ADRIAMYCIN IN A MULTIDRUG RESISTANT HUMAN CELL LINE

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Acknowledgements: The authors wish to thank André Tanel, Ahmed Bettaieb, Michel Marion and Denis Flipo for valuable technical assistance and Bertand Fournier for assistance with statistical analyses. This work was supported by grants from the Natural Science and Engineering Research Council of Canada (NSERC) (DAB) and the Cancer Research Society (DAB). K. Barot was the beneficiary of the Lapointe-Rosendahl and Mario-Plouffe scholarships provided by the Fondation UQAM.

Abbreviations: ADR: Adriamycin; BSA: bovine serum albumin; CAT: catalase; CHO: Chinese hamster ovary; DMEM: Dulbecco's modified Eagle's medium; FBS: fetal bovine serum; HT: hyperthermia; HeLa: human cervical adenocarcinoma cells; GST: glutathione S-transferase; GPx: glutathione peroxidase; MDR: multidrug resistance; MRP1: multidrug resistance-associated protein; SDS-PAGE: sodium dodecyl sulphate polyacrylamide gel electrophoresis; SEM: standard error of mean; P-gp: P-glycoprotein; PBS: phosphate-buffered saline.

Running Title: Cell killing by hyperthermia and Adriamycin in multidrug resistant cells

Abstract

Introduction: Apoptosis is a mechanism of cell death, which provides a physiological elimination of excess or damaged cells. It follows a characteristic program of events, including activation of the caspase cascade. A wide variety of toxic compounds (environmental pollutants, drugs) can induce cell death by apoptosis. Adriamycin is a widely used anticancer drug, however, its mechanisms of antitumour activity have been a long term matter of debate. In addition, the mechanisms of severe cardiac toxicity, which limit the usefulness of the drug, are not well understood. Multidrug resistance is a major obstacle in the successful use of cancer chemotherapy at clinical level. However, at present hyperthermia is used as a useful technique to overcome the problem of multidrug resistance. Purpose of using hyperthermia, is to increase the anticancer effect of Adriamcyin by sensitizing cell towards drug, at the same time decreasing the toxic effect of high dose of Adriamycin.

Objectives: The present study investigates the mechanism of cell death induced by Adriamycin and hyperthermia. The two main objectives of this project are: (1) to determine whether Adriamycin and hyperthermia alone can trigger apoptosis in human cervical adinocarcinoma cells over expressing MRP1 (HeLaMRP) relative to parental HeLa cells and (2) to determine whether hyperthermia can enhance the induction of apoptosis by Adriamycin.

Results: Treatment of cells with Adriamycin or hyperthermia alone or combined together resulted in activation of caspase-9 and caspase-3 as well as cleavage of inhibitor of caspase-activated DNase (ICAD), which leads to induction of apoptosis. Hyperthermia enhanced intracellular accumulation of Adriamycin, associated to a decrease in the mitochondrial membrane potential and translocation of pro-apoptotic proteins, Bax and cytochrome c between the cytoplasm and mitochondria of the cell.

Conclusion: Adriamycin is able to induce apoptosis by the mitochondrial pathway of apoptosis in both drug-sensitive and MRP1-overexpressing HeLa cells and hyperthermia alone is enable to induce apoptosis and could be a useful strategy to enhance the induction of apoptosis by Adriamycin in both drug-sensitive and MRP1-overexpressing HeLa cells.

Keywords: Hyperthermia, Multidrug resistance, MRP1, Apoptosis, Adriamycin

Introduction

Resistance to chemotherapy treatment is one of the major obstacles to the successful treatment of different types of cancer (Linn and Giaccone, 1995). Drug resistance can be classified in two categories: 1) intrinsic drug resistance whereby tumors already have a resistant phenotype to anticancer agents and 2) acquired drug resistance, wherein the resistant phenotype occurs after repeated exposure to cytotoxic agents. Furthermore, cancer cells often acquire cross-resistance to a variety of chemically and functionally unrelated compounds, a phenomenon known as multidrug resistance (MDR).

A variety of ATP binding cassette (ABC) transmembrane transporters are involved in MDR. The most important groups of proteins are the classical P-glycoprotein (P-gp) and the multidrug resistance associated protein (MRP) (Leonard et al., 2003). These transporters act as ATP-dependent efflux pumps, which actively transport a variety of chemicals and antitumour agents out of cells, thereby reducing their intracellular concentration (Leonard et al., 2003)

Multidrug resistance associated protein-1 (MRP1) is a human 190 kDa protein, encoded by the ABCC1 gene, which is located on chromosome 16 (Loe et al., 1996). The clinical cancers exhibiting high MRP1 expression include chronic and acute leukemias (Burger et al., 1994) and non-small cell lung carcinomas (Doubre et al., 2005). MRP1 is a primary active transporter of GSH-, sulphate- and glucuronide-conjugated organic anions (Jedlitschky et al., 1996). Several other substrates for MRP have been identified, such as glutathione disulfide (GSSG), steroid glucuronides (e.g. 17b-estradiol 17-(b-D glucuronide) (Leo et al., 1996) and cysteine leukotriene (LTC_4) (Hipfner et al., 1999). The exact mechanism of MRP1-mediated transport of cytotoxic compounds is not clear. However, several studies indicate that MRP1 can mediate efflux of several conjugated compounds by co-transport with glutathione

(GSH) or in a GSH-stimulated fashion by acting as a glutathione-S-conjugate export pump (GS-X pump) (Renes et al., 1999).

Overexpression of MRP1 is responsible for cellular resistance to several anticancer drugs, including Adriamycin, vinca alkaloids and epipodophyllotoxins (Hipfner et al., 1999). Adriamycin is a widely used anticancer agent, belonging to the family of anthracycline antibiotics (Gerwitz, 1999). The mechanism of action of Adriamycin is not entirely clear. However, it is known to intercalate into DNA, causes the generation of free radicals, induces lipid peroxidation, initiates DNA damage as a result of topoisomerase II inhibition, causes direct membrane effects and induces apoptosis.

Apoptosis is a mechanism that organisms have evolved to eliminate excess cells, or cells that are damaged, neoplastic, or infected with viruses (Hengartner, 2000). The principal mechanisms by which a cell can execute apoptosis are the mitochondrial, the death receptor and the endoplasmic reticulum pathways (Rao et al., 2004). Deregulation of apoptosis can lead to development of a variety of cancers (Pritchard and Watson, 1996).

Reversal of MDR offers the hope of increasing the efficacy of conventional chemotherapy. At the present time, several strategies are in use to reverse MDR, such as inhibitors of drug efflux pumps such as verapamil and cyclosporine A (Baird and Kaye, 2003), pegylated chemotherapy (Hau et al., 2004) and organ specific drug administration (Dong et al., 1994). These approaches generally increase drug delivery in cells and tissues. It is important to develop new approaches to reverse MDR.

Hyperthermia is widely used in the clinical treatment of cancer (Van der Zee, 2002), particularly in combination with chemotherapy and radiotherapy (Hehr et al., 2003). A wide range of *in vitro* and *in vivo* studies demonstrated that hyperthermia increases the cytotoxicity of a wide variety of chemotherapeutic agents, including Adriamycin, melphalan, BCNU, bleomycin and cisplatin (Raaphorst et al., 1996;

Bates and Mackillop, 1990, 1986; Honess, 1988; Bates et al., 1987). Hyperthermia could also be a useful strategy to overcome drug resistance. MDR cells overexpressing P-gp (Bates and Mackillop, 1986) or MRP-1 (Souslova and Averill-Bates, 2004) were sensitive to heat killing. To further support the usefulness of hyperthermia, drug-induced cell death by teniposide and topotecan (Hermisson and Weller, 2000), cisplatin (Raaphorst et al., 1996) and methotrexate (Herman et al., 1981) was increased by hyperthermia in cells exhibiting primary drug resistance. In MDR cells, hyperthermia enhanced drug sensitivity of melphalan (Bates and Mackillop, 1990) and liposomal Adriamycin (Gaber, 2002). Furthermore, hyperthermia combined with P-glycoprotein modulators, such as verapamil, cyclosporin A or PSC833, increased the effectiveness of drugs such as Adriamycin, melphalan and vinblastine, in the elimination of MDR cells (Averill and Su, 1999; Averill and Larrivée, 1998; Dumontet et al., 1998, Liu et al., 2001).

The present study explores the possibility of reversing resistance to Adriamycin using hyperthermia in human cervical adenocarcinoma (HeLa) cells overexpressing MRP1 (HeLaMRP). The objective of the study is to determine whether heat can increase the induction of apoptosis by Adriamycin in HeLa cells and in their MDR mutant overexpressing MRP1. Apoptotic events are evaluated at the level of mitochondria, caspase activation and cleavage of caspase substrates.

Materials and Methods

Tissue culture

HeLa (ATCC no. CCL-2) cells were grown in monolayer in Dulbecco's modified Eagle medium (DMEM) (Gibco Canada, Burlington, ON) which contains high glucose with L-glutamine (2mM) and pyridoxine hydrochloride. This medium was supplemented with 10% fetal bovine serum (FBS) (Gibco Canada) containing penicillin (50 units/ml) and streptomycin (50μg/ml) (Flow Laboratories, Mississauga, ON), sodium pyruvate (1.0mM), sodium bicarbonate (1.5g/L) and nonessential amino acids (0.1mM) (Souslova and Averill-Bates, 2004). The multidrug resistant HeLa-MRP cell line was a kind gift from Dr. Philippe Gros, McGill University, Montreal, QC (Kast and Gros, 1998). These cells are resistant to Adriamycin, etoposide, actinomycin D and vincristine. The culture medium for HeLa-MRP cells contained etoposide (VP-16) (250ng/ml), which was removed for the final passage prior to experiments. Cell lines were maintained in tissue culture flasks (Sarstedt, Saint-Laurent, QC) in a humidified atmosphere of 5% CO_2 at 37°C using a water jacketed incubator. Cells were grown to confluence and the culture medium was changed 24h prior to experiments. Cells were harvested using trypsin (0.5mg/ml)-EDTA (0.2mg/ml) (Gibco Canada) and washed again by centrifugation (1000g, 3min).

Adriamycin uptake

Freshly harvested HeLa and HeLaMRP cell suspensions were plated onto sterile coverslips in culture dishes at a concentration of $0.5x10^6$ cells per dish and incubated overnight at 37°C in an atmosphere containing 5% CO_2. Cells were incubated for 1h with Adriamycin (10 and 20μl), either alone at 37°C or combined with 42°C heat shock. Then, coverslips containing cells were washed twice with ice-cold PBS, inverted onto slides and viewed for fluorescence under UV illumination using a Zeiss microscope equipped with a mercury lamp (model IM, Carl Zeiss Canada Ltd., St. Laurent, QC). The UV illumination induced an orange-red fluorescence at sites of

Adriamycin accumulation (Larrivée and Averill, 2000). Quantification of fluorescence intensity in cells was carried out using Northern Eclipse Software (Empix Imaging, Mississauga, ON). At least 100 cells were counted per sample.

Heat and drug treatment

For the effect of heat alone, cells were incubated for 1 or 2h at temperatures ranging from 37 to 45°C, in temperature-controlled water baths (0±.0.02°C) (Haake D8, Fisher Scientific, Montreal, QC). For the effect of drug alone, cells were incubated for either 15h (caspase 9, Bax and cytochrome c) or for 18h (caspase 3, ICAD and Hoechst) at 37°C in an incubator containing 5% CO_2. For the combined effect of heat and drug, cells were incubated at 42°C (±0.1°C) during the 1st hour of the 15h or 18h drug treatments.

Subcellular fractionation and Western blot analysis of Bax, cytochrome c and ICAD

Following treatment with Adriamycin alone or combined with 42°C heat shock, cells were harvested, homogenized using a dounce homogenizer (50 strokes/sample) and then lysed in 500μl of lysis buffer (Samali et al., 1999) containing 100mM sucrose, 1mM EGTA, 20mM 3-N-morpholino-propanesulfonic acid (MOPS), 0.1mM dithiothreitol (DTT), 5% freshly added percoll, 0.01% digitonin, 1mM phenyl-methyl-sulfonyl fluoride (PMSF) and 100μl/10ml of cocktail of protease inhibitors (AEBSF, Aprotinin, Bestatin, E-64, Leupeptin and Pepstatin A) (Sigma-Aldrich, Inc., Saint-Louis, MO), pH 7.4. Debris, unbroken cells and nuclei were removed by centrifugation (10,000g, 10min), to provide whole cell lysates. For subcellular fractionation, supernatants were then centrifuged (15,000g, 15min) to separate mitochondria. Supernatants were then centrifuged (100,000g, 1h) to separate cytosolic and nucleosomal fractions (Jurkiewicz et al., 2004). Mitochondrial fractions

were resuspended in lysis buffer (300mM sucrose, 1mM EGTA, 20mM MOPS, 0.1mM DTT, 100ul/10ml of cocktail of protease inhibitors, pH 7.4). For the immunodetection of ICAD, whole cell lysates were used.

Protein concentrations were determined according to Bradford (1976). Proteins (30μg) from mitochondrial and cytosolic fractions, or whole cell lysates, were solubilised in sample buffer (Laemmli, 1970), heated for 5 min at 100°C and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (15% gel for Bax, cytochrome c; 10% gel for ICAD) using a constant voltage of 125V. Cellular proteins were transferred to polyvinyl difluoride (PVDF) membranes using a Milliblot Graphite Electroblotter I apparatus (Millipore, Bedford, MA). The transfer buffer contained 96mM glycine, 10mM Tris and 10% methanol. The transfer was carried out for 1.5 h at constant amperage of 80mA/gel. Hydrophobic or non-specific sites were blocked using skim milk in Tris-buffered saline (50mM Tris and 150mM Nacl) containing 0.1% Tween 20 (TBS-T) for 1h at room temp or overnight at 4°C. Membranes were washed and probed with mouse anti-cytochrome c monoclonal antibody (1:1000) (BD Biosciences, Mississauga, ON), rabbit polyclonal anti-Bax antibody (1:2000) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or polyclonal rabbit anti-ICAD antibody (1:1000) (Santa Cruz Biotechnology Inc.) for 1h at room temperature or overnight at 4°C. Membranes were washed and then incubated for 1h with horseradish peroxidase (HRP)-conjugated rat Mab-anti-rabbit IgG (1:1000) (Biosource, Camarillo, CA). Membranes were incubated in ECL-Plus chemiluminescence reagent (Amersham Bioscience Corp., Piscataway, NJ) for 1 min and films (Fuji medical X-ray film, Düsserldorf, Germany) were scanned with a Laser Scanning Densitometer (Alpha Innotech Corp., San Leandro, CA). Expression of Bax, cytochrome c and ICAD was quantified using IPGEL software. Purity of cytoplasmic and mitochondrial fractions was verified using antibodies to GSTπ_1 (Calbiochem, La Jolla, CA) and cytochrome c oxidase (Molecular Probes, Eugene, OR), respectively (data not shown).

Enzymatic assays of caspase-9 and -3 activities

Following incubation with Adriamycin and or heat, cells ($1x10^6$) were harvested, resuspended in PBS-BSA 1% containing 10mM glucose and then centrifuged in a microfuge (3min, 4000rpm). Cells were resuspended in 50μl of reaction buffer (20 mM piperazine-N,N'-bis (2-ethanesulfonic acid) (PIPES), 100mM NaCl, 10mM DTT, 1mM EDTA and 0.1% 3-[(3-cholamido-propyl)-dimethylammonio]-2-hydroxy-1-propanesulfonic acid (CHAPS), 10% sucrose, pH 7.2) (Stennicke and Salvesen, 1997). A 25μl aliquot of this solution was seeded into 96-well plates and the cells were then lysed by freezing at -20°C for 15min. Fifty μl of reaction buffer were added to each sample and then specific caspase substrates (25μl) (200μM) (Calbiochem, San Diego, CA) were added; Ac-Leu-Glu-His-Asp-7-amino-4-trifluoromethylcoumarin for caspase-9 and Ac-Asp-Glu-Val-Asp-amino-4-methylcoumarin for caspase-3. The kinetic reaction for caspase activity was followed for 30 min, at respective excitation and emission wavelengths of 400nm and 505nm for caspase-9 and 380nm and 460nm for caspase-3, using a spectrofluorimeter (Spectra Max Gemini, Molecular Devices, Sunnyvale, CA).

Morphological analysis of cell death

Following treatment with Adriamycin and/or 42°C hyperthermia, 50μg/ml of Hoechst 33258 (Sigma Chemical Co.) was added for 15min at 37°C, in order to stain apoptotic cells (blue-green fluorescence). Hoechst 33258 binds to condensed chromatin in the nucleus of apoptotic cells. Propidium iodide (Sigma Chemical Co.) (50μg/ml) was subsequently added to visualise necrotic cells (red fluorescence). Apoptotic and necrotic cells were observed by fluorescence microscopy (model IM, Carl Zeiss Canada Ltd, St. Laurent, QC) and photographs were taken using a Sony

digital 3CCD Color Video Camera and analysed using Northern Eclipse software (Empix Imaging, Mississauga, Ontario). At least 300 cells were counted per sample.

Statistics

Data are presented as means ± standard error of the mean (SEM) from at least 3 independent experiments performed with multiple estimations per point. For statistical analysis of data for cytochrome c, Bax, JC-1, drug accumulation and caspases, average values were compared to a control value. The control value was subtracted from each observation and a bilateral t-test was performed to see if the average value is null. A Bonferroni-Holm (sequentially rejective method) adjustment was performed to control for the family wise error (FWE) rate at 5%. A one way analysis of variance (ANOVA) and closed testing procedures adjustment using the macro SimIntervals (Westfall et al., 1999) were used for multiple comparisons between averages, except for ICAD and Hoechst tests, where the Dunnett bilateral test was performed for adjustment of P-values. Software used was JMP Statistical Discovery 4.0 (SAS Institute Inc., Cary, NC). Differences were considered statistically significant at $P < 0.05$.

Results

Adriamycin accumulation in parental and MDR HeLa cells: Effect of hyperthermia. The MDR phenotype is often associated with decreased drug accumulation. We determined whether this was the case for resistance to Adriamycin in HeLaMRP cells. Intense fluorescence was clearly distinguishable in the parental HeLa cells, following a 60 min exposure to Adriamycin (10 and 20 μM) at 37°C (Fig. 1A, 1C). In contrast, MDR cells displayed only faint Adriamycin fluorescence (Fig. 1E, 1H). Adriamycin accumulation was significantly lower in HeLaMRP cells compared to HeLa cells at 37°C (Fig. 1I). Hyperthermia (42°C) caused an increase in the intensity of Adriamycin fluorescence in HeLa parental cells (Fig. 1B, 1D, 1I), but demonstrated little effect in HeLaMRP cells (Fig. 1F, 1H, 1I).

Adriamycin and hyperthermia, alone or combined, induce the translocation of Bax and cytochrome c. An important molecular event in apoptosis is translocation of pro-apoptotic proteins such as Bax from the cytoplasm to mitochondria, thus interfering with anti-apoptotic functions of proteins like Bcl-2. As a consequence, release of pro-apoptotic proteins such as cytochrome c from mitochondria will be promoted. Adriamycin induced the translocation of Bax from the cytoplasm to mitochondria in HeLa (Fig. 2A, 2C, 2E, 2G) and HeLaMRP (Fig. 2B, 2D, 2F, 2H) cells, at 37°C. Bax expression decreased in the cytosolic fraction (Fig. 2A- 2D) with a corresponding increase in the mitochondrial fraction (Fig. 2E-2H). These changes were dependent on Adriamycin concentration and occurred at higher concentrations (2-10 μM) in MDR cells (Fig. 2D, 2H), compared to parental cells (0.5-2 μM) (Fig. 2C, 2G). Two μM Adriamycin induced more pronounced changes in Bax expression in HeLa cells compared to MDR cells. Hyperthermia (42°C) alone induced translocation of Bax from the cytosol to mitochondria to a similar extent in both cell lines. Hyperthermia increased the effect of Adriamycin on Bax expression in cytosolic and mitochondrial fractions in both cell lines. Levels of

cytochrome c in the cytoplasmic fraction increased in both cell lines with increasing Adriamycin concentration (0.5-2μM for HeLa, 2-10μM for HeLaMRP cells) at 37°C (Fig. 3A-3D). There was a corresponding decrease in cytochrome c expression in mitochondrial fractions (Fig. 3E-3H). Hyperthermia alone caused release of cytochrome c from mitochondria into the cytosol to a similar extent in both cell lines. Adriamycin-induced liberation of cytochrome c from mitochondria was increased by 42°C hyperthermia in both HeLa (Fig. 3C, 3G) and MDR (Fig. 3D, 3H) cells.

Caspase activation by hyperthermia and/or Adriamycin. A major downstream event from cytochrome c release in mitochondria-mediated cell death is activation of the initiator caspase 9. Hyperthermia induced activation of caspase 9 relative to unheated control cells at 37°C (Fig. 4A). Activation of caspase 9 occurred at 41-42°C in HeLa cells and at 41°C in HeLaMRP cells (Fig 4A). Caspase 9 activity decreased below control levels at 45°C for both cell lines (Fig.4A). Adriamycin caused an increase in the activity of caspase 9 in HeLa (Fig. 4B) and HeLaMRP (Fig. 4C) cells at 37°C. Caspase 9 activation occurred at higher drug concentrations (2-10μM) in MDR cells, compared to parental cells (0.5-2μM). Hyperthermia caused an increase in activation of caspase 9 by Adriamycin in both cell lines (Fig. 4B, 4C).

Caspase 3 is a downstream target of cleavage by caspase 9. Hyperthermia induced the activation of caspase 3 (Fig. 5A) relative to unheated control cells at 37°C. Activation of caspase 3 was observed at 41-42°C in HeLa and HeLaMRP cells (Fig. 5A). The activity of caspase 3 decreased below control levels at 45°C. Adriamycin (0-2μM for HeLa, 0-10μM for HeLaMRP cells) caused a concentration dependent increase in the activity of caspase 3 in HeLa and HeLaMRP cells at 37°C (Fig. 5B, 5C). Hyperthermia (42°C) caused an increase in activation of caspase 3 by Adriamycin in both cell lines (Fig. 5B, 5C).

Cleavage of caspase 3 substrate ICAD by Adriamycin and/or hyperthermia. ICAD is a well established cleavage target of caspase 3. Adriamycin caused a concentration dependent decrease in the expression of ICAD, which indicates cleavage of ICAD, in HeLa (Fig. 6A, 6C) and HeLaMRP cells (Fig. 6B, 6D). Hyperthermia (42°C) alone caused cleavage of ICAD and increased that caused by Adriamycin.

Morphological analysis of apoptosis induced by Adriamycin alone or combined with hyperthermia. One of the later events in the apoptotic cascade is chromatin condensation in the nucleus. This event can be triggered by activation of CAD, resulting from ICAD cleavage by caspases (Nicholson, 1999). Figure 7 illustrates induction of apoptosis (chromatin condensation) and necrosis by Adriamycin, whether alone or combined with 42°C hyperthermia. The number of apoptotic cells with condensation of chromatin increased steadily with Adriamycin concentration after 18h at 37°C for HeLa (Fig. 7A-7D, 7I) and HeLaMRP cells (Fig. 8E-8H, 8J). The HeLaMRP cells (Fig. 7J) were more resistant to induction of apoptosis by Adriamycin than HeLa cells (Fig. 7I) (note that higher Adriamycin concentrations were used for HeLaMRP cells). Hyperthermia (42°C) alone caused chromatin condensation in HeLa cells. Hyperthermia caused a significant increase in Adriamycin-induced apoptosis in HeLa cells (Fig. 7I), but a low effect was observed in MDR cells (Fig. 7J). Adriamycin caused a significant increase in the number of necrotic cells in HeLa cells at 37°C and 42°C at higher doses (2μM) (Fig. 7I). However, in HeLaMRP cells, there was a steady increase in the number of necrotic cells with Adriamycin concentration at 37°C and 42°C (Fig. 7J).

Comparison of expression of apoptotic proteins between HeLa and HeLaMRP cells. Some studies have reported differences in the expression of apoptotic proteins between sensitive and drug-resistant cells. We determined whether there were differences in endogenous levels of expression of apoptotic factors

between the parental and MDR HeLa cell lines. The previous figures show that endogenous levels of expression of Bax (Fig.2), cytochrome c (Fig. 3) and ICAD (Fig. 6) are very similar in HeLa and HeLa MRP cells, along with the basal activity of caspases 9 and 3 (Figs. 4, 5).

DISCUSSION

Clinical drug resistance remains one of the primary causes of suboptimal outcomes in cancer therapy (Linn and Giaccone, 1995) and understanding the cause for this phenomenon could answer many of the questions and complexities of cancer treatment. It was first documented experimentally in mouse leukemic cells that acquired resistance to 4-amino-N10-methyl-pteroylglutamic acid in a laboratory model in 1950 (Burchenal et al., 1950). The mechanisms of MDR with known clinical significance are the activation of transmembrane ABC transporter proteins such as P-gp and MRP1, which efflux a variety of chemical substances from cells, activation of enzymes of the glutathione detoxification system (Schroder et al, 1996) and alterations of genes and proteins involved in the control of apoptosis (Glisson et al., 1989).

This report demonstrates the induction of apoptosis by hyperthermia alone or combined with Adriamycin, in HeLa cells and in transfected HeLa cells overexpressing multidrug resistance associated protein. Hyperthermia (42oC) alone and Adriamycin alone induced apoptosis by the mitochondrial pathway. This was the case in both HeLa and HeLaMRP cells. Induction of mitochondrial apoptosis was manifested by a cascade of events including the translocation of Bax to mitochondria, which resulted in the liberation of cytochrome c into the cytoplasm and activation of initiator caspase 9. Caspase 9 in turn activated the effector caspase 3, which cleaved its substrate ICAD. Induction of apoptosis by either 42oC hyperthermia or Adriamycin was confirmed morphologically by the condensation of nuclear chromatin, which is a later event in the apoptotic cascade.

Higher concentrations of Adriamycin were required to induce apoptosis in HeLaMRP cells, given that these cells are drug resistant compared to the parental HeLa cells. A concentration of 2 μM Adriamycin induced lower levels of apoptosis in HeLaMRP cells than HeLa cells. MRP1-overexpressing HeLa cells were resistant to

Adriamycin-induced apoptosis at the level of all molecular events in the mitochondrial pathway including translocation of Bax and cytochrome c, activation of caspases, cleavage of ICAD and nuclear chromatin condensation. These findings are in agreement with previous work which showed that HeLaMRP cells exhibit resistance to cytotoxicity induced by Adriamycin, actinomycin D, etoposide and vincristine (Kast and Gros, 1998). We found that Adriamycin accumulation was lower in HeLa-MRP cells, which is likely explained by increased drug efflux by MRP1 in HeLaMRP cells. MDR cell lines often show decreased net accumulation of Adriamycin associated with energy-dependent outward efflux of cytotoxic agents (Inaba et al., 1979). Certain studies have reported that MRP1-overexpressing cells show increased drug efflux compared to the drug sensitive counterparts (Bakker et al., 1997).

In general, hyperthermia caused a similar extent of apoptosis in both drug sensitive and MDR HeLa cells. This was the case for all of the molecular events in the apoptotic cascade, from Bax translocation, caspase activation and cleavage of caspase substrates. This finding has important significance for the clinical application of hyperthermia in tumors containing drug resistant cells. Tumors often respond to initial treatments with chemotherapy, but subsequently do not respond to the drugs once resistance develops. These drug resistant tumor cells are sensitive to heat and could therefore be eliminated using hyperthermia (Bates and Mackillop, 1986; Souslova & Averill-Bates, 2004).

In general, hyperthermia (42oC) increased the level of apoptosis caused by Adriamycin, compared to 37oC, in both drug sensitive and MDR cells. It was previously reported that Adriamycin showed strong interactions with heat and enhancement of drug cytotoxicity was seen at non-lethal temperatures (38 –42°C) in other cell types (Bates and Mackillop, 1986, 1987). This study shows that hyperthermia increased intracellular Adriamycin accumulation in HeLa cells, but not in HeLaMRP cells. Heat appears to influence drug permeability to Adriamycin in

HeLa cells, but not in HeLaMRP cells. However, the exact mechanisms of cell death caused by hyperthermia and drugs are still a matter of debate. Heat would likely increase the generation of radicals produced by natural metabolic processes and also by free radical-generating drugs such as Adriamycin (Roti Roti and Laszlo, 1988). Adriamycin is known to generate oxidative stress with the subsequent formation of lipid peroxidation products (Gille and Nohl, 1997). Hyperthermia alone is able to cause oxidative stress by generation of reactive oxygen species (ROS) in cells (Lord-Fontaine and Averill, 1999) and an imbalance in the cellular redox equilibrium by depletion of cellular thiols (Mitchell and Russo, 1983). Hyperthermia can also cause GSH depletion by increasing the level of lipid peroxides (Anderstam et al., 1992), which could result in an enhancement of Adriamycin cytotoxicity due to less detoxification of drug. Furthermore, the rates of chemical and biochemical reactions increase with temperature. Thus the rates of reactions between Adriamycin and both intracellular and extracellular molecules, including cellular membrane components and the critical cellular targets, would also increase with temperature.

HeLaMRP cells have a severe redox imbalance due to lower activity of the enzyme glutathione peroxidase (GPx) and lower levels of glutathione (GSH), compared to HeLa cells (Souslova and Averill-Bates, 2004). GPx and GSH are important antioxidants which protect cellular targets such as DNA, proteins and membrane lipids against the damaging effects of oxidative processes (Halliwell and Gutteridge, 1999). GSH plays an important role in detoxification of ROS, electrophiles and oxyanions either by reduction of conjugation (Wang, 1998). Due to the redox imbalance, HeLaMRP cells would be more vulnerable to the oxidative stress caused by both heat and Adriamycin, relative to HeLa cells. This could account for the good sensitivity of HeLaMRP cells to heat and Adriamycin, despite the lack of heat-induced drug accumulation. This idea can be supported by several studies on the influence of the GSH redox pathway on cellular sensitivity to Adriamycin. It was shown that human ovarian cancer lines (Hamilton et al., 1985), MCF-7 breast tumour

cells (Dusre et al., 1989) and HL-60 leukemic cells (Raghu et al., 1993) were sensitized to Adriamycin through depletion of GSH.

The mechanisms of induction of apoptosis by toxic compounds such as Adriamycin are often dependent on different cell types. In support of our findings, several studies confirmed the ability of Adriamycin to trigger apoptosis by the mitochondria mediated pathway. Adriamycin was shown to induce apoptosis by favouring cytochrome c release and consequent formation of the apoptosome complex in cardiac cells. This occurred through up-regulation of Bax, which induces cytochrome c release by facilitating mitochondrial pore opening (Wang et al., 1998a,b), or by down-regulation of Bcl-XL, which is a member of the Bcl-2 protein family that blocks cytochrome c release (Kim et al., 2003). One study revealed that Adriamycin induced apoptosis in ATC cells by altering the acetylation state of histone. Adriamycin reduced histone deacetylase activity and induced hyperacetylation of histone 3, which leads to cell death (Rho et al., 2005). Another study suggests that subtoxic concentrations of Adriamycin can enhance TRAIL-induced apoptosis via depletion of antiapoptotic protein in the human prostate cancer cell line LNCaP (Kang et al., 2005). Recently, Eom and coworkers showed that different doses of Adriamycin activated different regulatory mechanisms to induce either apoptosis or cell death through mitotic catastrophe. For instance, high dose Adriamycin-induced apoptosis, but not low dose adriamycin-induced mitotic catastrophe, led to transient activation of NF-kappaB and strong, sustained activations of p38, c-Jun N-terminal kinase and caspases (Eom et al., 2005).

The development of strategies to overcome MDR has received considerable interest. Increased drug accumulation and drug resistance reversal with P-gp inhibitors have been well documented in vitro, but only suggested in clinical trials. Clinical trials on the design of early resistance reversal have shown statistically significant benefits with the use of P-gp inhibitors in combination with chemotherapy. Recent advances in medicine and science have provided multiple

agents for use in the struggle against MDR. Presently there are many improved chemotherapy drug and targeted therapies that act at a number of sites and by a variety of mechanisms to limit cancer cell proliferation. Conventional methods that are used to overcome MDR often involve the coadministration of chemosensitizers and anticancer drugs. Preclinical studies have described the role of hyperthermia as a technique to increase the activity of chemotherapeutic agents (Gerco et al., 1987). Combined with chemotherapeutic drugs, hyperthermia appears to be a useful strategy to combat the MDR phenotype mediated by both P-gp and MRP1 (Souslova and Averill-Bates, 2004; Larrivee and Averill, 1999; Bates and Mackillop, 1990).

Van der Zee and coworkers investigated the combined effect of hyperthermia on radiotherapy in advanced tumors of the bladder, cervix and rectum. They obtained a complete-response rate of 55%, which was expected to be only 39% without hyperthermia. The addition of hyperthermia seemed to be most important for cervical cancer, for which the complete-response rate was 83%, compared with 57% after radiotherapy alone. Furthermore, 3-year overall survival was 27% with radiotherapy alone, which increased up to 51% when it was combined with hyperthermia (Van der Zee et al., 2000). Literature reviews regarding response rates of addition of hyperthermia to radiotherapy in tumors of breast cancer, malignant melanoma and neck nodes suggest a clinical thermal enhancement ration of 1.5 to 1.7 (Review: Van der Zee, 2002).

Hyperthermia combined with chemotherapy also reveals promising results. For instance, simultaneous combination of cisplatin and hyperthermia in recurring cervical cancer, following irradiation, resulted in a 50% response rate, which was expected to be 15% without hyperthermia (De Wit et al., 1999). Several phase I and phase II clinical studies using combinations of hyperthermia and drug treatment have reported good response rates for treatment of limb melanoma (Engelhardt, 1987), pelvic tumors (Rietbrock et al., 1997), intra-peritoneal metastases (Alexander and Fraker, 1996) and other refractory tumors (Wiedemann et al., 1997). Several phase II

studies on hyperthermia in combination with pre- and/or postoperative chemotherapy in high-risk sarcomas have demonstrated quite impressive 5-year overall survival rates (Issels et al., 2001).

In conclusion, this study demonstrates that hyperthermia could be useful as a modifier of multidrug resistance involving MRP1. An important finding is that MDR cells overexpressing MRP1 do not exhibit cross resistance to heat. Adriamycin can cause cell death by the mitochondrial pathway of apoptosis involving cytochrome c release from mitochondria and the activation of caspases. Regional hyperthermia combined with Adriamycin could be a useful strategy for the elimination of tumours containing drug resistant cells via a targeted approach at the clinical level.

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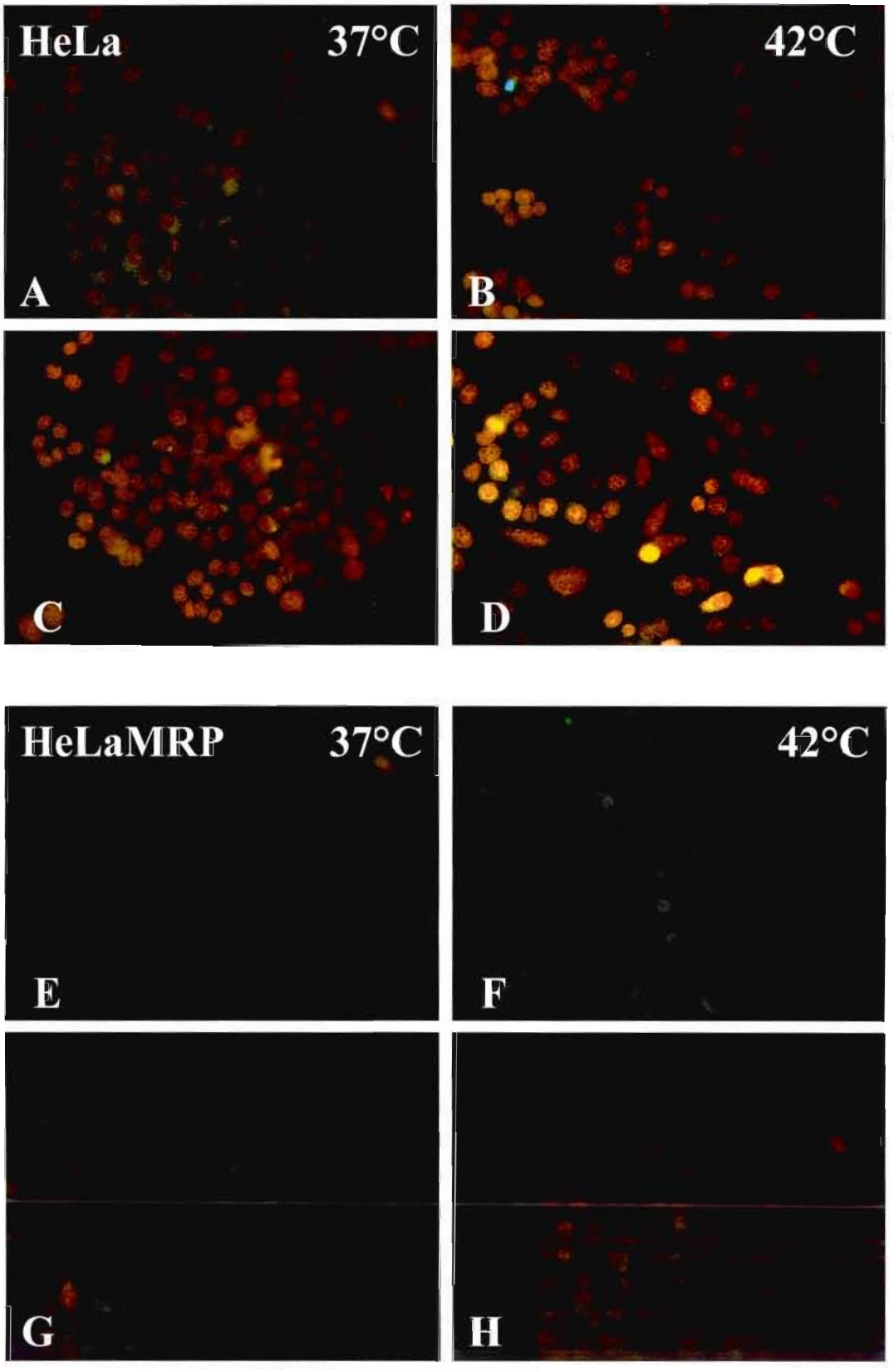


Figure 2.1 A-H

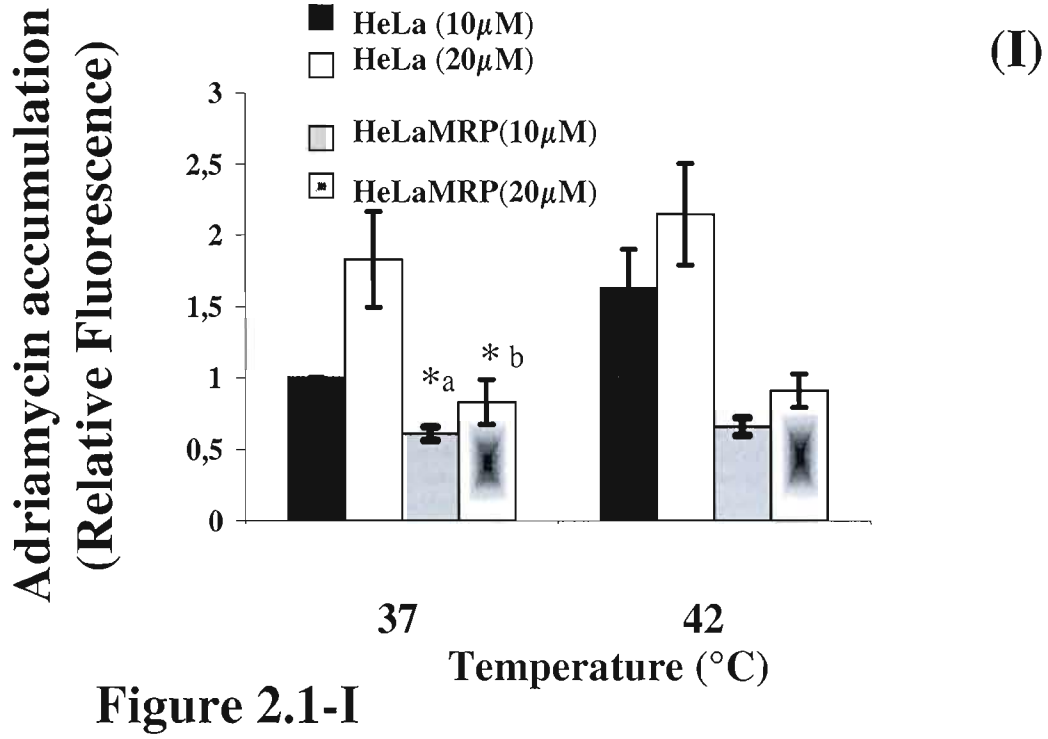


Figure 2.1-I

Figure 2.1: Adriamycin accumulation in HeLa and HeLaMRP cells: effect of 42°C hyperthermia. HeLa (A-D) and HeLaMRP cells (E-H) were incubated with 10μM (A, B, E and F) and 20μM (C, D, G and H) Adriamycin for 60 min in DMEM containing 10% FBS, at 37°C (A, C, E and G) or 42°C (B, D, F and H). Magnification x400. Quantification of images (I) was obtained for both cell lines, representing the relative fluorescence (average grey values). Values for fluorescence are expressed relative to Hela cells treated at 37°C with 10μM of Adriamycin, which was designated as 1. Means ± SEM are shown from at least three different experiments. Significant difference between HeLa and HeLa MRP cells: a. 10μM drug; b. 20μM drug, $p<0.05$ (*).

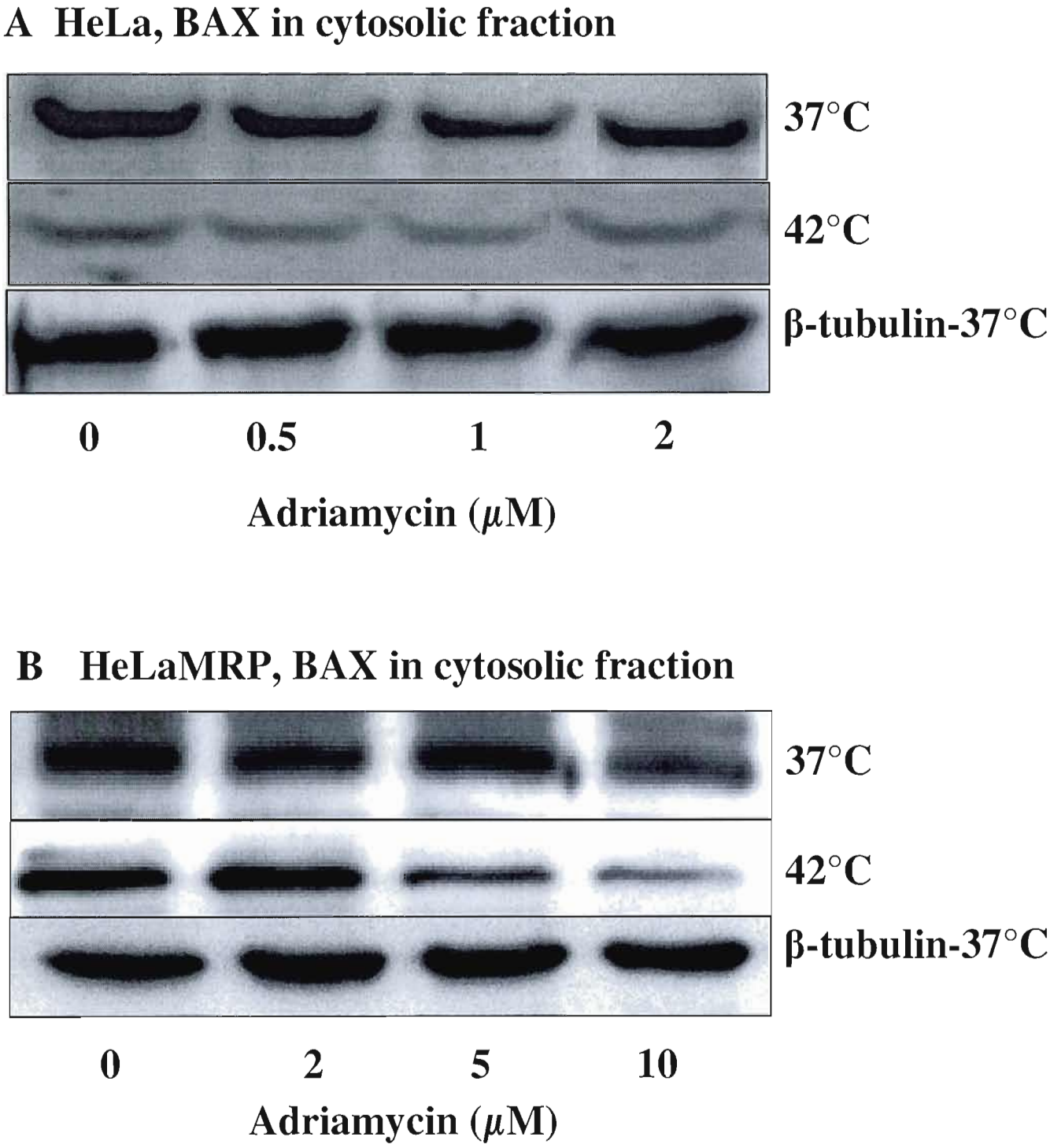

A HeLa, BAX in cytosolic fraction
37°C
42°C
β-tubulin-37°C
0
0.5
1
2
Adriamycin (μM)
B HeLaMRP, BAX in cytosolic fraction
37°C
42°C
β-tubulin-37°C
0
2
5
10
Adriamycin (μM)

Figure 2.2(A-B)

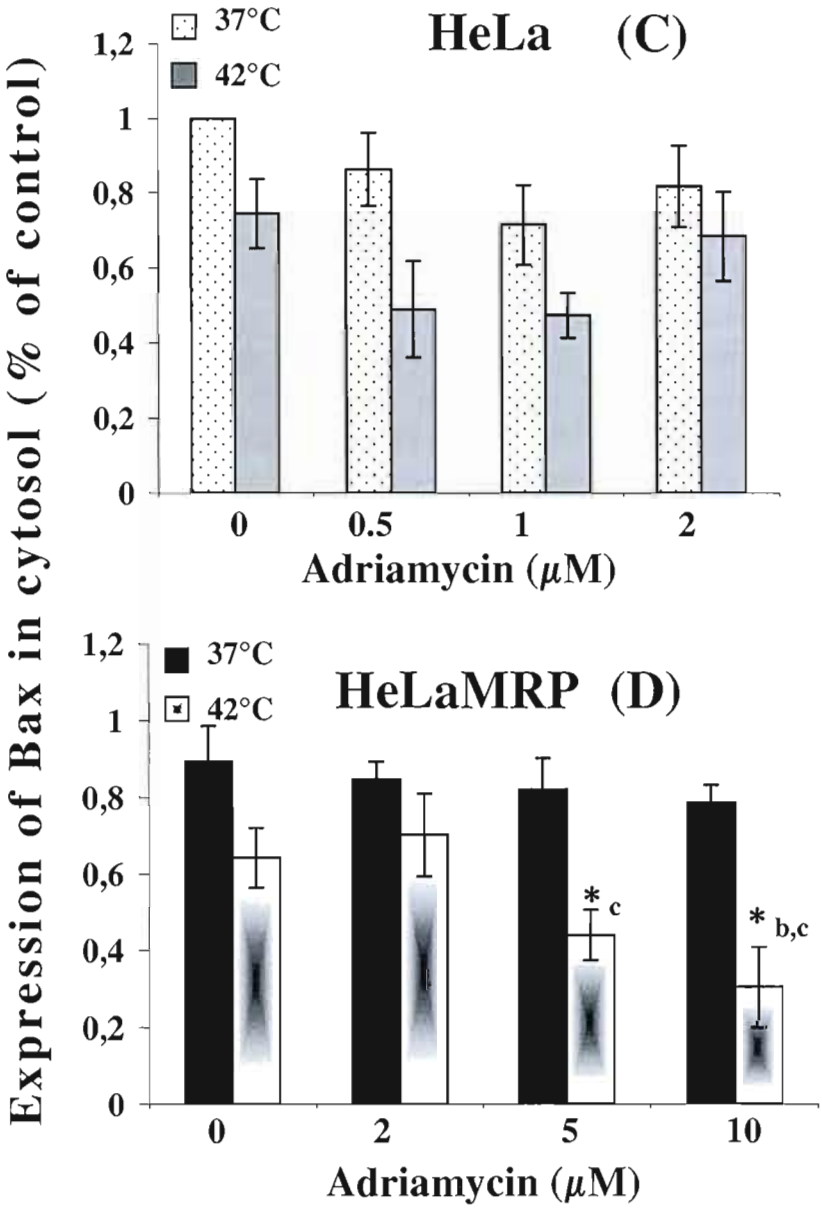
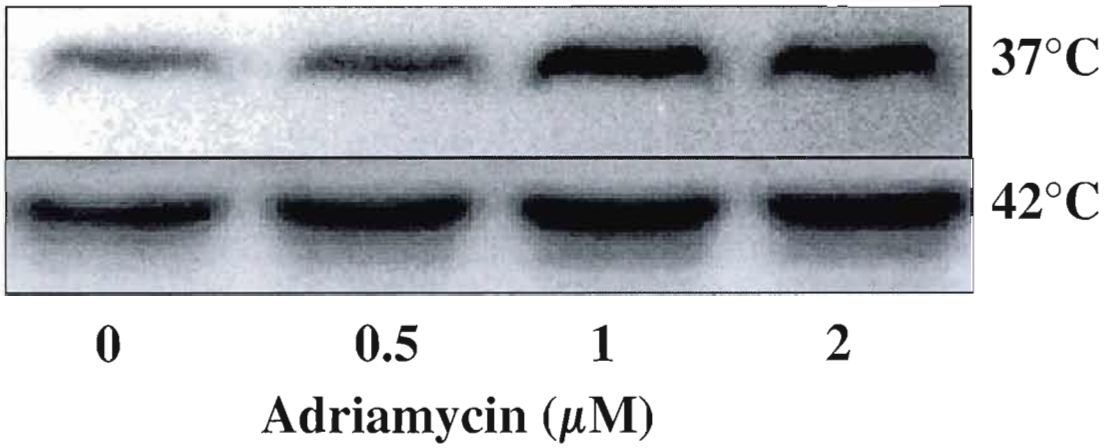

HeLa (C)
37°C
42°C
1,2
1
0,8
0,6
0,4
0,2
0
0
0.5
1
2
Adriamycin (μM)
HeLaMRP (D)
37°C
42°C
1,2
1
0,8
0,6
0,4
0,2
0
* c
* b,c
0
2
5
10
Adriamycin (μM)
Expression of Bax in cytosol (% of control)

Figure 2.2(C-D)

E HeLa, Bax in mitochondrial fraction



F HeLaMRP, Bax in mitochondrial fraction

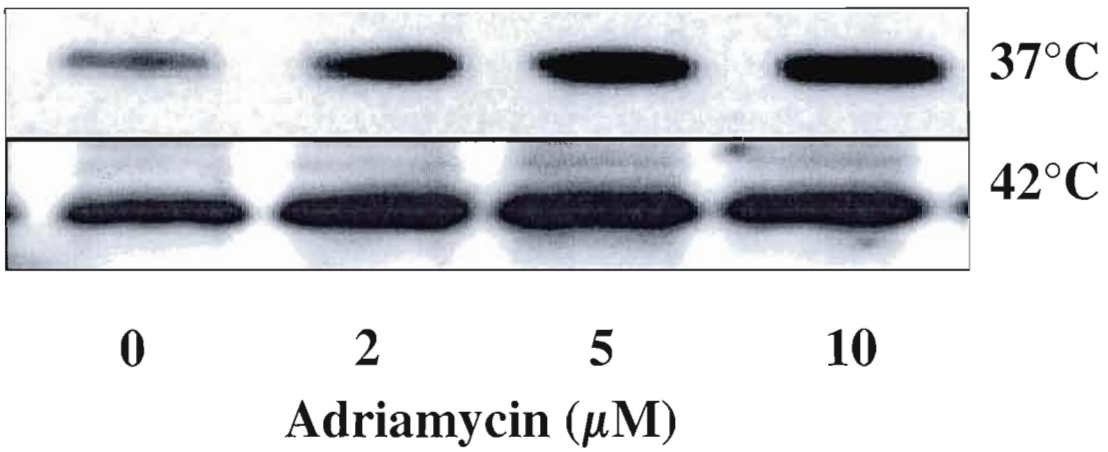


Figure 2.2(E-F)

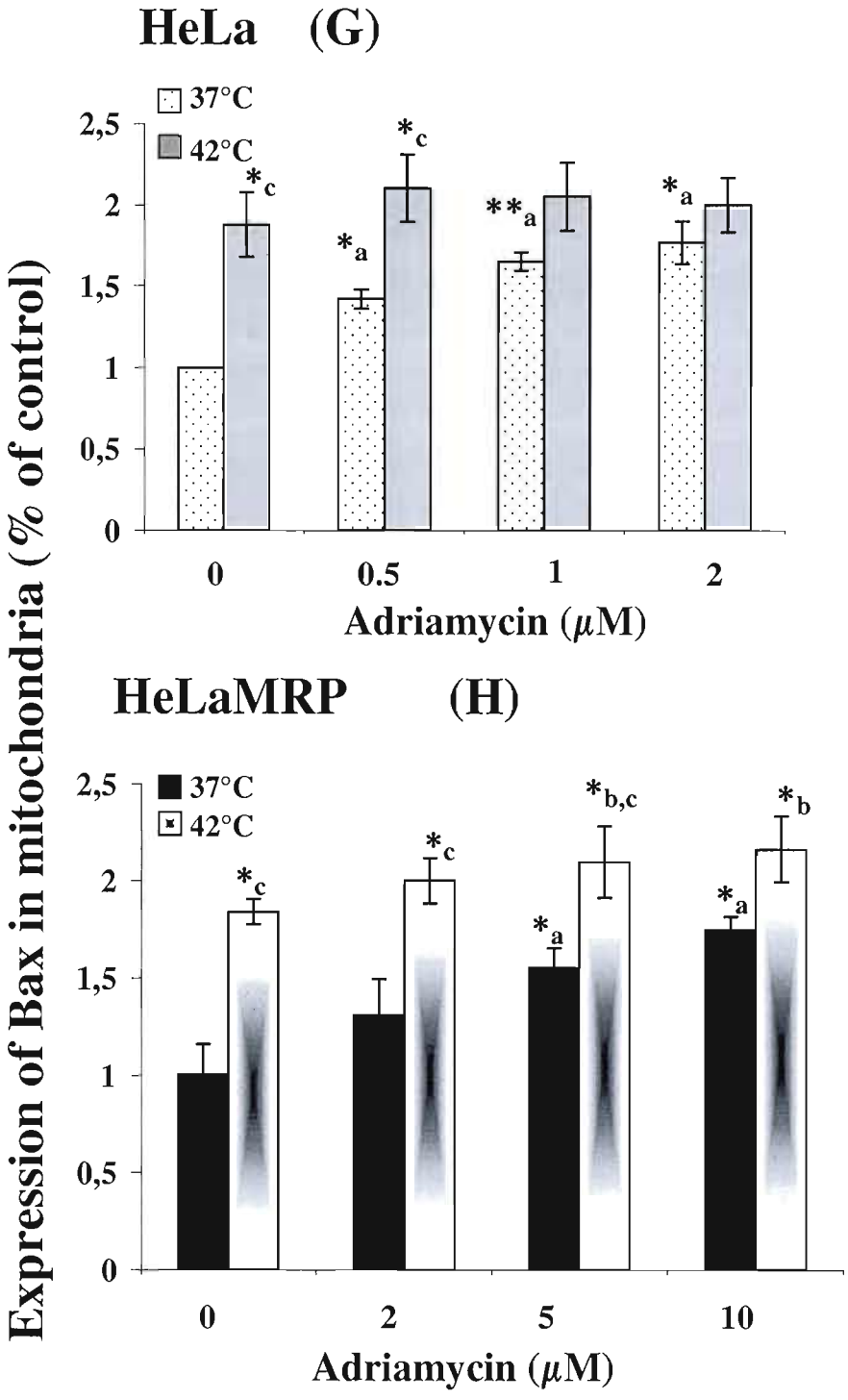
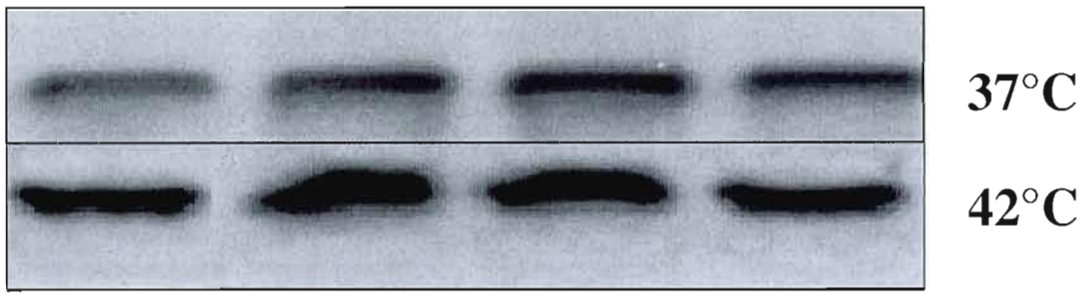

HeLa (G)
37°C
42°C
2,5
2
1,5
1
0,5
0
*c
*a
*c
**a
*a
0
0.5
1
2
Adriamycin (μM)
HeLaMRP (H)
37°C
42°C
2,5
2
1,5
1
0,5
0
*c
*c
*a
*b,c
*a
*b
0
2
5
10
Adriamycin (μM)
Expression of Bax in mitochondria (% of control)

Figure 2.2(G-H)

Figure 2.2: Induction of translocation of Bax from the cytoplasm to mitochondria by Adriamycin alone or combined with 42°C hyperthermia. HeLa (2A, 2E) and HeLaMRP cells (2B, 2F) were treated with different concentrations of Adriamycin (0-2μM for HeLa and 0-10μM for HeLaMRP) at 37°C for 15h or for 1h at 42°C and subsequently 14h at 37°C. Bax (21kDa) levels in cytoplasmic (2A, 2B) and mitochondrial fractions (2E, 2F) were analysed by immunoblotting. Protein levels were quantified by densitometry in HeLa (2C, 2G) and HeLaMRP (2D, 2H) cells. Bax levels were expressed relative to untreated HeLa cells at 37°C, designated as 1. Means ± SEM are shown from at least four independent experiments. a, significantly different from corresponding control (no drug) at 37°C. b, significantly different from corresponding treatment (no drug) at 42°C. c, significantly different at 42°C from corresponding conditions at 37°C, $p<0.05$(*) and $p<0.001$ (**).

A HeLa, cytochrome c in cytosolic fraction



B HeLaMRP, cytochrome c in cytosolic fraction

Figure 2.3 (A-B)

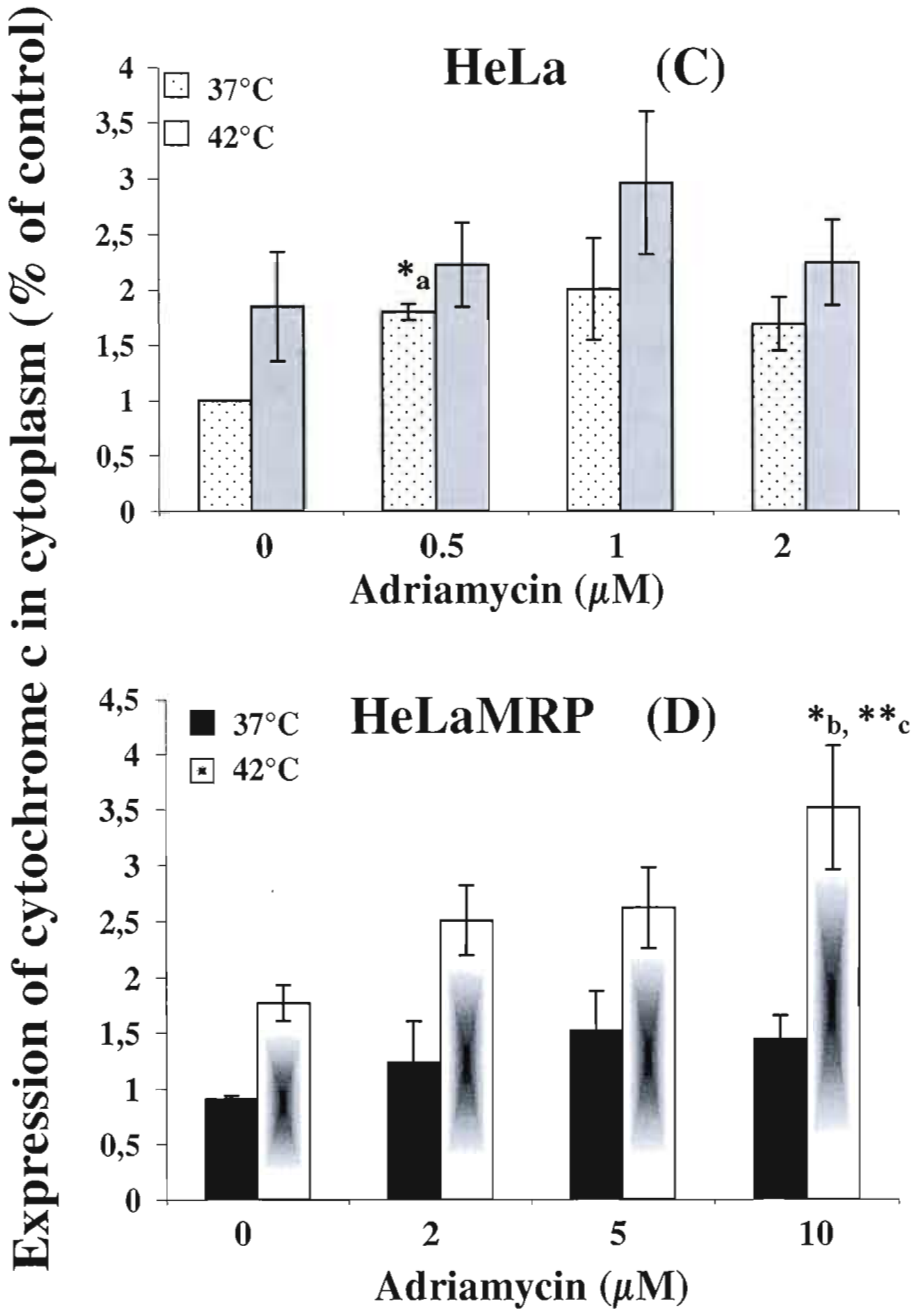

Expression of cytochrome c in cytoplasm (% of control)
HeLa (C)
37°C
42°C
*a
0
0.5
1
2
Adriamycin (μM)
HeLaMRP (D)
37°C
42°C
*b, **c
0
2
5
10
Adriamycin (μM)

Figure 2.3(C-D)

E HeLa, cytochrome c in mitochondrial fraction

F HeLaMRP, cytochrome c in mitochondrial fraction

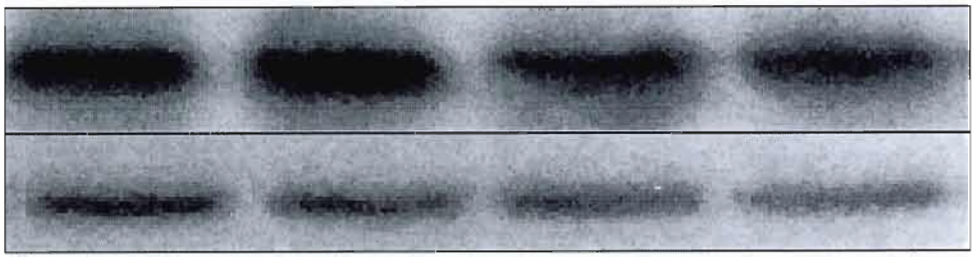


Figure 2.3(E-F)

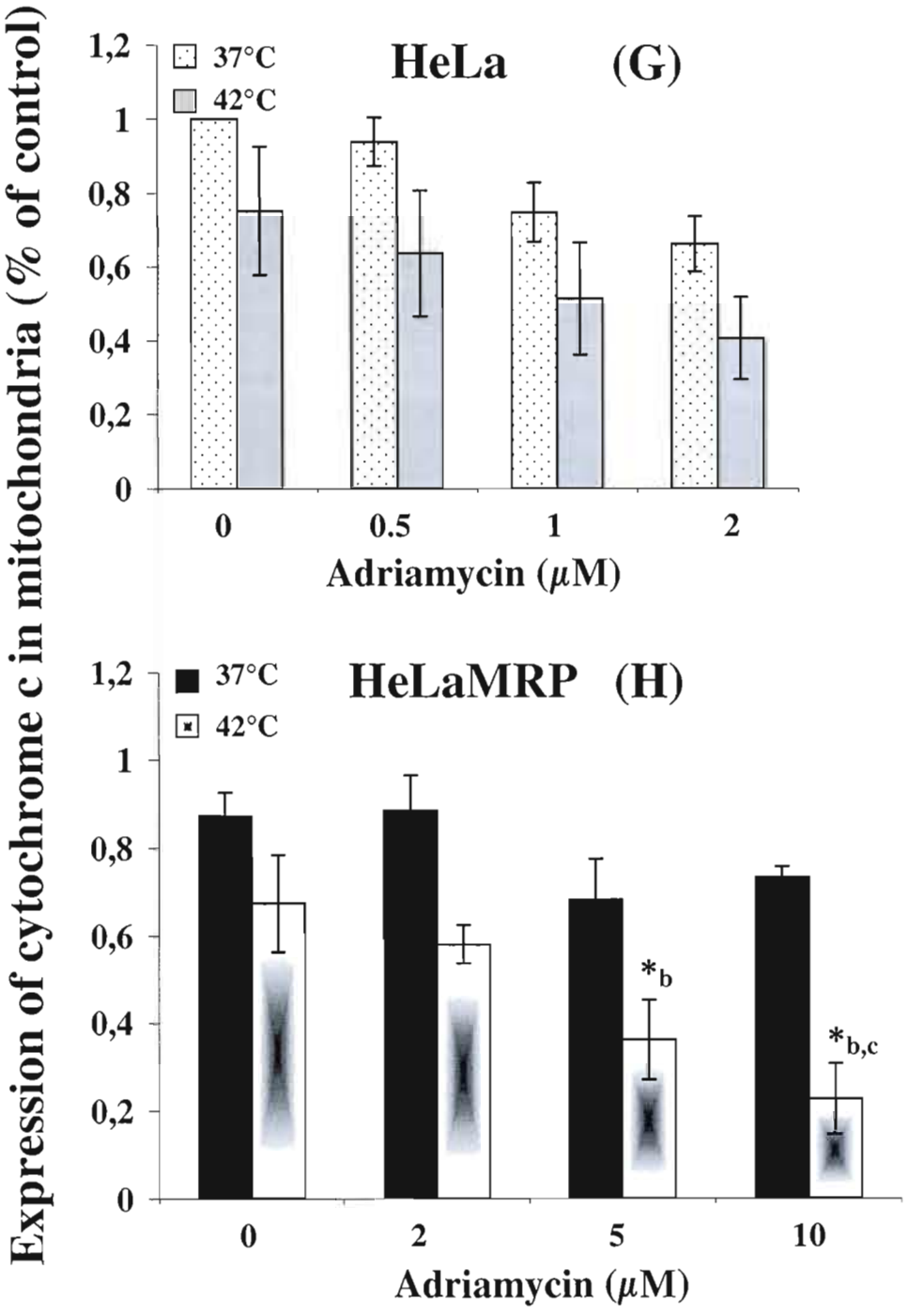

Expression of cytochrome c in mitochondria (% of control)
HeLa (G)
37°C
42°C
1,2
1
0,8
0,6
0,4
0,2
0
0
0.5
1
2
Adriamycin (μM)
HeLaMRP (H)
37°C
42°C
*b
*b,c
0
2
5
10
Adriamycin (μM)

Figure 2.3(G-H)

Figure 2.3: Induction cytochrome c release from mitochondria into the cytoplasm by Adriamycin alone or combined with 42°C hyperthermia. HeLa (3A, 3E) and HeLaMRP cells (3B, 3F) were treated with different concentration of Adriamycin (0-2μM for HeLa, 0-10μM for HeLaMRP) at 37°C for 15h or 1h at 42°C and 14h at 37°C. Following immunoblotting, proteins levels were quantified by densitometry in HeLa (3C, 4G) and HeLaMRP (3D, 3H) cells. Cytochrome c (16kDa) levels were expressed relative to untreated HeLa cells at 37°C, designated as 1. Means ± SEM are shown from at least four independent experiments. a, significantly different from corresponding control (no drug) at 37°C. b, significantly different from corresponding treatment (no drug) at 42°C. c, significantly different at 42°C from corresponding conditions at 37°C, $p<0.05$(*).

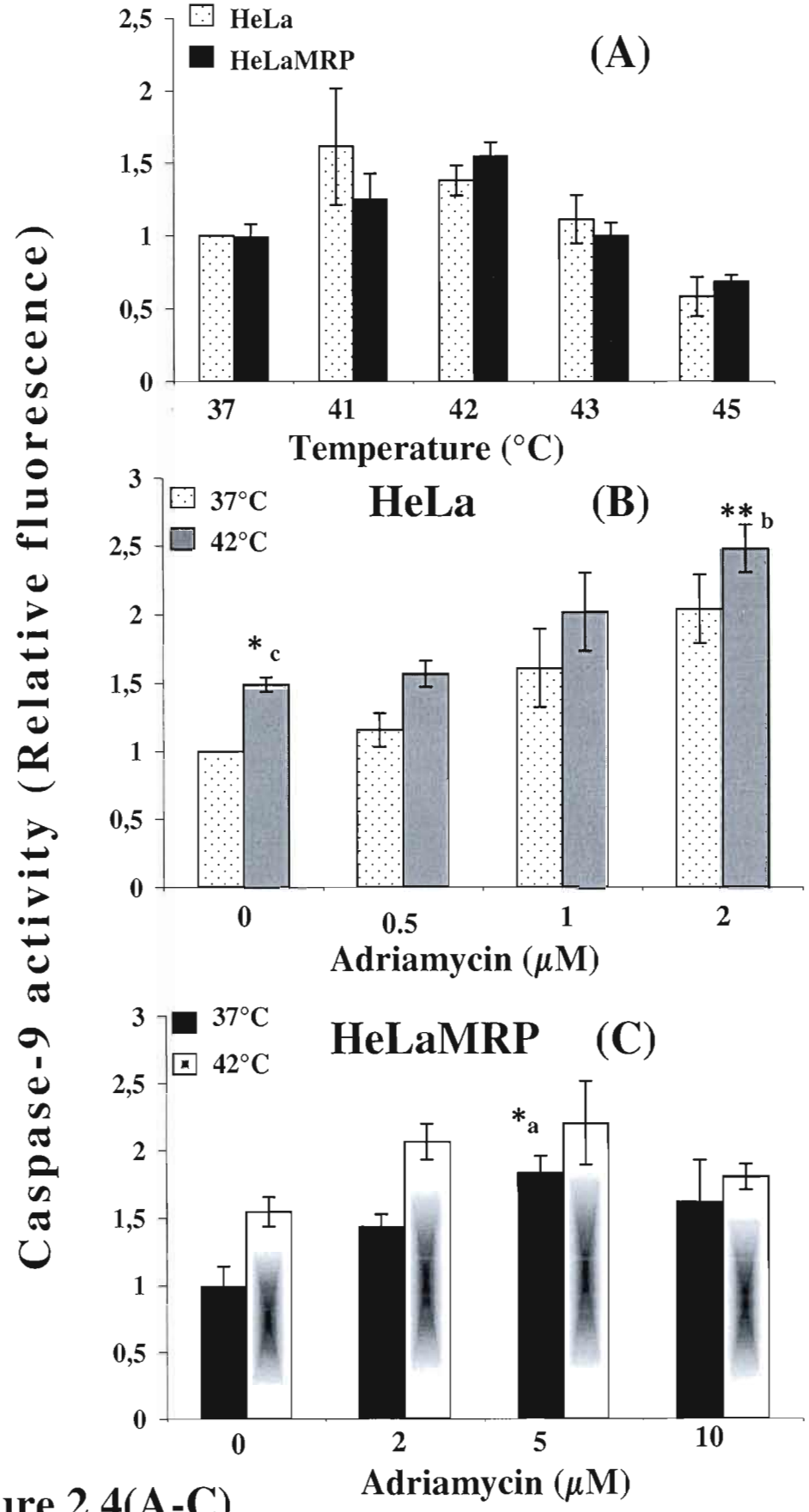

Caspase-9 activity (Relative fluorescence)
HeLa
HeLaMRP
(A)
2,5
2
1,5
1
0,5
0
37
41
42
43
45
Temperature (°C)
37°C
42°C
HeLa
(B)
** b
* c
3
2,5
2
1,5
1
0,5
0
0
0.5
1
2
Adriamycin (μM)
37°C
42°C
HeLaMRP
(C)
*a
3
2,5
2
1,5
1
0,5
0
0
2
5
10
Adriamycin (μM)

Figure 2.4(A-C)

Figure 2.4: Activation of caspase 9 by hyperthermia and/or Adriamycin. (A) For heat alone, HeLa (▨) and HeLaMRP cells (■) were heated (37 to 45°C) for 1h. For drug alone or drug combined with heat treatment, cells were incubated in monolayer with different concentrations of Adriamycin (0-10μM) for 15h at 37°C or 1h at 42°C + 14h at 37°C, (B) for HeLa and (C) HeLaMRP. Activities of caspase-9 were expressed relative to untreated HeLa cells at 37°C, designated as 1.0. Means ± SEM are shown from at least five independent experiments. a, significantly different from corresponding control (no drug) at 37°C. b, significantly different from corresponding treatment (no drug) at 42°C, $p<0.05$(*) and $p<0.001$ (**).

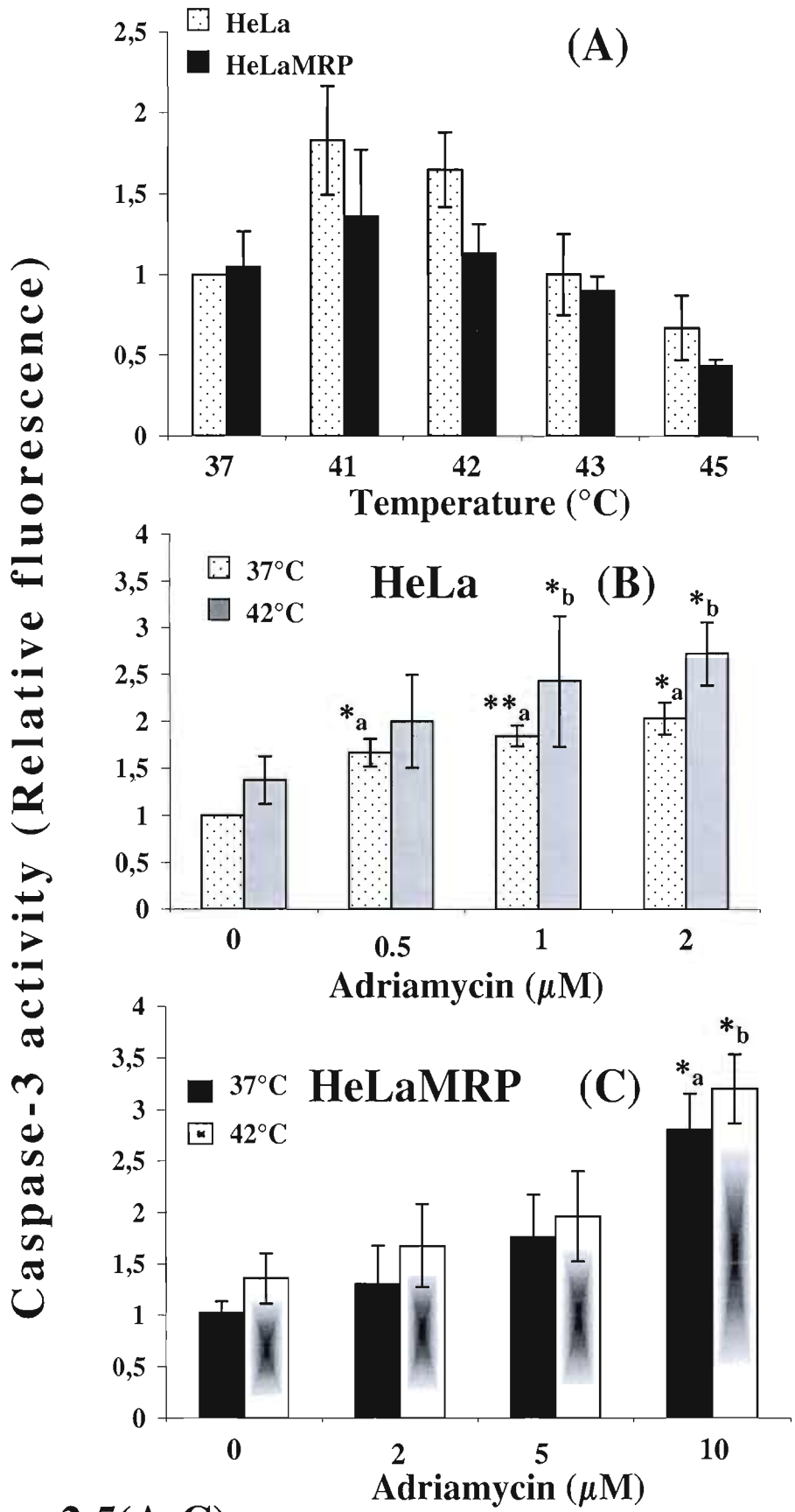
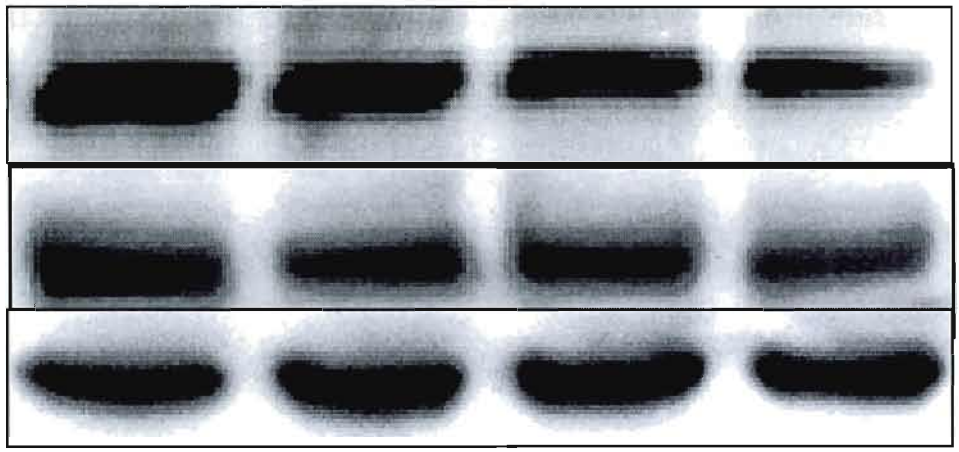

HeLa
HeLaMRP
(A)
2,5
2
1,5
1
0,5
0
37
41
42
43
45
Temperature (°C)
37°C
42°C
HeLa
(B)
*b
*b
*a
**a
*a
4
3,5
3
2,5
2
1,5
1
0,5
0
0
0.5
1
2
Adriamycin (μM)
37°C
42°C
HeLaMRP
(C)
*a
*b
4
3,5
3
2,5
2
1,5
1
0,5
0
0
2
5
10
Adriamycin (μM)
Caspase-3 activity (Relative fluorescence)

Figure 2.5(A-C)

Figure 2.5: Activation of caspase 3 by hyperthermia and/or Adriamycin. (A) For the effect of heat alone, HeLa (▨) and HeLaMRP cells (■) were heated (37 to 45°C) for 2h using temperature controlled water baths (±0.02°C). For drug alone or drug combined with heat treatment, cells were incubated in monolayer with different concentrations of Adriamycin (0 to 10μM) for 18h at 37°C or for 1h at 42°C + 17h at 37°C, (B) for HeLa and (C) HeLaMRP cells. Activities of caspase-3 were expressed relative to untreated HeLa cells at 37°C, designated as 1.0. Means ± SEM are shown from at least five independent experiments. a, significantly different from corresponding control (no drug) at 37°C. b, significantly different from corresponding treatment (no drug) at 42°C, $p<0.05$(*) and $p<0.001$ (**).

A HeLa, Expression of ICAD



B HeLaMRP, Expression of ICAD

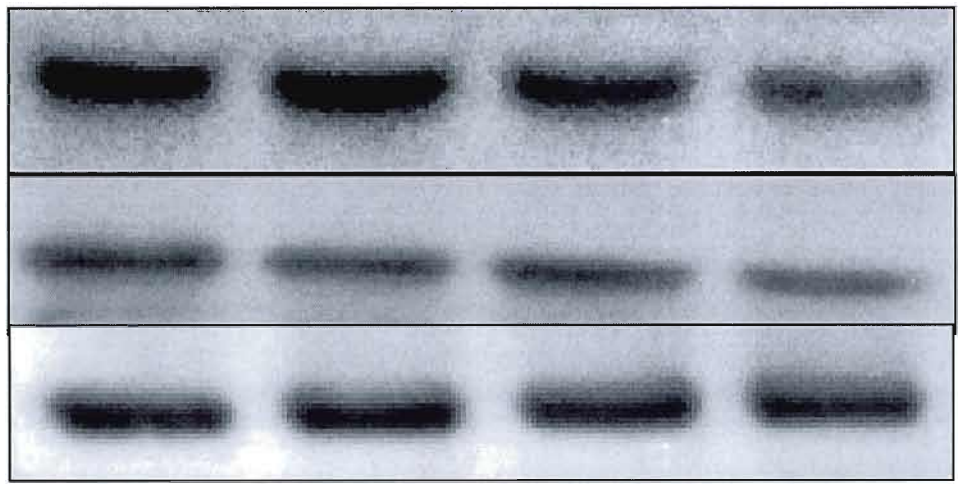


Figure 2.6(A-B)

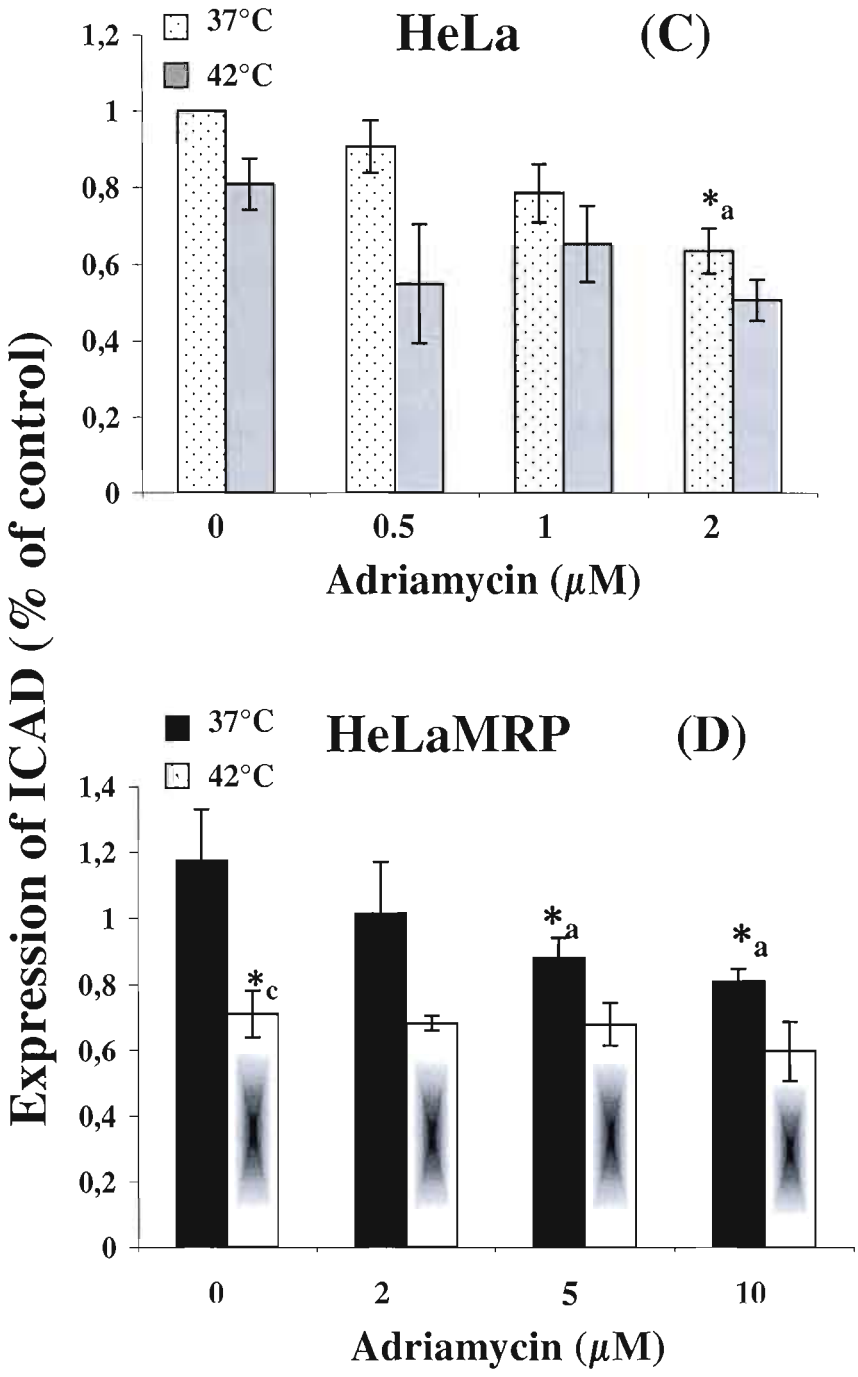

HeLa (C)
37°C
42°C
1,2
1
0,8
0,6
0,4
0,2
0
*a
0
0.5
1
2
Adriamycin (μM)
HeLaMRP (D)
37°C
42°C
1,4
1,2
1
0,8
0,6
0,4
0,2
0
*c
*a
*a
0
2
5
10
Adriamycin (μM)
Expression of ICAD (% of control)

Figure 2.6(C-D)

Figure 2.6: Induction of ICAD cleavage by Adriamycin and/or hyperthermia. HeLa (A) and HeLaMRP (B) cells were treated with Adriamycin for 18h at 37°C or for 1h at 42°C and 17 h at 37°C. Protein levels were detected by immunoblotting and quantified by densitometry in HeLa (C) and HeLaMRP cells (D) and expressed relative to untreated HeLa cells at 37°C, designated as 1. Means ± SEM are shown from at least four independent experiments. a, significantly different from corresponding control (no drug) at 37°C. c, significantly different at 42°C from corresponding conditions at 37°C, $p<0.05$(*).

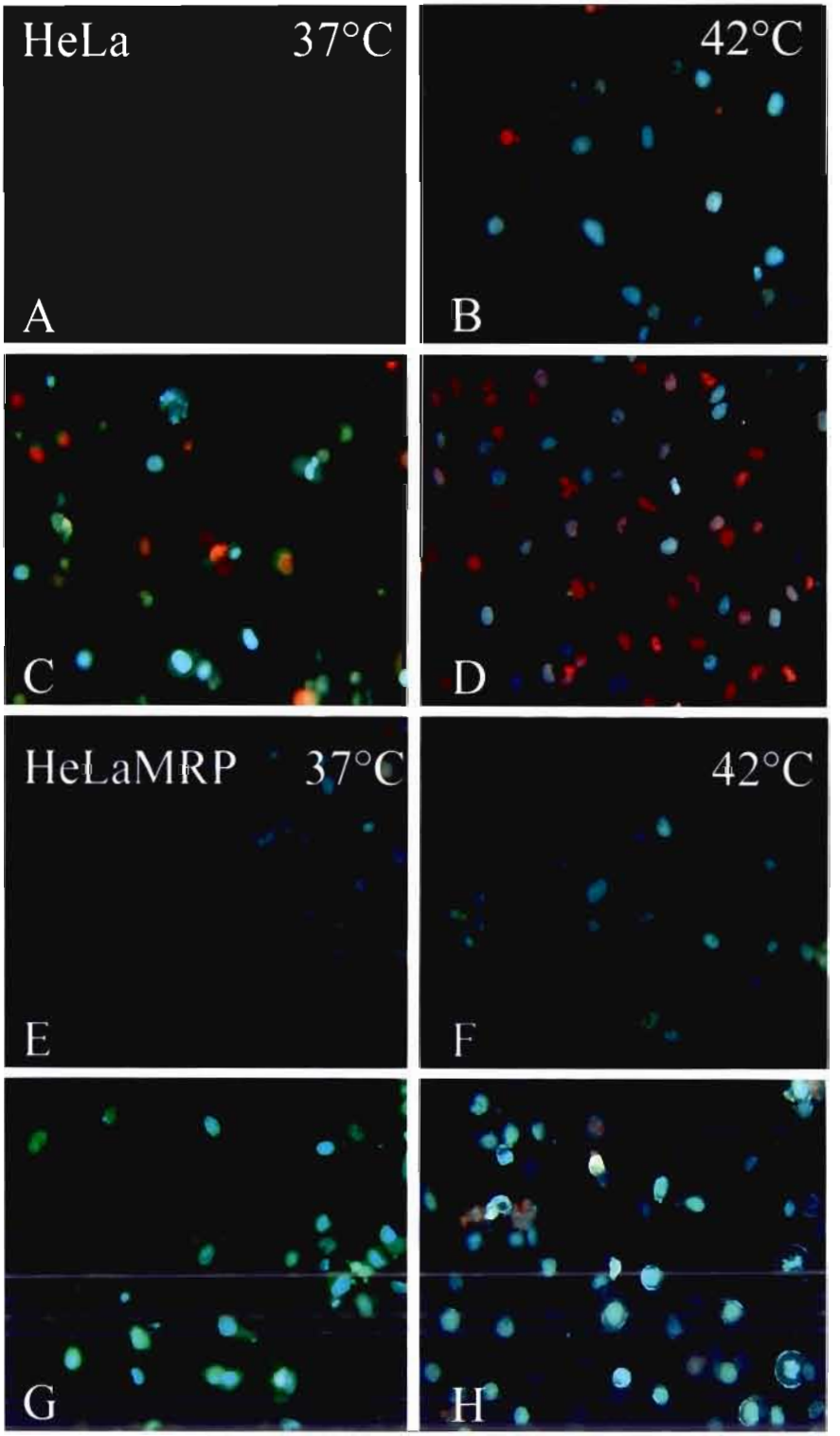


Figure 2.7(A-H)

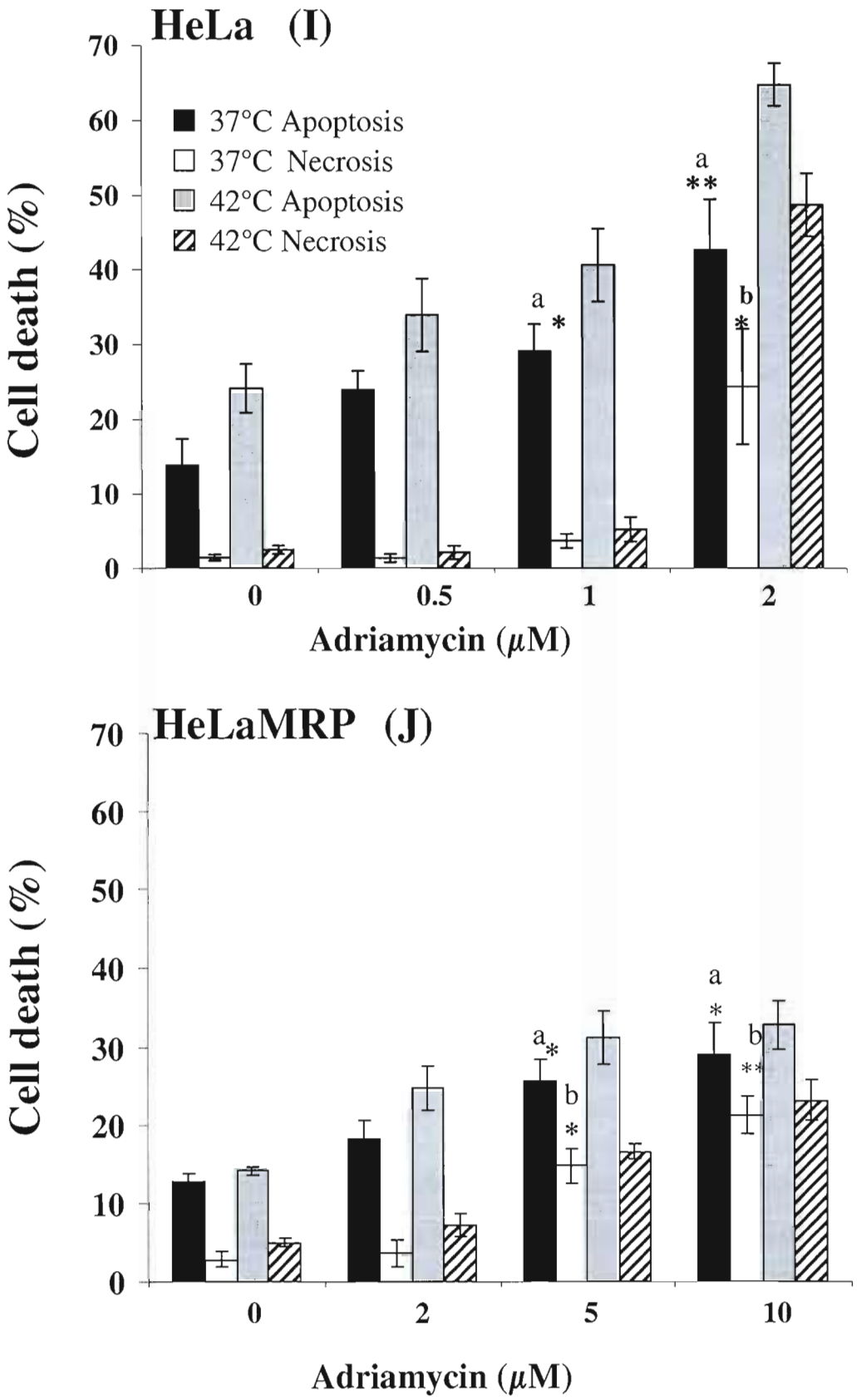

HeLa (I)
37°C Apoptosis
37°C Necrosis
42°C Apoptosis
42°C Necrosis
Cell death (%)
Adriamycin (μM)
HeLaMRP (J)
Cell death (%)
Adriamycin (μM)

Figure 2.7(I-J)

Figure 2.7: Induction of apoptosis by Adriamycin alone or combined with hyperthermia in HeLaMRP and HeLa cells. Cells were incubated with Adriamycin (0 to 2μM for HeLa, 0 to 10μM for HeLaMRP), either alone at 37°C for 18h or combined with 1h of 42°C hyperthermia for the first hour of drug treatment. (A) HeLa cells were incubated for 18h at 37°C or (B) for 1h at 42°C and then for 17h at 37°C, without drug treatment. HeLa cells were incubated with (C) 2μM Adriamycin at 37°C for 18h, or (D) with 2μM Adriamycin for 1h at 42°C and then for 17h at 37°C. (E) HeLaMRP cells were incubated for 18h at 37°C or (F) for 1h at 42°C and then for 17h at 37°C, without drug treatment. (G) HeLaMRP cells were incubated with a 10μM Adriamycin at 37°C for 18h, or (H) with 10μM Adriamycin for 1h at 42°C and then 17h at 37°C. Magnification: 320X. Quantification of images for (I) HeLa and (J) HeLaMRP cells, represents the percentage of apoptotic or necrotic cells, relative to total cells. Means ± SEM shown are from at least four different experiments. a, induction of apoptosis by Adriamycin relative to untreated control at 37°C. b, induction of necrosis by Adriamycin relative to corresponding control at 37°C, $p<0.05$(*) and $p<0.001$ (**). Comparison of curves: For necrosis at 37 versus 42°C in HeLa cells; $p<0.001$. For apoptosis in HeLa versus HeLaMRP cells at 37°C, $p<0.05$.

CHAPTER III

Conclusion

Chemotherapy is among the classical treatments of cancer. However, development of MDR by tumour cells is a major obstacle in the successful use of chemotherapy (Linn and Giaccone, 1995). For instance, fifty percent of human cancers are either totally resistant to chemotherapy or respond only transiently, after which they are no longer affected by commonly used anticancer drugs. Improved understanding of MDR could answer many of the questions and complexities of cancer treatment. Presently there are many strategies in use at the clinical level to combat MDR, although with limited success.

Coadministration of chemosensitizers and anticancer drugs is currently used as a method to overcome MDR. Verapamil, quinine and cyclosporine A are among the first generation of chemosensitizers that can reverse the MDR phenotype by reducing drug efflux from P-gp expressing cells (Georges *et al,* 1990). Several randomized clinical trials have shown benefits with these chemosensitizers. However, their clinical use is often limited due to their toxicity. Second generation agents such as valspodar and biricodar show better impact but were confounded by unpredictable pharmacokinetic interactions and interactions with other transporter proteins. Third generation P-gp inhibitors such as XR9576, LY335979, R101933 and ONT-093 have shown high effectiveness and specificity for P-gp in clinical trials. The continued development of these agents may establish the true therapeutic potential of P-gp-mediated MDR reversal (Thomas and Coley, 2003). To date, reversal of MRP1 mediated drug resistance has received little attention at the clinical level.

Hyperthermia is a recent type of cancer treatment in which body tissue is exposed to temperatures which are several degrees above body temperature (up to 45°C). The extensive amount of biological *in vitro* and *in vivo* experimental research on hyperthermia during the last two decades has established it to be a valuable new tool in cancer therapy (Liu and Wilson, 1998). Hyperthermia, usually with nominal injury to normal tissues, can damage and kill cancer cells by damaging proteins within the cells leading to the shrinkage of tumours (van der Zee, 2002; Hildebrandt et al., 2002). This fact has been supported by the work of Fajardo, who proved that most normal tissues are undamaged by treatment for 1h at temperatures of up to 44°C (Fajardo, 1984). Several studies using hyperthermia at the clinical level have demonstrated complete overall response rates of 13% (Hetzel *et al.,* 1987).

For hyperthermia in combination therapy, a large amount of medical evidence demonstrates remarkable improvement in response rates when it is used combined with radiation therapy and chemotherapy (Wust et al., 2002). Several phase I and II clinical studies have used combinations of hyperthermia and drug treatment. These studies have reported good responses for treatment of limb melanoma (Fraker, 2004), pelvic tumors (Hildebrandt et al., 2004; Rietbrock et al., 1997), intra-peritoneal metastases (Alexander and Fraker, 1996) and other refractory tumors (Wiedeman et al., 1997). Several phase II studies on hyperthermia in combination with pre- and/or postoperative chemotherapy in high-risk sarcomas have demonstrated quite impressive 5-year overall survival rates (Issels et al., 2001; Wendtner et al., 2001). Hyperthermia combined with cisplatin chemotherapy also reveals promising results, particularly in recurring cervical cancer, following irradiation (De Wit et al., 1999; Rietbroek et al, 1997). Van der Zee and coworkers found promising results for the added effect of hyperthermia on radiotherapy in advanced tumors of the bladder, cervix and rectum (Van der Zee et al., 2000). Literature reviews regarding respond rates of addition of hyperthermia to radiotherapy in tumor of breast cancer, malignant

melanoma and neck nodes suggest a clinical thermal enhancement ration of 1.5 to 1.7 (Review: Van der Zee, 2002).

Our previous studies at the cellular level demonstrated that hyperthermia combined with chemotherapy and chemosensitizers could be a useful strategy to combat the multidrug resistance phenotype mediated by P-gp (Turcotte and Averill-Bates, 2001; Larrivée and Averill, 2000, 1999; Averill and Su, 1999; Averill and Larrivée, 1998; Bates and Mackillop, 1990). Our findings demonstrated that over expression of P-gp can be modulated by hyperthermia combined with melphalan and ethacryinc acid, verapamil and cyclosporine A (Turcotte and Averill-Bates, 2001; Averill and Larrivée, 1998; Larrivée and Averill, 2000). When hyperthermia was used in combination with cyclosporine A in multidrug resistant $CH^{R}C5$ Chinese hamster ovary cells, this caused an increase in melphalan uptake and a decrease in melphalan efflux out of MDR cells, leading to an overall increase in intracellular drug accumulation. The combined effect of hyperthermia and verapamil increases intracellular accumulation of drugs such as Adriamycin in certain multidrug-resistant cell lines. This combination alters membrane permeability to Adriamycin and consequently enhances the cytotoxicity of the drug.

Recent data showed that hyperthermia administered with appropriate scheduling with drug caused a modest increase in etoposide-induced apoptosis in both the drug sensitive parental cell line (e.g. HeLa) and multidrug resistant (e.g. Hela-MRP) cells (Souslova and Averill-Bates, 2004). Cytotoxicity measurements showed that folated liposomes combined with hyperthermia were found to be over 3-fold more effective than the free drug (Adriamycin) for growth inhibition of human cervical carcinoma derived KB31 cells (Gaber, 2002). In an effort to overcome chemoresistance of human malignant glioma cells, Hermisson and Weller (2000) analyzed the modulation of drug-induced cell death by hyperthermia in 4 human malignant glioma cells lines, LN-18, LN-229, T98G and U87MG. The results of this study showed that hyperthermia might be a useful approach to overcome

chemoresistance of these cell lines. Although hyperthermia does not reverse resistance in MRP1-HeLa cells, we were able to induce apoptosis independently of the susceptibility of the cell line or treatment, and therefore hyperthermia renders resistant cells a bit more susceptible to the drug. However, the magnitude of cell death in MRP1-HeLa cells is still much lower than for drug sensitive cells (and higher drug concentrations are still required).

The findings of the present study show that Adriamycin activated the mitochondrial pathway in HeLa cells and that 42°C hyperthermia caused an increase in Adriamycin induced apoptosis. This study also evaluated the effect of hyperthermia combined with Adriamycin on the induction of apoptosis in MDR cells with overexpression of MRP1. In this study, we used higher concentrations of Adriamycin for induction of apoptosis in HeLa-MRP cells, which can be explained by the fact that these cells are resistant to cytotoxicity induced by Adriamycin, compared to the parental cells. This is in agreement with findings that HeLaMRP cells exhibit resistance to actinomycin D, etoposide, vincristine and Adriamycin induced cytotoxicity (Kast and Gros, 1998). Adriamycin-induced apoptotic cell death was accompanied by caspase-9 and caspase-3 activation, as well as the cleavage of the caspase-3 substrate ICAD, which was preceded by mitochondrial cytochrome c release. These results suggest that the release of mitochondrial cytochrome c and the sequential activations of caspase-9 and caspase-3 are important events in the signal transduction pathway of Adriamycin-induced apoptotic cell death in both HeLa and HeLaMRP cells.

We found that hyperthermia alone was equally effective in inducing apoptosis in HeLa and HeLaMRP cells. This finding is extremely important since the MDR cells are sensitive to heat killing. Therefore, hyperthermia could be and effective strategy for eliminating MDR cells (Bates and Mackillop, 1986). The effect of hyperthermia was investigated by Zang and coworkers, in MDR mediated by P-gp in K562/ADM and Tca8113 cell lines. The result of this study was that 41°C

hyperthermia reduced MDR1 and MRP expression and enhanced intracellular drug concentration (Zang *et al.,* 2003). There are several possible explanations for that, such as Adriamycin is known to generate oxidative stress with subsequent formation of lipid peroxidation products (Gille and Nohl, 1997; Ollinger and Brunmark, 1994). Hyperthermia alone is able to cause oxidative stress by generation of ROS in cells (Lord-Fontaine and Averill, 1999; Flanagan, 1998; Lin, 1991) and an imbalance in the cellular redox equilibrium by depletion of cellular thiols (Mitchell and Russo, 1983). GPx and GSH are enzymes which protects the membrane lipids against oxidation (Halliwell and Gutteridge, 1999). To exacerbate the redox imbalance, activity of the antioxidant enzyme GPx was also lower and additionally level of glutathione (GSH) was depleted in HeLaMRP cells (Souslova and Averill-Bates, 2004). GSH is a critical factor in MRP1-mediated drug resistance (Versantvoort *et al.,* 1995; Zaman *et al.,* 1995). It plays an important role in detoxification of ROS, electrophiles and oxyanions either by reduction or conjugation (Wang, 1998; Meister, 1994; Meister and Anderson, 1983). Hyperthermia possibly caused more depletion of GSH in HeLaMRP cells, therefore it would be more vulnerable to the oxidative stress caused by Adriamycin. This finding can be supported by several studies on the influence of GSH redox pathway on cell sensitivity to Adriamycin have shown that human ovarian cancer lines (Hamilton *et al.,* 1985), MCF-7 breast tumour cells (Dusre *et al.,* 1989) and HL-60 leukemic cells (Raghu *et al.,* 1993) were sensitized to Adriamycin through depletion of GSH.

In conclusion, this study demonstrates clearly that hyperthermia could be useful as a modifier of multidrug resistance mediated by MRP1. Adriamycin can cause cell death by the mitochondrial pathway of apoptosis involving cytochrome c release from the mitochondria and the activation of caspases in human cervical adenocarcinoma cells (HeLa and HeLaMRP). Based on cellular studies, hyperthermia combined with Adriamycin could be a useful strategy for elimination of tumour cells

at the clinical level. However further investigation of mechanisms of toxicity induced by heat and drugs are required.

Future perspectives

We have found that Adriamycin can cause cell death by the mitochondrial pathway of apoptosis involving cytochrome c release from the mitochondria and the activation of caspases. However, the possible involvement of the death receptor pathway of apoptosis should also be taken into account. Future perspectives of our study will be the investigation of the role of death receptors in Adriamycin-induced apoptosis. Apoptosis could also occur via death receptor pathways without the involvement of cytochrome c. With respect to the death receptor-mediated pathway, Adriamycin was shown to increase apoptosis by recombinant Fas ligand (rFsaL) in neonatal rat cardiomyocytes (Yomaoka *et al.,* 2000).

To determine the involvement of death receptor mediated cell death by Adriamycin alone and combined with hyperthermia in HeLa and HeLaMRP cells, the activity of initiator caspase-8 was measured. Hyperthermia induced the activation of caspase-8 (Fig. 1A) relative to unheated control cells at 37°C. Activation of caspase 8 was observed at temperatures of 41-45°C in HeLa and HeLaMRP cells (Fig 1A). HeLaMRP cells were slightly less sensitive to heat (Fig. 1A). Adriamycin (0-2μM for HeLa, 0-10μM for HeLaMRP cells) increased caspase-8 activity in HeLa cells but caspase-8 activity was decreased by Adriamycin in HeLaMRP cells (Fig. 1B, C). Hyperthermia was unable to increase caspase-8 activation by Adriamycin in HeLaMRP cells. Future studies will determine the reasons for differing responses of caspase-8 in Adriamycin treated HeLa and HeLa MRP cells.

The MRP-transfected HeLa cells display an increase in resistance to Adriamycin that is proportional to the levels of integral membrane MRP expression. Overexpression of the human MRP protein causes a form of multidrug resistance similar to that conferred by P-glycoprotein, although the two proteins are only distantly related. In contrast to P-glycoprotein, human MRP has also been shown to be a primary active transporter of a structurally diverse range of organic anionic conjugates, some of which may be physiological substrates. Consequently, the use of higher concentrations of Adriamycin may lead to the induction of apoptosis.

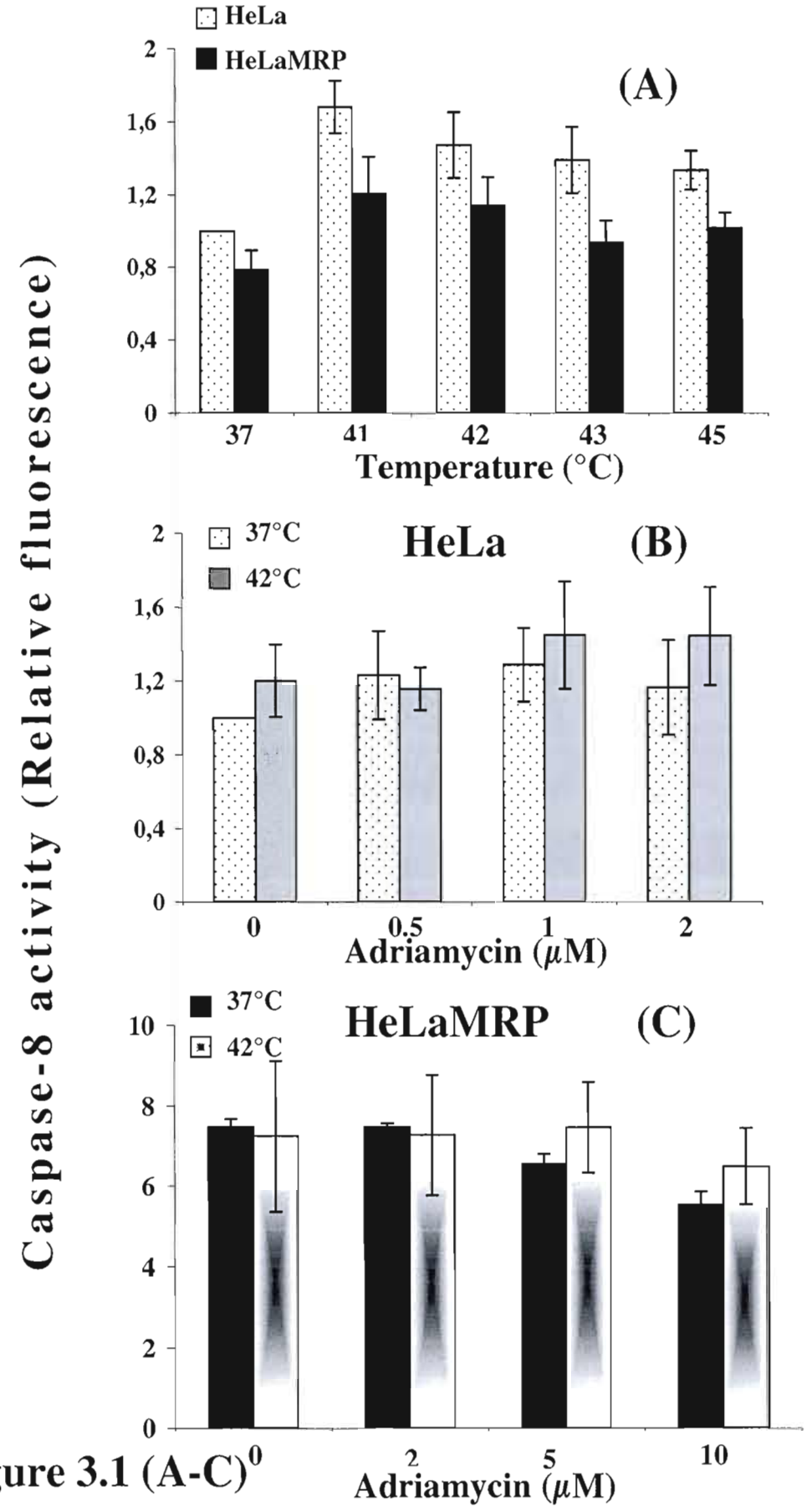

HeLa
HeLaMRP
(A)
2
1,6
1,2
0,8
0,4
0
37
41
42
43
45
Temperature (°C)
37°C
42°C
HeLa
(B)
0
0.5
1
2
Adriamycin (μM)
37°C
42°C
HeLaMRP
(C)
10
8
6
4
2
0
0
2
5
10
Adriamycin (μM)
Caspase-8 activity (Relative fluorescence)

Figure 3.1 (A-C)

Figure3.1: Activation of caspase 8 by hyperthermia and/or Adriamycin. (A) HeLa (▨) and HeLaMRP cells (■) were heated (37 to 45°C) for 2h. HeLa (B) and HeLaMRP (C) cells were incubated in monolayer with different concentrations of Adriamycin for 18h at 37°C or for 1h at 42°C and 14h at 37°C. Activities of caspase-8 were expressed relative to untreated HeLa cells at 37°C, designated as 1.0. Means ± SEM are shown from at least three independent experiments.

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