PHARMACOLOGICAL INTERVENTIONS IN HEAT STRESS BASED ON AN ANIMAL MODEL

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Acknowledgements

Extending the capability of a lab with a new project is no easy task, especially when you are on your own. This project gave me the opportunity to immerse myself completely into the world of biomedical research, something that has helped to chart my course in life. All of this would not be a reality today without the help of my supervisors, colleagues and family. I would like to give my heartfelt thanks to Assistant Professor Kerwin Low Siew Yang for his support, suggestions, encouragement, guidance, concern and his help in reviewing the manuscript and making time for me, despite his hectic schedule; to Associate Professor Shabbir M Moochhala for his guidance, encouragement, words of inspiration and for the access to the equipment at the Defence Medical Research Institute (DMRI) laboratory; to Ms Shoon Mei Leng for her support, advice, assistance and concern; to my colleagues at the Department of Pharmacology and DMRI for their suggestions, assistance and kind advice; to my family for putting up with my requests and being so understanding; to Billy *le loup*, for just being there; and especially to Trixie Ann for her care, concern, patience and love.

Summary

It is known that heat shock proteins are able to reduce the degree of injury sustained by tissues following exposure to heat stress. This study looked into the use of a suitable pharmacological agent to induce heat shock proteins in an animal model, hence conferring thermotolerance to the animal. This was done in tandem with the development of a suitable model of heat stress. As restraint was a known inducer of heat shock proteins, a free moving animal model was designed, utilizing the CorTemp temperature sensor.

In order to verify that the CorTemp sensor was as effective as the more commonly used conventional rectal probes, rats were either implanted with the sensor or rectal probe and were then exposed to a heat stress of 45^{o}C for 25 minutes. Rats implanted with the CorTemp sensors were free moving, while those with the rectal probes had to be restrained. Results showed that there were no statistically significant differences in the recorded temperatures during heat stress exposure. However, rats in the free moving animal model were able to cool faster, compared to those that were restrained. The free moving rats were able to cool themselves by various behavioral responses to heat stress, such as lying prostate and spreading their saliva about themselves. Hence the use of the CorTemp sensors in the free moving model of heat stress proved to be effective in measuring temperature, as well as permitting the animals to carry out their natural behavioral responses, unlike those in restraint.

Herbimycin A, a benzoquinoid ansamycin antibiotic, was shown to be capable of inducing the production of heat shock proteins in rat tissues. Hence the hypothesis that herbimycin A was able to induce hsp70 in a rat model, and subsequently protect the animal

from exposure to heat stress was studied. The results from western blot studies showed that herbimycin A was capable of inducing hsp70 to peak levels in the liver, lung, heart and kidney tissues of the rat, 12 hours post IP administration. Densitometry data showed that the overexpression of hsp70 by herbimycin A was significantly greater than that from vehicle and saline treated rats. Rats exposed to heat stress 12 hours post herbimycin A administration showed significantly lower peak core temperatures, compared to vehicle and saline treated rats. Histological studies using H&E staining in tissues collected from animals 24 hours after exposure to heat stress showed no major morphological changes in all the four tissues, for all three treatment groups. However, TUNEL stains of the same tissues collected the same time showed a greater degree of apoptotic nuclei ($P < 0.05$) in the tissues of the vehicle and saline treated rats, compared to herbimycin A treated rats. From western blotting and densitometry results, it was observed that caspase 3 activation was greater in the liver, lung, heart and kidney tissues of the vehicle and saline treated rats, compared to herbimycin A treated rats, 24 hours after heat stress. Hence it can be seen that herbimycin A was able to reduce the degree of apoptosis in these tissues following heat stress, unlike the vehicle and saline treated rats. The findings of this study thus support the hypothesis that herbimycin A is able to induce hsp70 in a rat model, and subsequently protect the rat from heat stress holds.

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List of Abbreviations

AIDS	acquired immunodeficiency disease syndrome
ARDS	adult respiratory distress syndrome
AIF	apoptosis inducing factor
Apaf-1	apoptotic protease activation factor 1
ATP	adenosyl triphosphate
ADP	adenosyl diphosphate
C	carboxyl (terminus)
CARD	caspase recruitment domain
CIOMS	Council for International Organization of Medical Sciences
CRC	Clinical Research Center
DD	death domain
DED	death effector domains
DMSO	dimethyl sulphoxide
EGF	endothelial growth factor
FADD	Fas associated death domain protein
HSE	heat shock elements
HSF	heat shock factor
HSBP1	heat shock factor binding protein 1
HSP	heat shock proteins
H & E	hematoxcylin & eosin staining

HRP	horseradish peroxidase
HIV	human immunodeficiency virus
IAPs	inhibitory apoptotic proteins
IP	intra peritonealy
IL-1	interleukin 1
JNK1	c-Jun N-terminal Kinase
N	amino (terminus)
NSAIDs	Nonsteriodal anti-inflammatory drugs
NFκB	nuclear factor kappa b
POAH	preoptic anterior hypothalamus
PG	prostaglandins
Smac	second mitochondria derived activator of caspase
TNF- α	tumor necrosis factor-α
TRADD	TNF receptor death domain protein
TUNEL	terminal transferase-mediated d-UTP nick end labeling
UTR	untranslated regions

Chapter 1

Introduction

1.1 Thermoregulation and hyperthermia

Body temperature is a balance between heat production and heat dissipation. Heat is generated internally as a byproduct of metabolism. When the ambient temperatures exceed body temperature, heat is also taken in from the external environment (Simon, 1993). Exposure to heat stress can result in the activation of thermoregulatory centers in the brain and spinal cord. There is general agreement that the primary control of body temperature in mammals lies at the preoptic anterior hypothalamus (POAH), with its array of thermo sensitive neurons that receive afferent neural input from cold and warm sensors in both the periphery and other parts of the central nervous system (Adler and Geller, 1988). These centers in turn activate appropriate physiological and behavioral responses to maintain the core temperature at the temperature set point designated at the hypothalamus, which is usually fixed at a level of approximately 37^{o}C. The responses include cutaneous vasodilation to transfer heat from the body core to the body surface by circulation and evaporative heat loss from the body surface.

Body temperature increases when the rate of heat production exceeds the rate of heat dissipation. Hyperthermia occurs when thermoregulatory mechanisms are overwhelmed by excessive metabolic production of heat, excessive environmental heat, or impaired heat dissipation. In hyperthermic states, the hypothalamic set point is normal but peripheral mechanisms are not able to maintain a body temperature that matches the set point. In contrast, fever occurs when the hypothalamic set point is increased by the action of circulating pyrogenic cytokines, causing peripheral mechanisms to conserve and generate heat until the body temperature rises to the elevated set point (Dinarello et al, 1988). Under certain conditions of hyperthermia, when the heat loss responses are unable

to cope with the external heat load, core body temperature rises, leading to the occurrence of a possible clinical heat stroke (Alzeer et al., 1999).

Heat stroke is a systemic disorder characterized by neurological abnormalities, such as delirium, convulsions, coma, usually in combination with multiple organ failure, hemorrhage and necrosis in the heart, liver, kidney and brain, often resulting in death (Simon, 1993). Heat stroke has played a sad role in human history. Examples include the failure of a Roman military expedition to North Africa due to an outbreak of heat stroke in 24 BC to recent reports of athletes, miners, urban dwellers, and soldiers all suffering a significant morbidity during periods of heat stress (Romanovsky and Blatteis, 1998).

1.2 Hyperthermia treatment

Despite the apparent clinical significance of hyperthermic disorders, the arsenal of therapeutic measures used in heat stress to control the patient's body temperature is usually limited to physical cooling. Immersion in ice water, with or without massage was shown to be effective in rapid cooling of the body (Costrini, 1990). In the case of exertional hyperthermia, administration of intravenous fluids is able to reduce core temperature, and at the same time, replenish the dehydrated body with much needed fluids (Shapiro and Seidman, 1990). However, it should be noted that employing cooling would shift blood from the dilated peripheral vessels to the center, so that overenthusiastic fluid replacement may lead to circulatory overload. Hence it is recommended that infusion should be avoided until the effect of cooling has been observed (Knochel, 1989).

Besides employing physical means to lower elevated body temperatures, drugs have played an important role in this aspect as well. Nonsteriodal anti-inflammatory drugs (NSAIDs) are able to inhibit the synthesis of prostaglandins, following exposure to

pyrogens, both exogenous and endogenous. Hence NSAIDs are able to effectively arrest a pyretic febrile response (Vane, 1971). During conditions of hyperthermia, no resetting of the set point in the hypothalamus takes place. Thus the rise in body temperature observed in hyperthermia is not due to a change in set point, unlike in a febrile response (Simon, 1993). Hence, the use of NSAIDs cannot be expected to attenuate the rise in body temperature during heat stress. Dantrolene sodium, the preferred drug for the treatment of malignant hyperthermia, acts directly on skeletal muscle (Britt, 1984). It is thought to increase calcium intake, or inhibit its release, through the sarcoplasmic reticulum, which reverses muscle rigidity and consequently, body heat production (Britt, 1984). However, it has also been shown that dantrolene sodium is not generally effective in the treatment of heat stroke in dogs (Amsterdam et al, 1986) or humans (Bouchama et al, 1991a).

Recently, it was demonstrated that heat shock proteins (HSP) expression was able to protect against death following heatstroke in rats (Yang et al, 1998). It has been suggested that HSPs play a role in thermotolerance and acclimatization, and recently their use is gaining ground, both as a treatment for hyperthermia and other disease states (Morimoto and Santoro, 1998).

1.3 Heat shock proteins

The observation that an increase in temperature of a few degrees above the physiological level induces the synthesis of a small number of proteins in *Drosophila* salivary glands led to the discovery of a universal protective mechanism which prokaryotic and eukaryotic cells utilize to preserve cellular function and homeostasis (Linquist and Craig, 1988). This complex physiological defense mechanism, known as the heat shock

response, involves the rapid induction of a specific set of genes encoding cytoprotective proteins, also known as HSPs (Santoro, 1999).

HSPs are highly conserved, ubiquitous, and abundant in nearly all sub-cellular compartments. They are divided into different families, that which exhibit apparent masses of approximately 8, 28, 58, 70, 90 and 110 kDa (Welch, 1992). HSPs consist of both stress inducible and constitutive family members. Constitutively synthesized HSPs perform housekeeping functions. For example, they function as molecular chaperones by helping nascent polypeptides assume their proper conformation. HSPs are also involved in antigen presentation, steroid receptor function, intracellular trafficking, nuclear receptor binding and apoptosis (Kiang & Tsokos, 1998; Sharp et al., 1999). Inducible HSPs prevent protein denaturation and incorrect polypeptide aggregation during exposure to physiochemical insults, such as an elevation of temperature, and stressors such as heavy metals (arsenite, cadmium), ethanol, oxygen radicals, and peroxides (Lindquist, 1986; Minowada & Welch, 1995). Exercise (Locke et al, 1990) and restraint (Udelsman et al, 1993) have also been shown to induce the expression of HSPs in mammals.

Initially, stress-induced HSP accumulation was associated with thermotolerance, the ability to survive otherwise lethal heat stress, and later with tolerance to a variety of stresses, including hsp27 and hsp70 in ischemia (Marber et al, 1995), hsp 64 in ultraviolet irradiation (Barbe et al, 1988), and cytokines such as tumor necrosis factor-α (TNF- α) (Jaattela & Wissing, 1993). The fact that overexpression of various HSPs, such as hsp8 which plays a role in denatured protein removal and hsp90 which inhibits short term protein synthesis (Welch, 1992), confers tolerance in the absence of conditioning stress and that inhibition of HSP accumulation through blocking antibodies impairs stress tolerance strongly support the hypothesis that HSPs themselves confer the stress tolerance.

The mechanism by which the HSPs confer stress tolerance is not completely understood but may relate to the important role of HSPs in the processing of stress-denatured proteins (Mizzen & Welch, 1988). HSPs are also thought to manage the protein fragments occurring as the result of stress-induced arrest of protein translation, during protein synthesis (Chirico et al, 1988; Palleros et al, 1991). The maintenance of structural proteins may also be a key to HSP-associated stress tolerance. For example, hsp27 prevents actin microfilament disruption under stress conditions (Lavoie et al, 1993). This effect on the cytoskeleton may be important not only in individual cell tolerance to stress through cytoskeletal stabilization but may also be integral to the protection of the whole organism through the maintenance of endothelial and epithelial barrier functions.

1.4 Thermotolerance vs. acclimatization

The ability of the HSPs to confer thermotolerance in both cultured cells and in animals is well documented (Li, 1985; Weshler et al, 1984). Thermotolerance refers to an organism's ability to survive an otherwise lethal heat stress from a prior heat exposure sufficient to cause the cellular accumulation of HSPs. Regardless of stimuli, the features of thermotolerance are essentially the survival of the cell or organism exposed to an otherwise lethal heat stress, in conjunction with the synthesis of HSPs. The thermotolerant state lasts for a relatively short duration, often in the range of hours to days, and it correlates with the presence of elevated cellular HSPs and declines with the decrease in HSPs over time. The requirement of HSPs for thermotolerance and the role of HSPs in protein folding, assembly, and transport support the hypothesis that the thermotolerant state is dependent on one or all of these HSP-related functions, especially through the management of both denatured proteins and of partially synthesized protein fragments.

In marked contrast to thermotolerance, heat acclimatization refers to the organism's ability to perform work in elevated environmental temperatures as well as to continue work under elevated but non-lethal core temperatures. Unlike thermotolerance, where cell or organism survival is the measured end point, acclimatization is determined through a work heat-tolerance test demonstrating the organism's ability to achieve and maintain thermal equilibrium at a given work rate in the heat. In addition, heat acclimatization results from a series of elevations in core temperature, generated by performing work in the heat (Baum et al, 1976; Fruth et al, 1983). Passive hyperthermia is normally associated with only partial acclimatization. Unlike thermotolerance, which undergoes a rapid decay correlating with a decline in HSPs, heat acclimatization can be maintained for prolonged periods as long as the organism continues to undergo periodic elevations in core temperature. Finally, it should be noted that unlike thermotolerance, there is no cellular model of heat acclimatization.

Heat acclimatization not only reduces resting core temperature and provides for greater heat transfer to the skin or heat-dissipating capacity but also allows the organism to tolerate a higher core temperature. Increased heat dissipation occurs through systemic alterations including a decrease in sweating threshold, an increased sweating output at a given core temperature, a reduced threshold for cutaneous vasodilation, and greater skin blood flow at a given core temperature (Baum et al, 1976; Fruth et al, 1983; Nadel et al, 1974). The ability to work at higher core temperatures seen in both rats (Fruth et al, 1983) and humans (Maron et al, 1977; Pugh et al, 1967), however, mirrors the thermotolerant state and suggests that cellular mechanisms of adaptation such as those related to HSPs may be at work.

1.5 Hsp70

The 70kDa HSP (hsp70) is one of the most extensively studied HSP, whose structure has been widely conserved through evolution from bacteria to man, hence indicating an important role in the survival of the organism (Linquist and Craig, 1988; Morimoto and Santoro, 1998). Included in this family are the hsc70 (heat shock cognate, the constitutive form), hsp70 (the inducible form, also known as hsp72), grp75 (a constitutively expressed mitochondrial glucose regulated protein) and grp78 (a constitutively expressed glucose regulated protein found in the endoplasmic reticulum) (Welch, 1992).

All members of the *hsp*70 family of proteins contain two major domains, namely an ATP (adenosyl triphosphate) binding site at the N (amino) terminus and a peptide binding region at the C (carboxyl) terminus of the molecule. The ATP binding site, which is associated with a weak ATPase activity, is the most conserved region of the different *hsp*70 members, as well as *hsp70s* of different species (Morimoto, 1991; Mckay et al, 1994). Additionally, hsp70 has a high affinity for hydrophobic peptides, and this affinity is increased after hydrolysis of ATP (Hightower and Sadis, 1994). It is proposed that hsp70 associates with ATP and binds to the hydrophobic domains of proteins. The binding affinity is increased by the hydrolysis of ATP into ADP (adenosyl diphosphate), prolonging the time of the hsp70-polypeptide interaction. The ATPase activity of hsp70 is apparently stimulated by other HSPs such as hsp40 (DnaJ) (Liberek et al, 1995). After the exchange of ADP for ATP, which is enhanced by another molecular chaperone, GrpE (Georgopoulos, 1992), the peptide is released, and the cycle begins again (Hightower and Sadis, 1994). The released polypeptide is directed to the cellular protein folding pathway that is composed of several other chaperone proteins. The fact that the interaction between

hsp70 and polypeptides is reversible is of crucial importance to the process of folding and translocation.

During stress, hsp70 seems to interact with the hydrophobic domains of proteins that become exposed as a consequence of the insult, such as an elevation in temperature. When cells return to normal conditions, these denatured polypeptides fold back or are targeted to the proteolytic subcellular compartments. There are several examples in which HSPs are capable of refolding artificially denatured proteins. It was demonstrated that heat inactivated RNA polymerase could be reactivated by the cooperation of the bacterial hsp70 (Dnak) and the two chaperones hsp40 (DnaJ) and GrpE (Skowyra et al, 1990; Ziemienowicz et al, 1993).

At the level of gene organization, the *hsp*70 family in humans is complex. The constitutive forms, hsc70 and grp78, are encoded by single genes. However, there are several genes that contain sequences encoding the inducible form of the *hsp*70 family. The fact that many genes encode for the same polypeptide may indicate the vital importance of this gene product. There are three copies of hsp70 in chromosome 6, two in chromosomes 1 and 14, and one in chromosome 5. The highest homology is observed between genes in the same chromosome, suggesting that they may be derived from gene duplication. The hsp70 gene contains a single exon, whereas hsc70 is composed of several exons (Wu et al, 1985; Tavaria et al, 1995). Multiple copies of the hsp70 gene have also been observed in other species, such as yeast (Ingolia et al, 1982), fruit fly (Holmgren et al, 1979), mouse (Lowe and Moran, 1986), and rat (Fagnoli et al, 1990). Another interesting characteristic of the *hsp*70 family is that they have a high degree of homology in the sequence containing the open reading frame, whereas the untranslated regions (UTR) are considerably different. This observation may be important in the regulation of these genes.

1.6 Regulation of heat shock response

HSPs appear to play a direct role in the autoregulation of the heat shock response. In eukaryotic cells, heat regulation of HSP genes requires the activation and translocation of heat shock factor (HSF), a transregulatory protein. HSF recognizes the modular sequence elements referred to as heat shock elements (HSE), located within the HSP gene promoter (Wu, 1995; Morimoto et al, 1996). An HSF multi gene family has been identified in vertebrates, and at least three HSFs (HSF1-3) have been isolated from the human, mouse, and chicken genomes, while an additional factor, HSF4, has been described in human cells (Rabindran et al, 1991; Sarge et al, 1991; Schuetz et al, 1991; Nakai & Morimoto, 1993; Nakai et al, 1997). HSFs from different organisms share a number of structural features, including a conserved DNA-binding domain, which exhibits a winged helix-turn-helix motif, located near the amino terminus (Harrison et al, 1994).

In mammalian cells, HSFs are co-expressed, negatively regulated, and activated upon specific environmental and physiological events (Morimoto et al, 1996; Voellmy, 1996). HSFs 1 and 3 function as stress-responsive activators and both are required for maximal heat shock responsiveness (Tanabe et al, 1998), whereas HSF2 is activated during embryonic development and differentiation (Sistonen et al, 1992; Schuetz et al, 1991). HSF4 was discovered in human cells and appears to be preferentially expressed in the human heart, brain, skeletal muscle, and pancreas (Nakai et al, 1997). Unlike the other HSFs, HSF4 constitutively binds to DNA, but lacks the properties of a transcriptional activator, and it has been suggested to be a negative regulator of the heat shock response (Nakai et al, 1997). The presence of different HSFs in larger eukaryotes suggests that interaction among these factors may play an important role in the protection of complex

organisms that are exposed to diverse forms of developmental and environmental changes. In larger eukaryotes, HSF1 is present in both unstressed and stressed cells. However, in the absence of stress, HSF1 is expressed as an inert monomer bound to hsp70 and other chaperones, and it lacks transcriptional activity (Morimoto et al, 1996; Shi et al, 1998). Both the DNA-binding activity and the transcriptional transactivation domain are repressed through intramolecular interactions and constitutive serine phosphorylation (Morimoto et al, 1996; Voellmy, 1996).

How is it that eukaryotic cells are able to sense a change in environmental temperature and activate HSF1? It is commonly held that the stress signal is the consequence of the flux of non-native proteins which, in turn, results in the cellular requirement for molecular chaperones, including hsp70, hsp90, and the co-chaperone Hdj1, to prevent the appearance and aggregation of misfolded proteins (Fig. 1). Chaperones bound to HSF1 would then be sequestered by cellular damaged proteins. As a consequence of the appearance of non-native proteins and release of interacting chaperones, HSF1 DNA-binding activity is de-repressed and monomers oligomerize to a trimeric state, translocate to the nucleus where they become inducibly phosphorylated at serine residues, and bind to HSE located upstream of HSP genes, resulting in stress-induced transcription (Morimoto et al, 1996; Voellmy, 1996). Inducible phosphorylation appears to be essential for transcriptional activation. For example, chemicals such as salicylates and the NSAIDs aspirin and indomethacin cause HSF1 trimerization, nuclear translocation, and binding to the HSE of the endogenous hsp70 gene; however, they are unable to trigger HSF1 phosphorylation, thus inducing a transcriptionally inert DNA-binding trimeric state, where expression of HSP genes is not detected (Lee et al, 1995; Amici et al, 1995; Cotto et al, 1996). On the other hand, salicylate or NSAID-treated cells

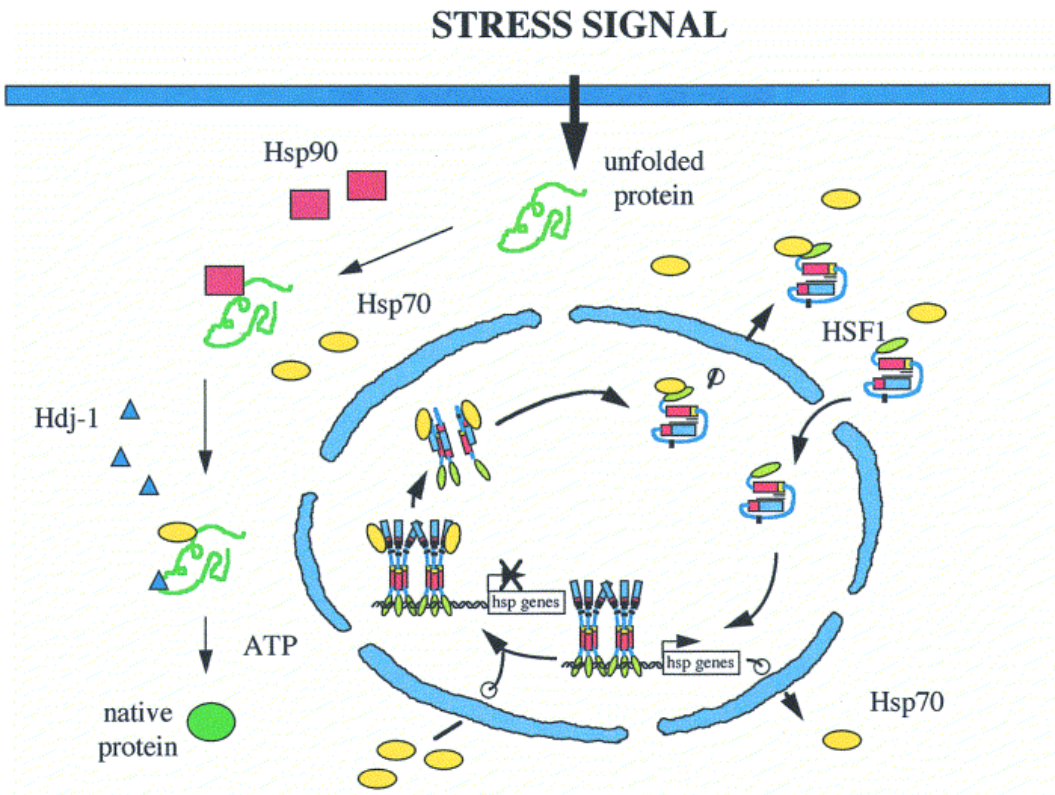


Fig. 1. Stress Response (adapted from Santoro, 2000).

are primed for subsequent exposure to heat shock and other stresses, leading to the enhanced transcription of heat shock genes (Lee et al, 1995; Amici et al, 1995). Moreover, alterations of HSF1 phosphorylation by exposure to the calcium ionophore A23187 lead to inhibition of HSP gene expression (Elia et al, 1996). Whereas inducible phosphorylation is believed to be important for transcriptional activation (Morimoto et al, 1996), the kinase (or kinases) involved is still unknown. The identification of the signaling pathway controlling this activity would be a major advance in the understanding of the regulation of the heat shock response in mammalian cells. As the synthesis of HSP increases to levels proportional to the appearance of non-native proteins, hsp70 and other chaperones relocalize to the nucleus and bind to the HSF1 transcriptional transactivation domain, thereby repressing transcription of heat shock genes (Morimoto & Santoro, 1998; Shi et al, 1998). Attenuation of the heat shock response is also dependent on the negative regulatory effects of heat shock factor binding protein 1 (HSBP1), which binds to the region of HSF1 corresponding to the heptad repeat, leading to dissociation of the trimers and refolding to the inert monomeric state, thus completing the cycle (Satyal et al, 1998).

Whereas HSF1 is considered the rapidly activated stress-responsive factor, the co-expressed HSF2 is activated in response to distinct developmental cues or differentiation stimuli. HSF2 was shown to be converted from an inert dimer to an active trimer during hemin-induced erythroid differentiation in K562 human erythroleukemia cells (Sistonen et al, 1992). Unlike the rapid activation and attenuation of HSF1, HSF2 requires a period of 16 to 24 hours to be activated and remains in the trimeric activated state through 72 hours.

Like HSF2, chicken HSF3 is also found as an inert dimer; however, HSF3 shares many characteristics with HSF1, such as negative regulation, activation to trimer, and sequence-specific binding to HSE (Nakai & Morimoto, 1993). HSF3 is activated mainly

upon exposure to extreme temperatures and under conditions of severe stress, and its kinetics of activation exhibits a delayed response as compared to HSF1 (Nakai & Morimoto, 1993; Tanabe et al, 1998). As anticipated above, HSF3 appears to be an important co-regulator of HSF1, enhancing the cellular ability to tightly regulate the heat shock response (Tanabe et al, 1998). HSF4, which lacks the leucine zipper in the C terminus portion of the protein, was shown to constitutively bind to DNA, but to be unable to activate transcription (Nakai et al, 1997). The fact that transient transfection of HSF4 in HeLa cells, which do not express this factor, results in a reduction of HSP synthesis has suggested that HSF4 is a negative regulator of the heat shock response, whose function is to repress the expression of HSP genes (Nakai et al, 1997).

1.7 Cytoprotective role of HSP in diseases

Altered expression of HSPs has been extensively documented in association with a diverse array of diseases including ischemia and reperfusion damage, cardiac hypertrophy, fever, inflammation, metabolic diseases, infection, cell and tissue trauma, aging and cancer (Feige et al, 1996; Suzue and Young, 1996). Oxygen free radicals and other oxidative intermediates have been implicated in reperfusion damage and in the response to environmental agents such as xenobiotics and aromatic hydrocarbons. The ability to detect and respond to oxidized proteins may also be relevant to events leading up to and during stroke and neurotransmitter toxicity. The question, however, for many of these pathologies is whether the expression of HSPs is an adaptation to the particular pathophysiologic state or reflects the sub optimal cellular environment that is associated with a particular disease state.

An important feature of HSPs is their role in the cytoprotection and repair of cells and tissues against the deleterious effects of stress and trauma. Over expression of one or more HSP genes is sufficient to protect against otherwise lethal exposures to heat, cytotoxic drugs, toxins, and TNF- α (Parsell and Lindquist, 1994). Yeast cells engineered to over express hsp70 or hsp104 cross protect against lethal heat shock, H_2O_2, heavy metals, arsenite, anoxia, and ethanol toxicity (Parsell and Lindquist, 1994). In vertebrates, modulation of the heat shock response or the expression of specific HSPs can either limit or prevent the pathology associated with certain chronic diseases. The induction of hsp70 during cardiac hypertrophy associated with both ischemia and reperfusion could reflect the appearance of damaged proteins during myocardial adaptation; likewise, the elevated synthesis of chaperones could reflect the attempt by myocardiocytes to repair protein damage and survive the stress. Over expression of hsp70 confers myocardial protection, as observed by resistance to myocardial ischemic stress and reperfusion damage (Mestril et al, 1994; Marber et al, 1995; Plumier et al, 1995). If activation of the heat shock response in the myocardium is proportional to protein damage, a potential strategy would be to enhance the expression of chaperones such as hsp70, which in turn allows a more rapid reestablishment of normal cardiac protein synthesis and myocardial function. In the case of inflammation, HSP protect mammalian cells from TNF- α and β mediated cytotoxicity (Jaattela et al, 1992), and were shown to suppress astroglial-inducible nitric oxide synthase expression (Feinstein et al, 1996) In a rodent model for adult respiratory distress syndrome (ARDS), heat shock induced hsp70 accumulation within the lung has been associated with decreased pulmonary inflammation and prevention of lethality (Villar et al, 1993) The cytoprotective role of hsp70 has also been documented in the areas of metabolic disorders (Williams et al, 1993), and infection (Amici et al, 1994). These observations suggest new

therapeutic strategies relying upon the development of drugs that are able to increase the expression of HSPs.

1.8 Pharmacological regulators of the heat shock response

Small molecules that either enhance the expression or function of HSPs could play a role in the chronic or acute treatment of certain human diseases. Such a pharmacological approach to hsp70 induction and cardiac protection is suggested by the hsp70 induced protection by simulated ischemia in rat neonatal cardiomyocytes treated with the hsp70 inducer herbimycin A (Morris et al, 1996). Bimoclomol, a hydroxylamine derivative, has cytoprotective activity during ischemia and wound healing (Vigh et al, 1997). Other classes of small molecules with heat shock regulatory properties include NSAIDs, cyclopentenone prostaglandins, serine protease inhibitors, and inhibitors of the ATP-dependant ubiquitin dependant proteosome (Jurivich et al, 1992; Mathew et al, 1998; Santoro et al, 1997).

Pretreatment with NSAIDs, such as salicylates and indomethacin, decreases the temperature threshold of the heat shock response and confers cytoprotection. Exposure to aspirin or indomethacin at concentrations comparable to clinical levels results in the priming of human cells for subsequent exposure to heat shock and other stresses, the enhanced transcription of heat shock genes, and cytoprotection from thermal injury (Amici et al, 1995). However, it must be noted that NSAIDs are not able to induce hsp70 in the absence of stress. The cyclooxygenase cyclopentenone metabolites, such as prostaglandins (PG), PGAs and PGJs, are characterized by their ability to induce hsp70 synthesis for extended periods, such as 12 to 24 hours, in the absence of cytotoxicity (Santoro et al, 1989). HSP induction by prostaglandins is mediated by cycloheximide-sensitive activation

of the inducibly phosphorylated form of HSF1, which activates transcription of heat shock genes (Amici et al, 1992). As shown by structure activity relationship studies, the induction of heat shock gene expression by prostaglandins requires a reactive –unsaturated carbonyl group in the cyclopentenone ring. Cyclopentenone prostanoids confer upon treated cells a potent activity against a wide range of DNA and RNA viruses, including the human immunodeficiency virus (HIV-1) (Santoro, 1996; Rozera et al, 1996). The antiviral response is dependant on the synthesis of heat shock proteins, which are involved in controlling virus replication at multiple levels (Santoro, 1997; Santoro, 1996).

1.9 Animal models of heat stress

Theoretically, the most accurate and beneficial information concerning heat stress should be obtained from humans by subjecting them to heat stress conditions. Obviously, it is neither ethical nor feasible to expose humans to such strenuous conditions. The only information from human subjects is obtained after actual attacks of heat stroke (i.e., case reports), which does not allow manipulation of exposure conditions and various designs of experiments. Several case reports in this respect are available in literature (AL-Hadrami and Ali, 1989; Seraj et al., 1991). Moreover, there are a few instances where human volunteers have been exposed to high temperatures for certain studies (Harris et al., 1990). Nevertheless, such studies are limited by several ethical and logical constraints.

Investigators in heat related studies have employed several animal species. Rats have been used to study some aspects of the temperature regulation mechanisms in humans (Hubbard et al., 1977; Kielblock et al., 1982; Kregel et al., 1988). Dogs have been similarly used (Shapiro et al., 1973; Bynum et al., 1977; Magaznic et al., 1980; Assia et al., 1989). The dog model gives an opportunity to monitor several parameters

concurrently, over a longer time period; however, the supply, housing, and husbandry of this species poses difficulties. MacCurmic et al. (1980) used chicks exposed to high temperatures in some of their studies. Shih et al. (1984) used rabbits as an experimental animal model for heat stress, and sheep have also been successfully employed (Khogali et al., 1983; Hales et al., 1987; Tayeb and Marzouki, 1989). In addition, cows (Richards, 1985), baboons, and monkeys (Gathriam et al., 1988; Eshel et al., 1990) have been utilized to examine some of the effects of heat exposure. Usually, baboons are ideal from the point of similarity of biological changes to that expected in humans. This may have been anticipated in view of the close phylogenetic relationship to humans; however, the cost, availability, and the inconvenience in the manipulation of the baboon limits its usefulness and does not justify its regular use in investigations. As cows and chicks have not been extensively used in heat stress studies, no information is available about the effect of heat exposure in these species. Further, costs and/or handling of such animals do not encourage their use in routine studies.

Rats and rabbits are the most convenient and readily available standard laboratory animal species and they have been used successfully in several heat stress studies (Hubbard et al., 1977; Shih et al., 1984; Shido and Nagasaka, 1990). Of these two species, the rat has advantages of economy and simple husbandry.

1.10 Temperature sensors

The commonly used method of recording body temperature in rats is by means of a temperature probe. The use of a probe would usually entail the use of a restraining device when continuous measurement of temperature is desired. Often, periodic measurements of temperature are carried out, thus doing away with the need for restraint. However, such

methods would require frequent handling of the animals. It is well known that both handling and restraint are able to induce hsp70 in animals (Udelsman et al., 1993). Thus it should be noted that the use of an animal model that entails the use of a restraining device, or frequent handling would invariably interfere with the investigation of hsp70 expression, especially in an investigation that looks into pharmacological mediators of the heat shock response. Today, advent of biotelemetry has provided a solution to this issue.

Biotelemetry is able to evaluate continuous spontaneous locomotor activity, along with blood pressure, heart rate and temperature. The telemetry system usually comprises of a transmitter implanted in the peritoneal cavity of the animal, and a receiver placed beneath its cage. The receiver detects the radio waves emitted by the transmitter, and the data is presented in the desired format on a computer. Implantable telemetry compared to the conventional methods, has a number of advantages, such as elimination of stress from tethers, handling, restraint and human contact. Furthermore, maintenance is not required once telemetry devices are implanted. Thus this method allows for automated continuous monitoring for days, weeks, or months. The measurements are free from the effects of anesthesia, and the data obtained by telemetry do not contain 'cable' artifacts common in tether systems (Gegout-Pottie et al, 1999).

In this study, a free moving rat model of heat stress was developed, based on an ingestible thermometric system. The ingestible sensor (CorTemp) contains a temperature-sensitive quartz crystal oscillator (Fig. 2a). The telemetered signal is inductively coupled by a radiofrequency coil system to an external receiver, attached to a clear Perspex cage, in which the rats are placed. The sensors, covered with a protective silicon coating, are 10 mm in diameter and 20 mm long and are energized by an internal

silver-oxide battery. As the sensors are too large to be ingested by a rat, they were instead implanted abdominally in the rats, outside of the peritoneal cavity.

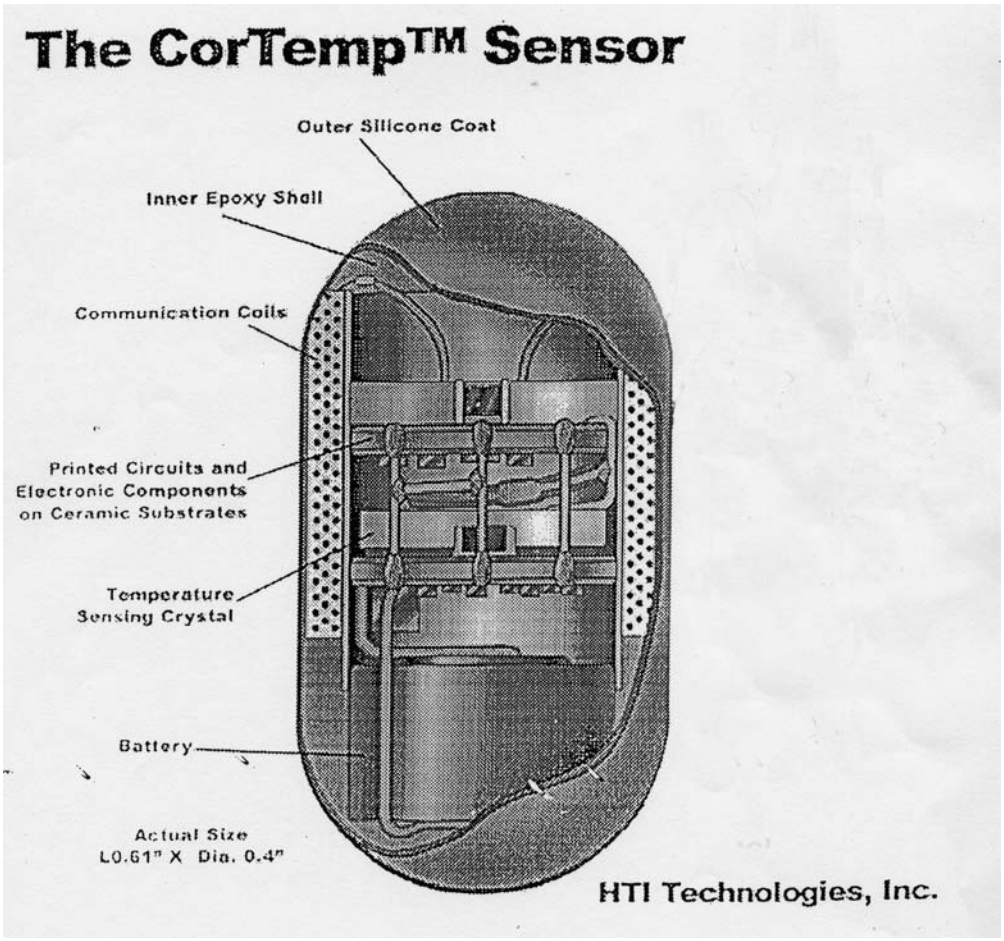


Fig. 2a. CorTemp temperature sensor.

1.11 Herbimycin A and hsp70

The objectives of this study were primarily, to find a suitable pharmacological agent that was capable of conferring thermotolerance to a rat animal model prior to heat stress exposure, as well as to develop a suitable rat model of heat stress. Herbimycin A (Fig. 2b) was selected as the pharmacological agent of choice in this study, based on its ability to induce hsp70, without the need for a concurrent heat stress exposure (Javadpour et al., 1998). The use of herbimycin A in an in vivo rat model is limited to the work done by Javadpour et al (1998), who showed that herbimycin A attenuated ischemia reperfusion induced pulmonary neutrophil infiltration. In this study, we hypothesized that herbimycin A is able to induce hsp70 in a rat model, and subsequently protect the animal from exposure to heat stress. Parameters such as body temperature, HSP induction based on western blots, terminal transferase-mediated d-UTP nick end labeling (TUNEL), Hematoxcylin & Eosin staining (H & E) were studied to test our hypothesis.

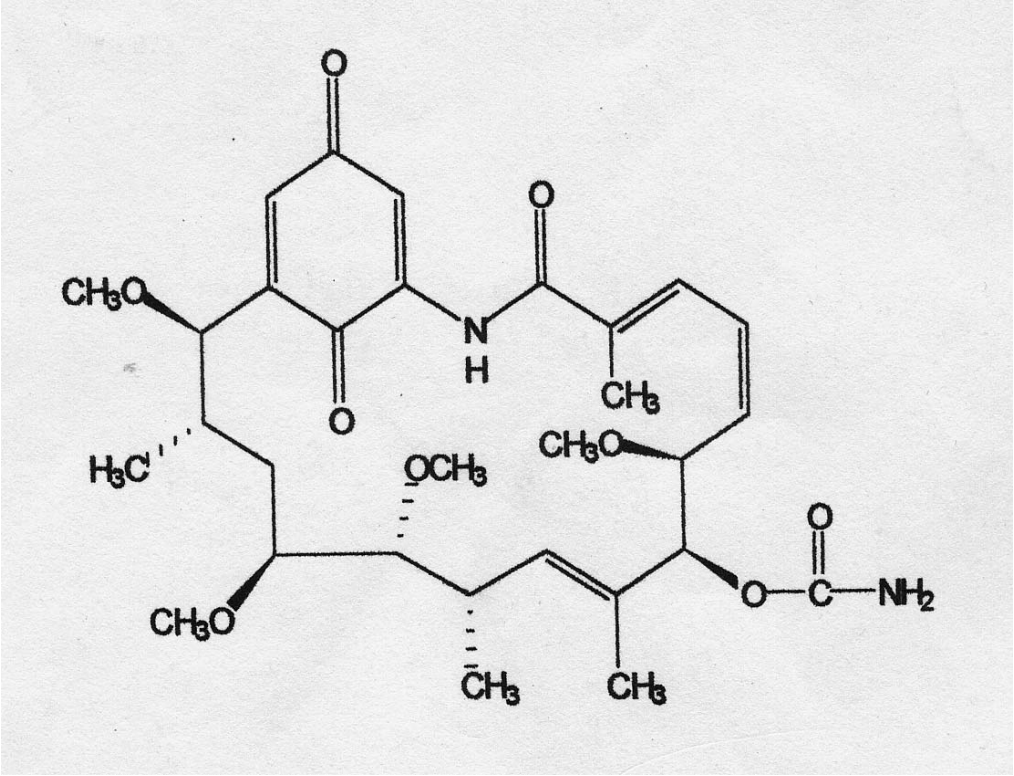


Fig. 2b. Herbimycin A.

Chapter 2

Methods

2.1 Animals

Male Sprague Dawley rats (250-300g) were used in this study. The animals were handled in accordance with the guidelines of the Council for International Organization of Medical Sciences (CIOMS) ethical code for animal experimentation (Howard-Jones, 1995). All animals were held in an air-conditioned animal housing facility, with a 12-hour light/dark cycle. Water and rat chow were provided *ad libitum*.

To analyze the comparability of the temperature data acquired by the sensors and rectal probes, 4 groups of rats (n = 6) were used, namely; rats with implanted sensors exposed to heat stress, rats with rectal probes exposed to heat stress, rats with implanted sensors kept at room temperature and rats with rectal probes kept at room temperature. To determine the peak time of hsp70 expression, rats (n = 4 per time point) were treated with herbimycin A (refer to "Drug treatment" under section 2.3 of methods) and sacrificed at 6, 12, 18 and 24 hours after treatment and hsp70 expression in tissues was analyzed by Western blotting. From these results (Fig. 4), it was determined that maximum hsp70 induction occurred at 12 hours post herbimycin A treatment. Vehicle treated rats (n = 4) and saline treated rats (n = 4) were sacrificed 12 hours after treatment and hsp70 expression in their tissues were analyzed by Western blotting for comparison. 3 groups of rats were used to evaluate the effectiveness of herbimycin A in conferring thermotolerance, namely; herbimycin A treated rats (n = 6), vehicle treated rats (n = 6) and saline treated rats (n = 6). The animals were drug treated and exposed to heat stress 12 hours later. Following heat stress, the rats were allowed to recover for 24 hours, before perfusion for TUNEL (n = 6) and H&E (n = 6) staining was done.

2.2 Implantation of temperature sensor

Rats were anaesthetized with Clinical Research Center (CRC) cocktail (i.p. 0.33 ml /100 g), containing 1 part Hypnorm (Jansen Pharmaceutica, Beerse, Belgium), which contains fentanyl (0.315 mg/ml) and fluanisone (10 mg/ml), 1 part Dormicum (Roche, Basel Switzerland), which contains midazolam (5 mg/ml), and 2 parts water for injection, before implantation. A small incision was made abdominally, above the right leg, and the sensor was placed under the skin, in contact with the peritoneal cavity. Baneocin (250 IU / bacitracin zinc. B.P., 5000 IU neomycin, as sulphate B.P., Biochemie GmbH, Vienna, Austria) was applied to the incisions, after they were closed with sutures. The operated animals were allowed to recover for 48 hours after the operation, before being used in the study.

2.3 Drug treatment

1 mg of herbimycin A (Sigma) was dissolved in 1 ml of (DMSO), and then diluted to 6 ml with 0.9% saline. A vehicle of 1 ml of DMSO diluted to 6 ml with 0.9% saline was used. Doses of 2 ml/kg of herbimycin A solution, vehicle or saline to were administered intra peritonealy (IP) to each of the groups. The dose of herbimycin A was selected based on that reported by Javadpour et al (1998) where it was found to be effective in inducing hsp70.

2.4 Western blotting

At the pre-determined time points, the rats were decapitated, and the liver, heart, lung and kidney were rapidly removed and washed in ice-cold saline. The organs were cut into suitable small pieces and stored in cryo vials that were rapidly frozen in liquid

nitrogen. The frozen tissues were stored at -80^0C until protein extraction and quantitation was done. For the analysis of apoptosis inhibition, rats from the three treatment groups were sacrificed 24 hours after heat stress. Their tissues were harvested and stored as described above.

During protein extraction, the frozen tissues were first powdered in a mortar and pestle, using liquid nitrogen. The powdered tissues were then suspended in an extraction buffer, containing phosphate buffered saline, with 1 μg/ml of aprotinin (Sigma) and 0.01% Triton X (BioRad). The tissue suspension was then homogenized in ice for 2 minutes (Heildolphe), followed by sonication (Misonix) in ice for 1 minute. The samples were then centrifuged at 14 000 rpm for 10 minutes and the supernatant was recovered. Protein content in the supernatant was quantitated based on the Bradford assay (BioRad).

Equal protein amounts of 30 μg were separated on a one-dimensional 7.5% polyacryamide gel (BioRad), under standard denaturing conditions according to the method of Laemmli (1970). Briefly, protein samples were diluted in a denaturing buffer (Laemmli, 1970), heated at 100^0C for 5 minutes and allowed to cool, before equal protein loads of 30 μg were loaded into each well. The loaded protein samples were then separated by electrophoresis at 150mAmp. The separated proteins were then transferred onto a PVDF membrane at 100mAmp, 2 hours (BioRad). The membranes were then blocked with a solution of 5% non-fat milk for 30 minutes, before overnight probing with a mouse monoclonal antibody specific for hsp70 (C92, 1:1000 dilution) at 4^0C. The C92 antibodies were obtained from StressGen Biotechnologies. After incubation overnight, the membranes were washed with Tris buffered saline and probed with a goat anti-mouse IgG horseradish peroxidase (HRP) conjugated secondary antibody (1:10000 dilution) for 1 hour. The secondary antibodies were obtained from StressGen Biotechnologies as well.

The membranes were then washed thoroughly with Tris buffered saline, and they were developed using the enhanced chemiluminescence-Western blot detection kit (Amersham-Pharmacia-Biotech) at a darkroom and exposed to X-ray film (Kodak). Densitometry analysis of the bands obtained was done using Fluor S (BioRad).

In the analysis of apoptosis inhibition, Goat polyclonal IgG antibodies (L-18, 1:500 dilution) specific for the p20 subunit of the 32 kDa cysteine protease of caspase 3, were obtained from Santa Cruz Biotechnology and were used as the primary antibody. Bovine anti-goat IgG-HRP from Santa Cruz Biotechnology was used as the secondary antibody. The protein samples were extracted as described above. Equal amounts of protein (30 μg, denatured as above) were loaded into a one-dimensional 12% polyacryamide gel (BioRad). The separated proteins were then transferred onto a PVDF membrane at 300mAmp, 3 hours (BioRad). All the remaining steps were similarly followed as for hsp70 analysis.

2.5 Heat stress protocol

A heat stress protocol of exposure to 45 ^{o}C for 25 minutes at 55% humidity in a climatic chamber (Fig. 3a) (Cold-Heat-Climate-Test chamber, Weiss Technik) was used in this study. A previous study in our lab determined that an exposure to such conditions enabled rats to attain a colonic temperature of greater than 41 ^{o}C (Sachidhanandam et al, 2002). The rats with rectal probes (YSI) were held in a restrainer, with the rectal probes inserted about 5 cm past the rectum, and connected to a six-channel thermistor thermometer (Cole Parmer), which was used to read off colonic temperatures, as in the previous study. Rats with the implanted sensors were allowed to move freely in a Perspex container throughout the duration of the experiments. A receiver was attached to the

outside of the container to record the signals from the sensors in this group of rats. Both set ups are shown in Fig. 3b.

Fig. 3a. Front view of the climatic chamber.

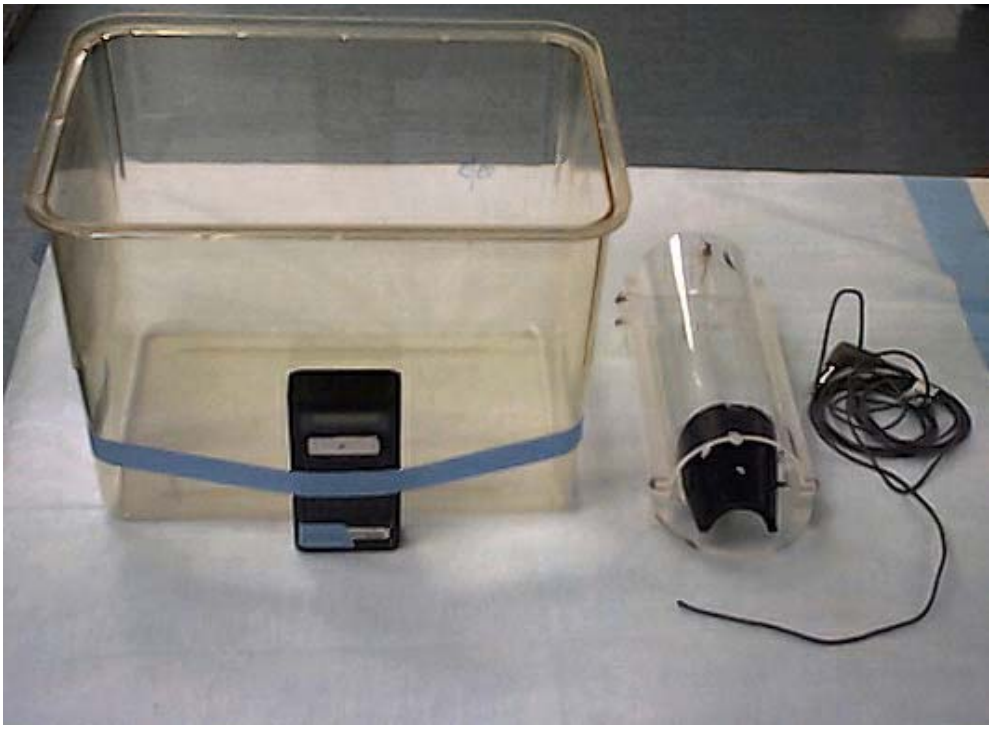


Fig. 3b. Set up for CorTemp sensor (left) and rectal probe (right).

Following heat stress, the animals from the heat stress groups were allowed to recover at room temperature (25 ^{o}C) for another 20 minutes before being returned to their cages. Non-heat stressed groups were kept at room temperature for a duration of 45 minutes. Temperatures were recorded at 5-minute intervals for all groups, during the entire duration of the experiments.

2.6 H&E and TUNEL staining

Following heat stress, the rats were allowed to recover for 24 hours, before perfusion was carried out. During perfusion, the rats were anesthetized with CRC cocktail (i.p. 0.33 ml/100 g), and were pinned down onto a wax plate. A thoracotomy was done to expose the heart. Ringer's solution was allowed to drip slowly from the reservoir to a needle via a tube. The needle was inserted into the left ventricle of the heart, and the resulting build up in blood pressure was released via a puncture made to the right atrium. About 50 ml of Ringer's solution was allowed to pass through each rat, before infusion of 10% formalin, which was used as the fixative. Infusion was stopped when the whole animal was completely perfused with the fixative, indicated by stiffening of the tail. The liver, lung, heart and kidney were then removed and cut into suitable sizes and placed in cassettes. The cassettes were then post fixed by storing in 10% formalin for 24 hours, before tissue processing.

Tissue processing was done using an automated set-up. The processed tissues were then embedded in paraffin wax and cut to 4 μm sections, using a microtome. The cut sections were mounted onto slides and allowed to dry overnight at 37 ^{o}C. The slides were dewaxed in two changes of xylene (5 minutes each), followed by two changes in 100% ethanol. The slides were then immersed in 90%, 80% and 70% ethanol respectively for

three minutes each, and then washed in water. The slides were dipped into gill's haematoxylin for 5 seconds and washed in water, before being dipped into eosin solution for 30 seconds. After washing in water, the slides were rehydrated in 70%, 80%, 90% and 100% ethanol sequentially. Finally, the slides were immersed in two changes of xylene, and then covered with coverslips.

In the case of TUNEL staining, the slides were dewaxed as described above, and the staining was carried out according to the protocol for paraffin embedded tissue provided in the TdT-FragEL^{TM} DNA Fragmentation Detection Kit (Cat# QIA33, Oncogene Research Products). The slides were then covered with a coverslip and observed under a microscope. Photographs of fields of interest were taken.

2.7 Statistics

SPSS version 10 was used in the analysis of the body temperatures obtained. The data from the various groups were analyzed by analysis of variance (ANOVA), followed by post hoc Bonferroni tests. Error bars represent the standard error of the mean (S.E.M.). Densitometry data from the western blots were analyzed in a similar manner.

Chapter 3

Results

3.1. Time expression of hsp70 in herbimycin A treated rats

Figures 4a to 4d show the western blot results obtained from the liver, lung, heart and kidney respectively, from herbimycin A treated rats, sacrificed at 6, 12, 18 and 24 hours post drug administration. From these results, it can be clearly observed that peak hsp70 expression, indicated by intense bands, was seen 12 hours post herbimycin A administration, in all four tissues. Hsp70 expression fell with time, as shown by the decreasing intensity of the bands at 18 and 24 hours post drug administration. Figures 5a to 5d show the densitometry data based on the western blots in figures 4a to 4d. The data show that hsp70 expression was significantly greater at 12 hours, when compared against expression at 6, 18 and 24 hours ($P < 0.05$). Based on the densitometry results, it can be observed once more that peak hsp70 expression was seen at 12 hours post herbimycin A administration.

3.2. Herbimycin A and hsp70

Figures 6a to 6d shows the western blot expression of hsp70 in the liver, lung, heart and kidney respectively, in herbimycin A, vehicle and saline treated rats 12 hours post drug administration. In all four tissues, herbimycin A was able to induce the expression of hsp70. Both vehicle and saline treated rats showed no obvious difference in hsp70 expression in the tissues studied. Neither was able to increase the expression of hsp70. Figures 7a to 7d show the densitometry results based on the western blot in figures 6a to 6d. Expression of hsp70 was significantly greater ($P < 0.05$) following herbimycin A treatment, as compared to administration of vehicle and saline, for the liver, lung, heart and kidney tissues. No statistically significant differences were observed in the expression of hsp70, between vehicle and saline treated rats for all four tissues.

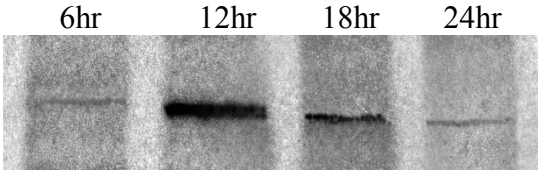


Fig. 4a.

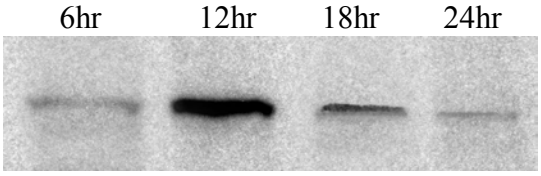


Fig. 4b.

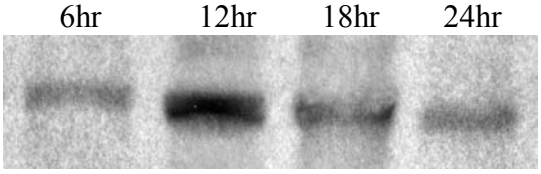


Fig. 4c.

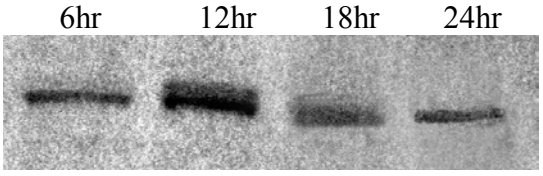


Fig. 4d.

Fig. 4. Western blots of hsp70, from the liver (Fig. 4a.), lung (Fig. 4b.), heart (Fig. 4c.) and kidney (Fig. 4d.) of herbimycin A treated rats. The tissues were harvested at 6, 12, 18 and 24 hours post herbimycin A administration. 30 μg of protein was loaded per lane. The doublets in Fig. 4c and 4d could be due to the expression of other *hsp70* family members after herbimycin A treatment.

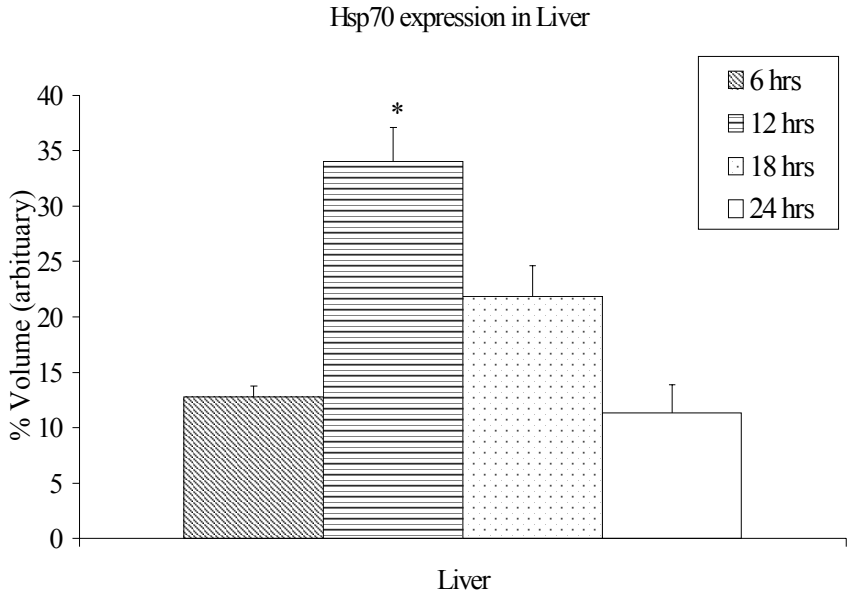

Hsp70 expression in Liver
6 hrs
12 hrs
18 hrs
24 hrs
*
% Volume (arbituary)
40
35
30
25
20
15
10
5
0
Liver

Fig. 5a.

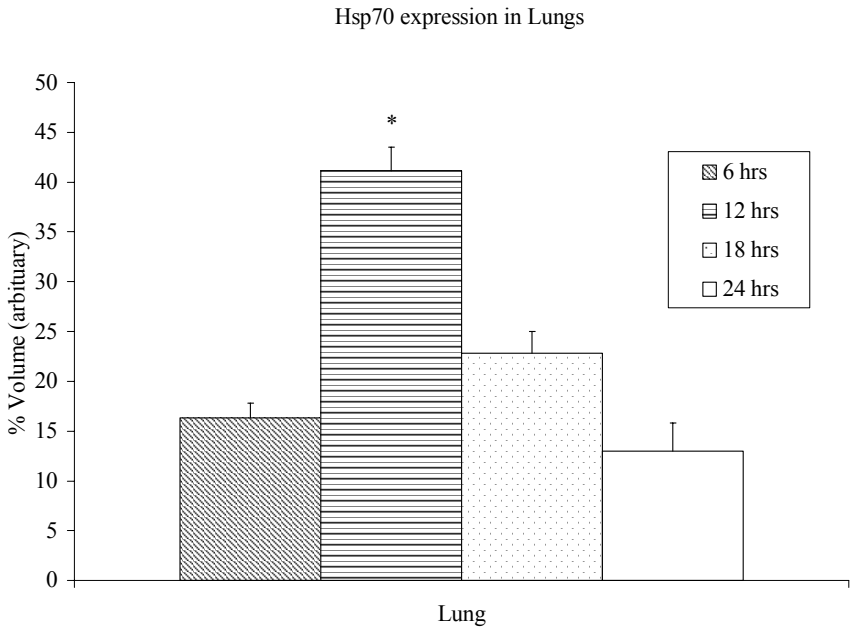

Hsp70 expression in Lungs
6 hrs
12 hrs
18 hrs
24 hrs
*
% Volume (arbituary)
50
45
40
35
30
25
20
15
10
5
0
Lung

Fig. 5b.

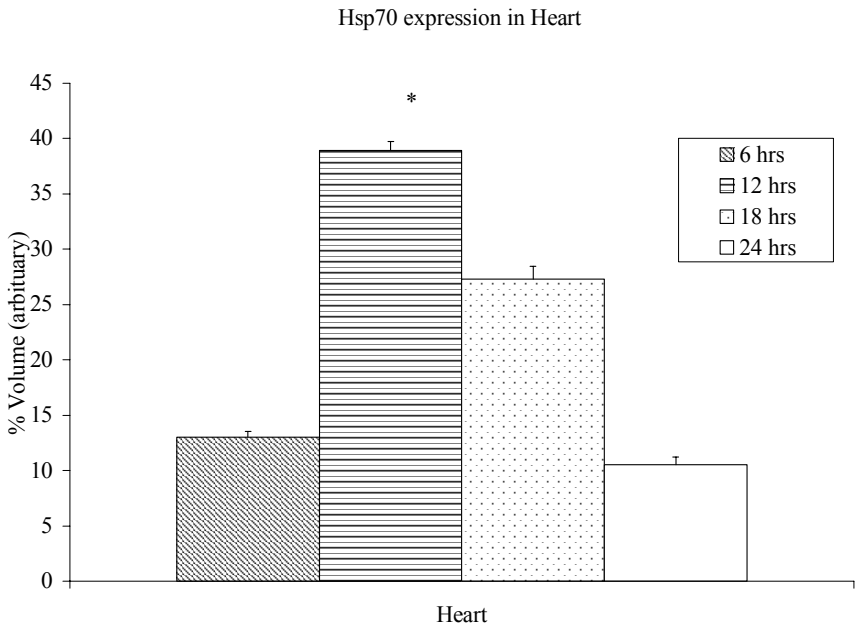


Fig. 5c.

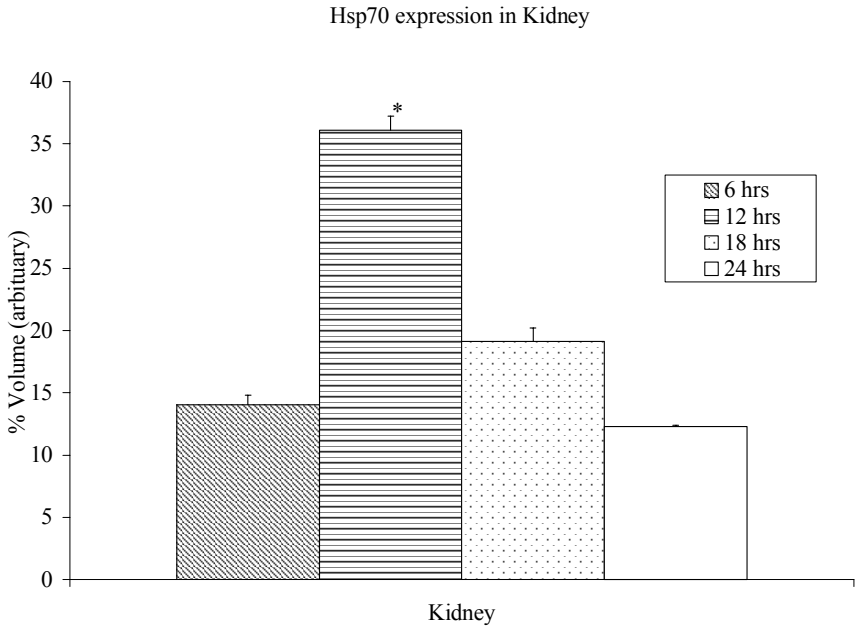


Fig. 5d.

Fig. 5. Densitometric analysis of hsp70 expression over time, from western blot data, in the liver (Fig. 5a.), lung (Fig. 5b.), heart (Fig. 5c.) and kidney (Fig. 5d.), of herbimycin A treated rats. Each bar is the mean ± S.E.M. (* denotes $P < 0.05$ when compared against 6, 18 and 24 hours).

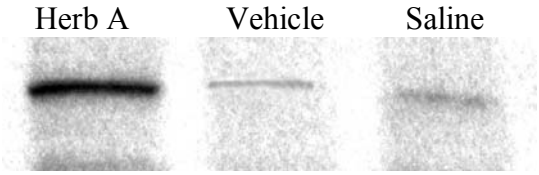


Fig. 6a.

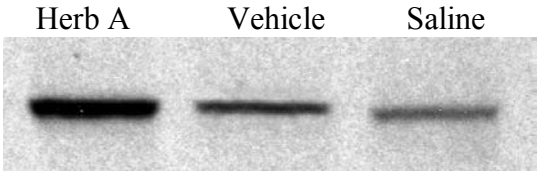


Fig. 6b.

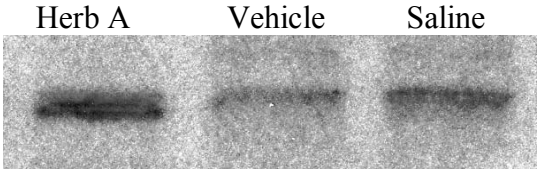


Fig. 6c.

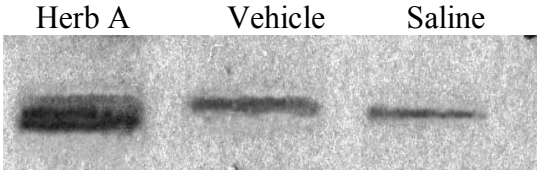


Fig. 6d.

Fig. 6. Western blots of hsp70 from the liver (Fig. 6a.), lung (Fig. 6b.), heart (Fig. 6c.) and kidney (Fig. 6d.) of herbimycin A (Herb A), vehicle and saline treated rats. The tissues were harvested 12 hours post drug administration. 30 μg of protein was loaded per lane. The doublets in Fig. 6c and 6d could be due to the expression of other *hsp70* family members after herbimycin A treatment.

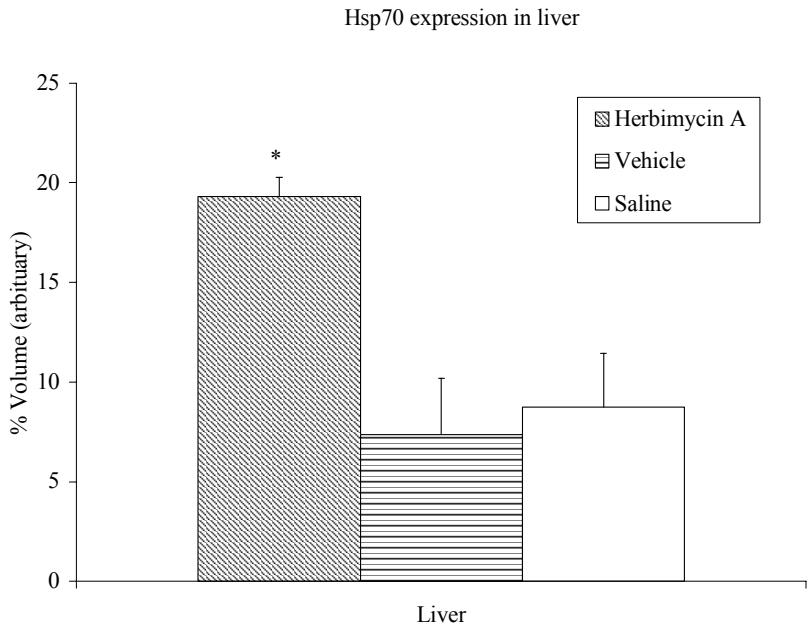

Hsp70 expression in liver
25
20
15
10
5
0
% Volume (arbituary)
*
Herbimycin A
Vehicle
Saline
Liver

Fig. 7a.

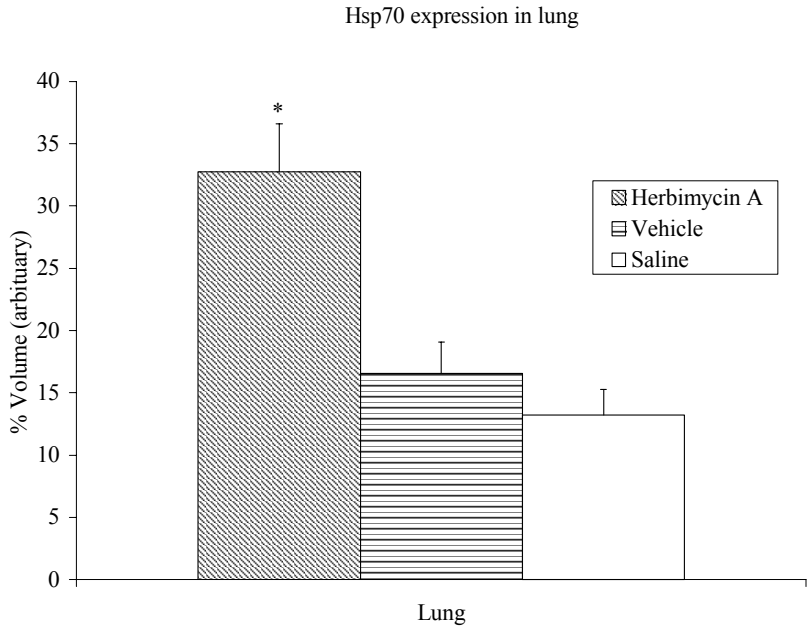

Hsp70 expression in lung
40
35
30
25
20
15
10
5
0
% Volume (arbituary)
*
Herbimycin A
Vehicle
Saline
Lung

Fig. 7b.

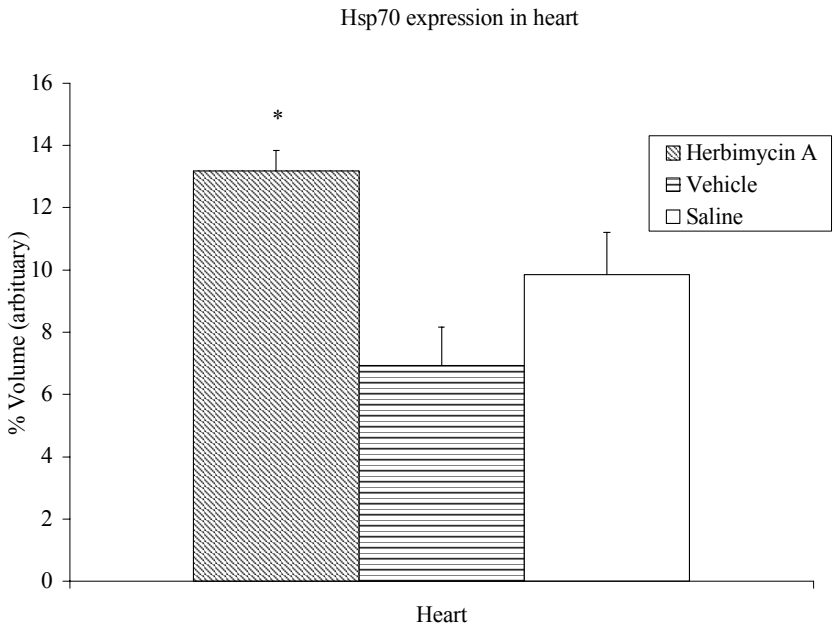


Fig. 7c.

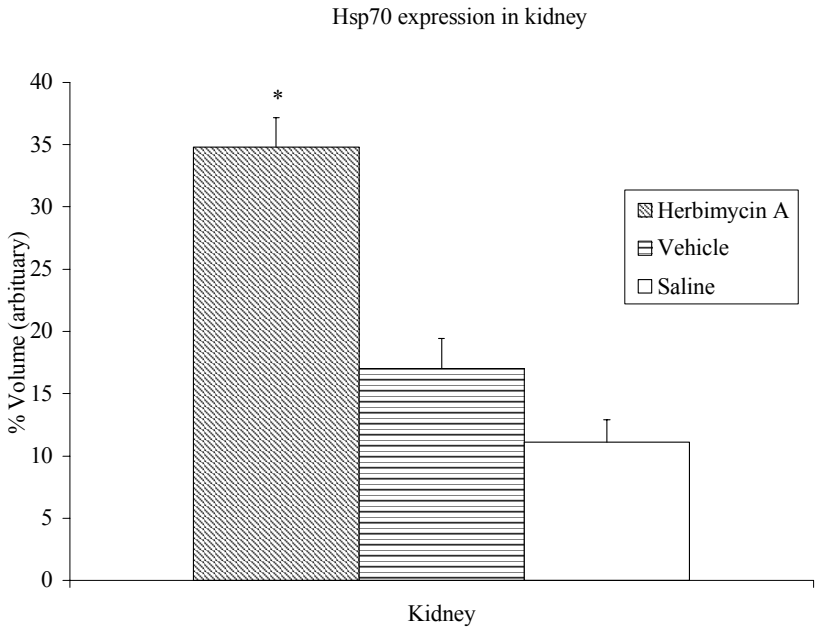


Fig. 7d.

Fig. 7. Densitometric analysis of hsp70 expression from western blot data, in the liver (Fig. 7a.), lung (Fig. 7b.), heart (Fig. 7c.) and kidney (Fig. 7d.), of herbimycin A, vehicle and saline treated rats. Each bar is the mean ± S.E.M. (* denotes $P < 0.05$ when compared against vehicle and saline treated rats).

3.3. CorTemp pill vs. YSI probe

Figure 8 shows the temperature data obtained from rats that were exposed to heat stress and had their temperatures recorded with the CorTemp pill (free moving animal model) or YSI probe (restrained animal model). Both groups showed no statistically significant differences in core temperature, from t = 0 to t = 40 minutes. Core temperatures of both groups rose with time, with peak temperatures at t = 30 minutes. The temperatures then fell with time until the last recording at t = 45 minutes. The rats in the free moving animal model attained a peak temperature of 41.92 ± 0.20 ^{0}C, while the rats in the restrained animal model attained a peak temperature of 41.94 ± 0.22 ^{0}C, both at t = 30 minutes. At t = 45 minutes, rats in the free moving animal model attained lower core temperature of 39.06 ± 0.70 ^{0}C ($P < 0.05$), as compared to the rats in the restrained animal model, with core temperatures of 40.83 ± 0.31 ^{0}C.

3.4. Heat stress and temperature

Figure 9 shows the temperature data of herbimycin A, vehicle and saline treated rats during and after exposure to heat stress. Peak core temperatures were attained by all three groups at t = 30 minutes. Herbimycin A treated rats attained lower peak temperatures of 41.16 ± 0.14 ^{0}C ($P < 0.05$), when compared to vehicle and saline treated rats that attained 41.76 ± 0.16 ^{0}C and 41.85 ± 0.18 ^{0}C respectively. Herbimycin A treated rats had lower temperatures ($P < 0.05$), as compared to vehicle and saline treated rats at t = 25 minutes, and as compared to vehicle treated rats at t = 30 minutes. Both the vehicle and saline treated showed no statistically significant differences in their core temperatures throughout the duration of recording.

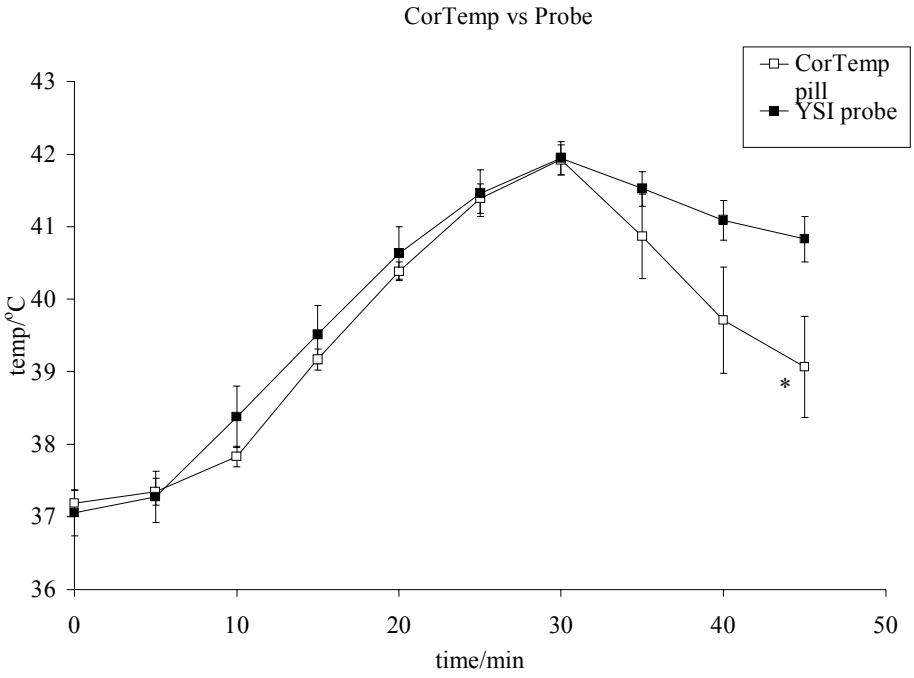


Fig. 8. Temperature vs time course, of core body temperature of rats exposed to 45 ^{o}C heat stress for 25 minutes, using CorTemp pill and YSI probe. Each point is the mean ± S.E.M. (*: $P < 0.05$ compared to YSI probe).

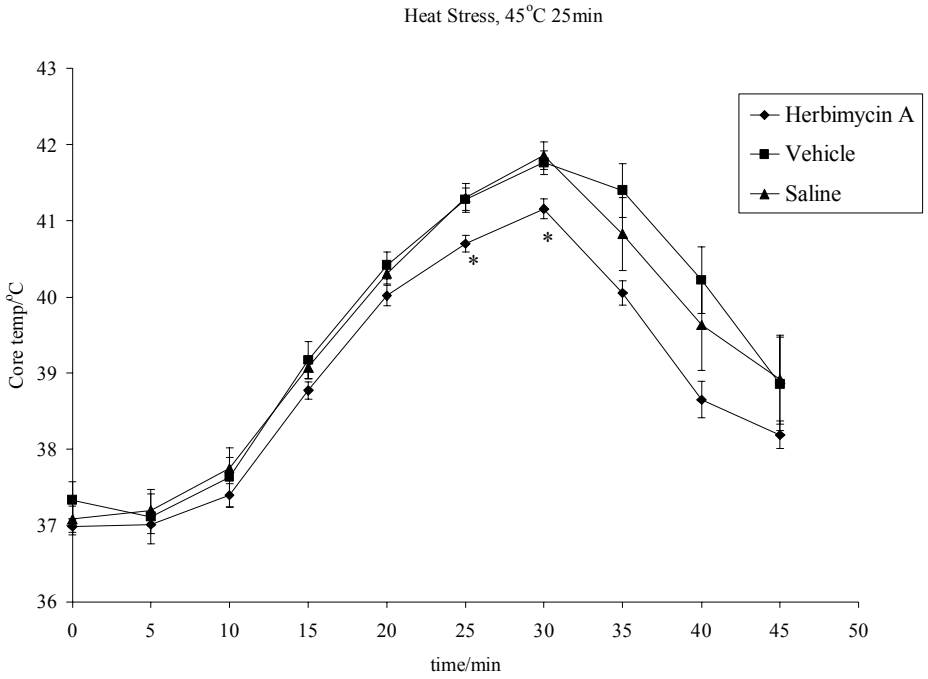


Fig. 9. Temperature vs time course, of rats treated with herbimycin A, vehicle and saline, exposed to 45 ^{o}C heat stress for 25 minutes. Each point is the mean ± S.E.M. (*: $P < 0.05$ compared to YSI probe).

3.5. H&E staining

Figures 10a to 10c show the H&E stained sections of the liver from herbimycin A, vehicle and saline treated rats respectively, 24 hours after heat stress exposure. No differences in morphology were observed between all three treatments groups. Similarly, the lung, heart and kidney sections (Figs. 11 to 13 respectively) of all three treatment groups also did not show any differences in morphology. Signs of necrosis, such as cellular swelling and cytoplasmic vacuolization, were absent in the analyzed liver, lung, heart and kidney tissues, from all three treatment groups.

3.6. TUNEL staining

Figures 14a to 14c show the TUNEL stains of the liver from herbimycin A, vehicle and saline treated rats respectively, 24 hours after heat stress exposure. Apoptotic nuclei, with DNA fragmentation, are stained brown, and can be clearly observed in the vehicle (Fig. 14b) and saline (Fig. 14c) treated rat liver sections, compared to the herbimycin A (Fig. 14a) treated rat liver sections. Similarly for the lung, heart and kidney sections (Fig. 15 to 17 respectively) of the vehicle and saline treated rats, apoptotic nuclei were widespread in the tissues, as compared to the respective tissues from the herbimycin A treated rats. A scoring system was designed whereby the total number of apoptotic cells were expressed as a percentage of the total number of cells in each field, per tissue, per treatment. These data are presented in figure 18. From figure 18, it can be seen that the percentage of apoptotic nuclei in the tissues of the herbimycin A treated rats was significantly lower ($P < 0.05$), as compared to that from the vehicle and saline treated rats. Furthermore, there was no statistically significant difference in the percentage of apoptotic nuclei, between the liver, lung, heart and kidney tissues of vehicle and saline treated rats.

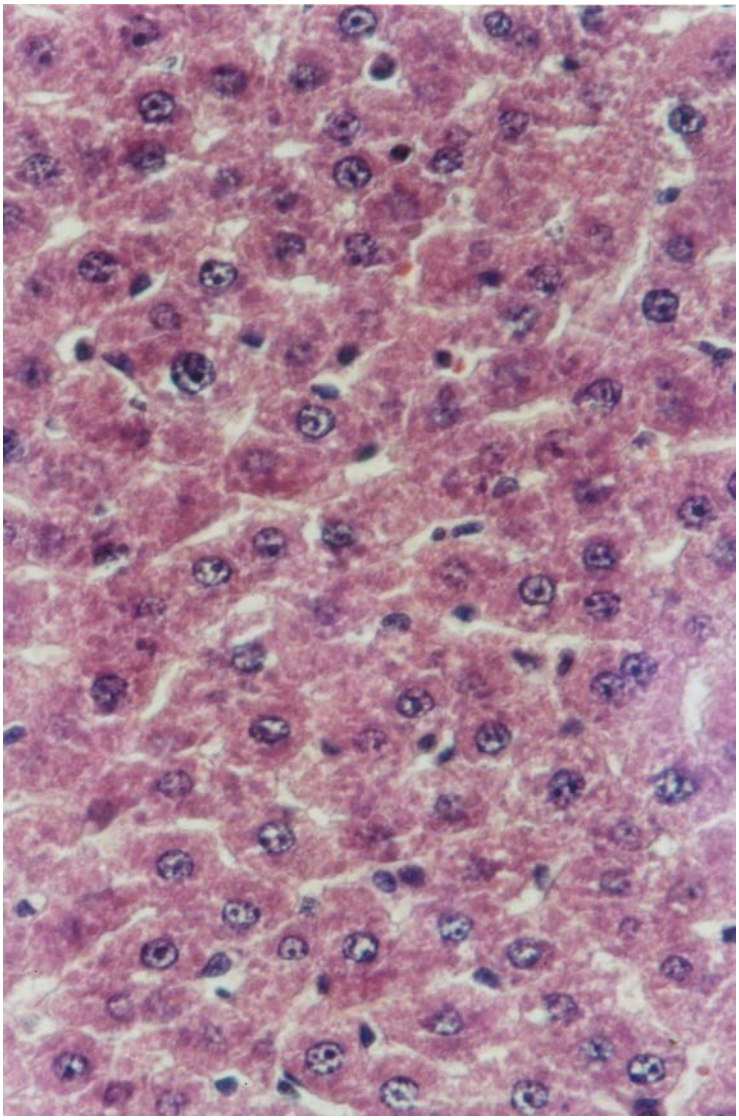


Fig. 10a. Liver biopsy of herbimycin A treated rat.

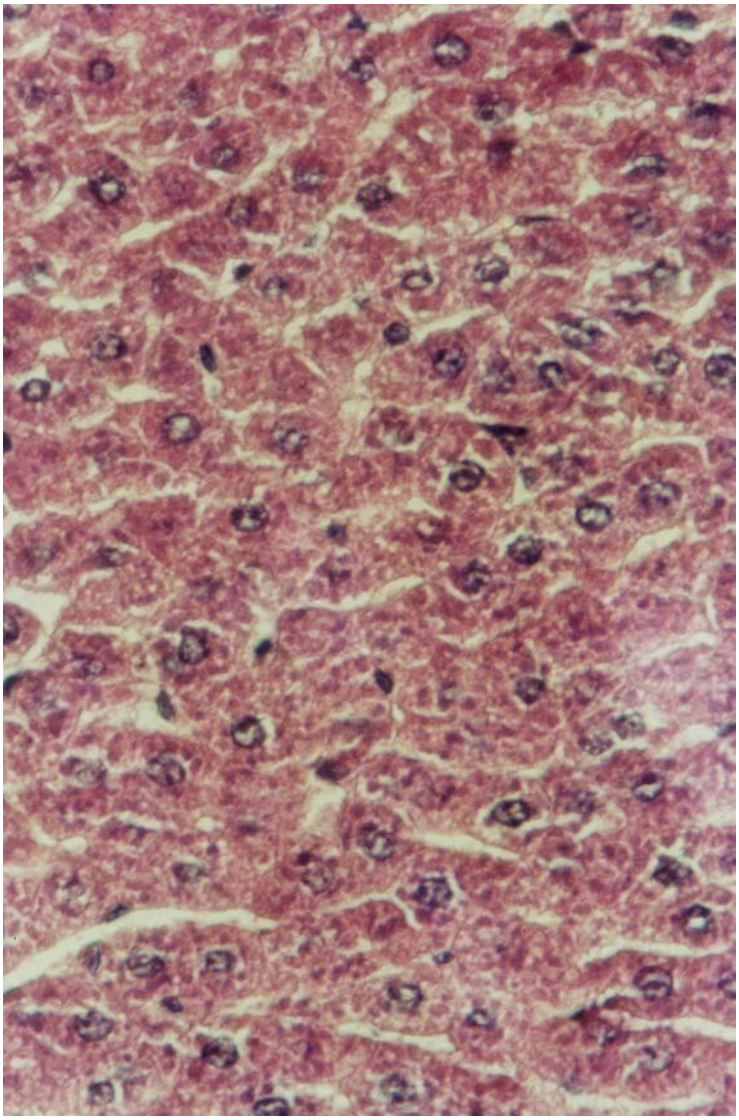


Fig. 10b. Liver biopsy of vehicle treated rat.

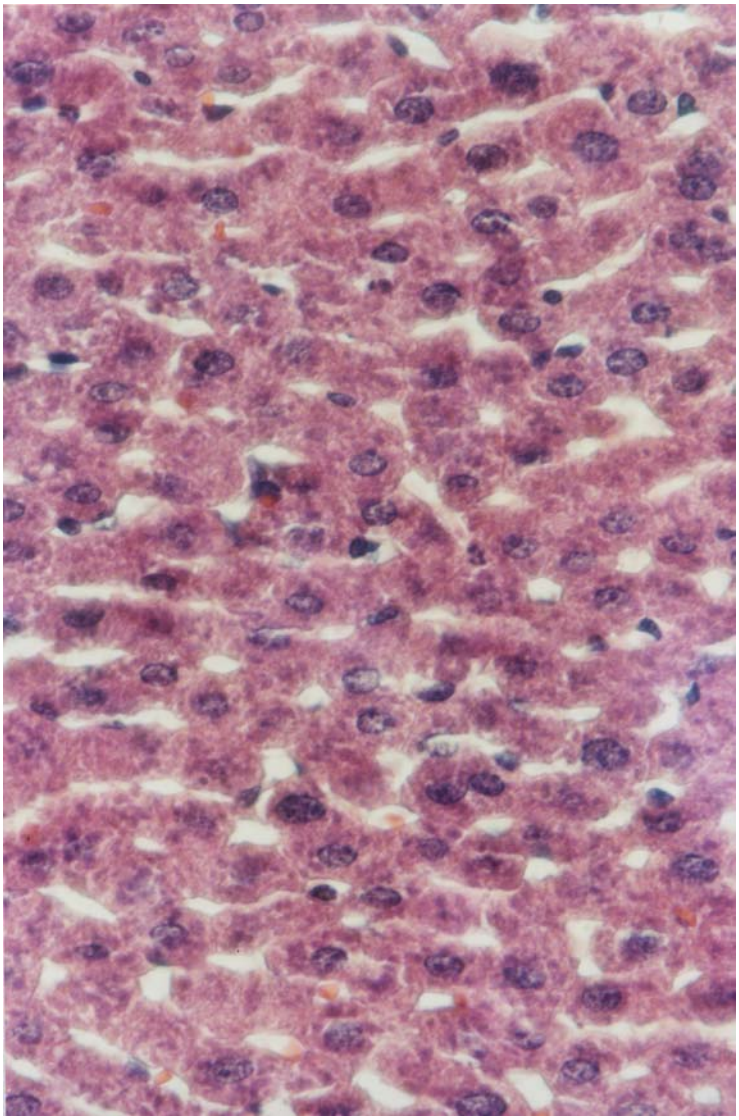


Fig. 10c. Liver biopsy of saline treated rat.

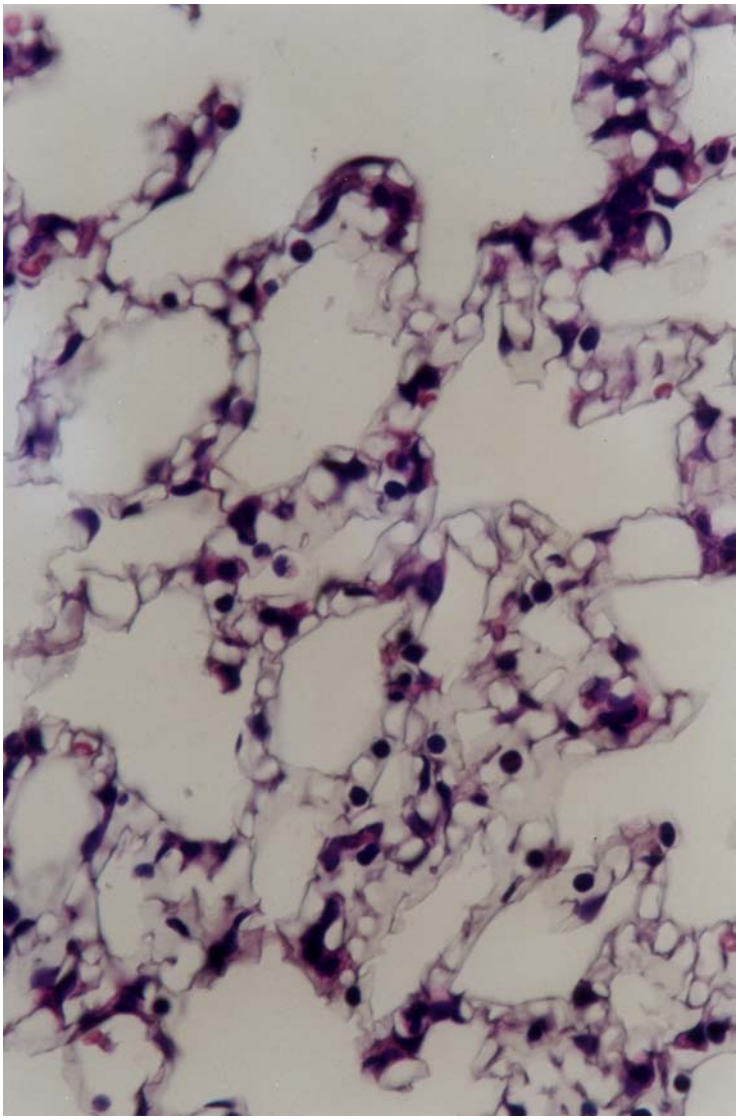


Fig. 11a. Lung biopsy of herbimycin A treated rat.

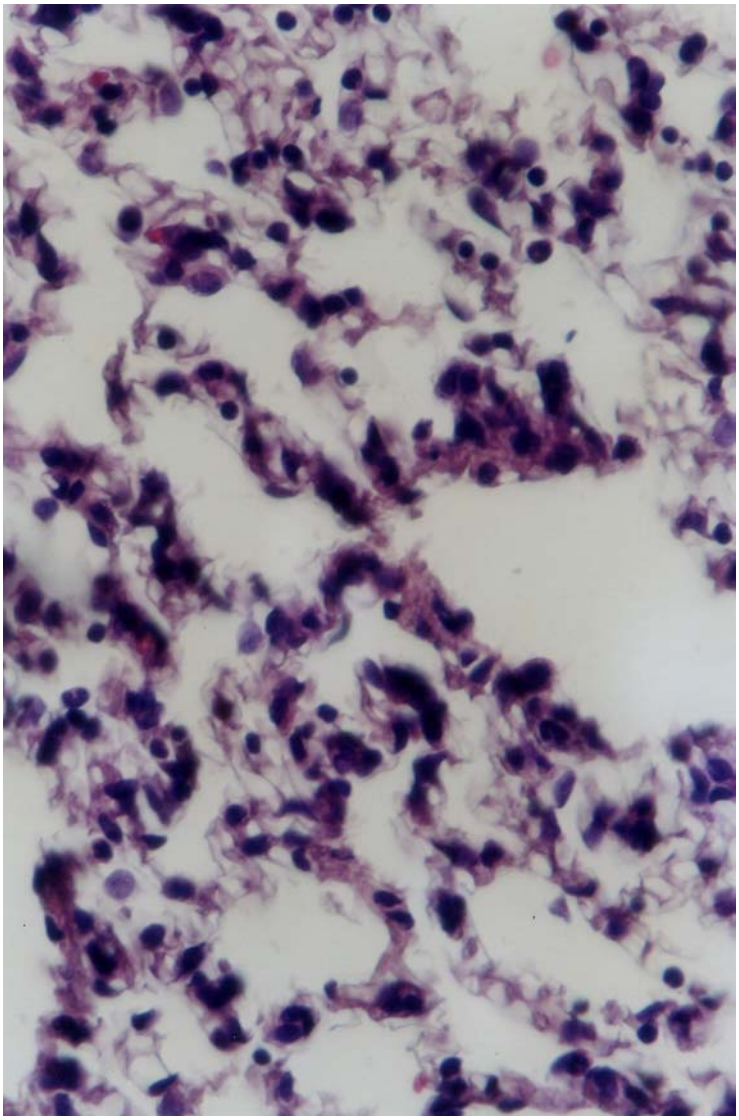


Fig. 11b. Lung biopsy of vehicle treated rat.

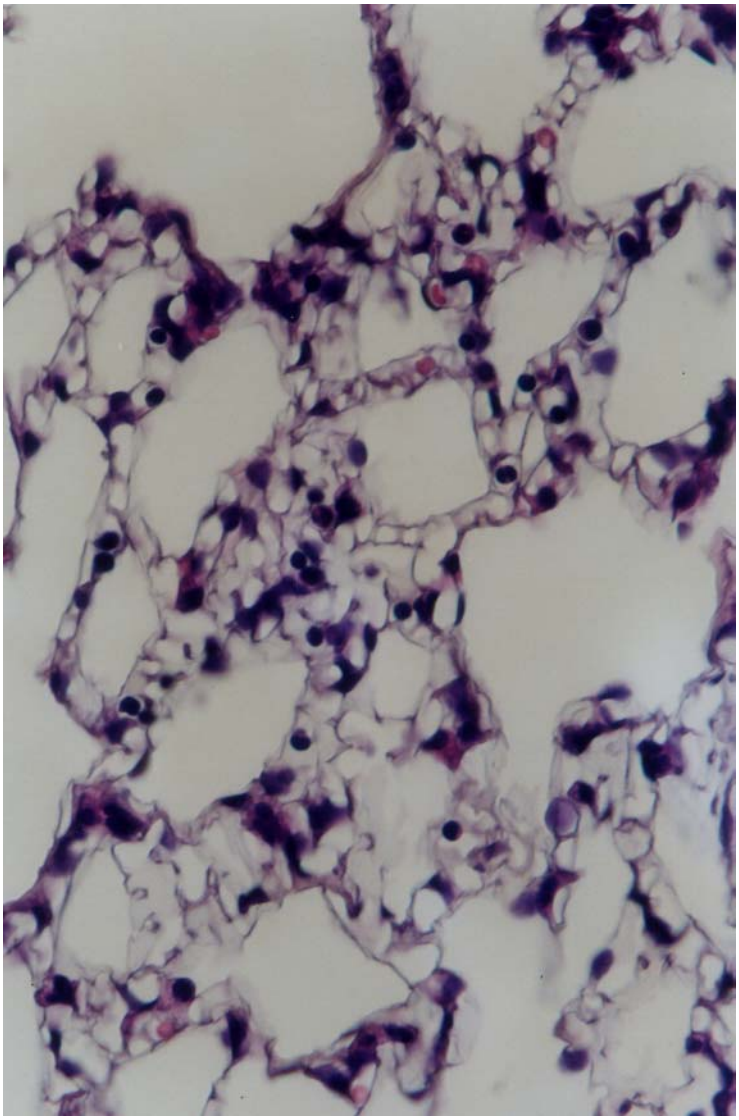


Fig. 11c. Lung biopsy of saline treated rat.

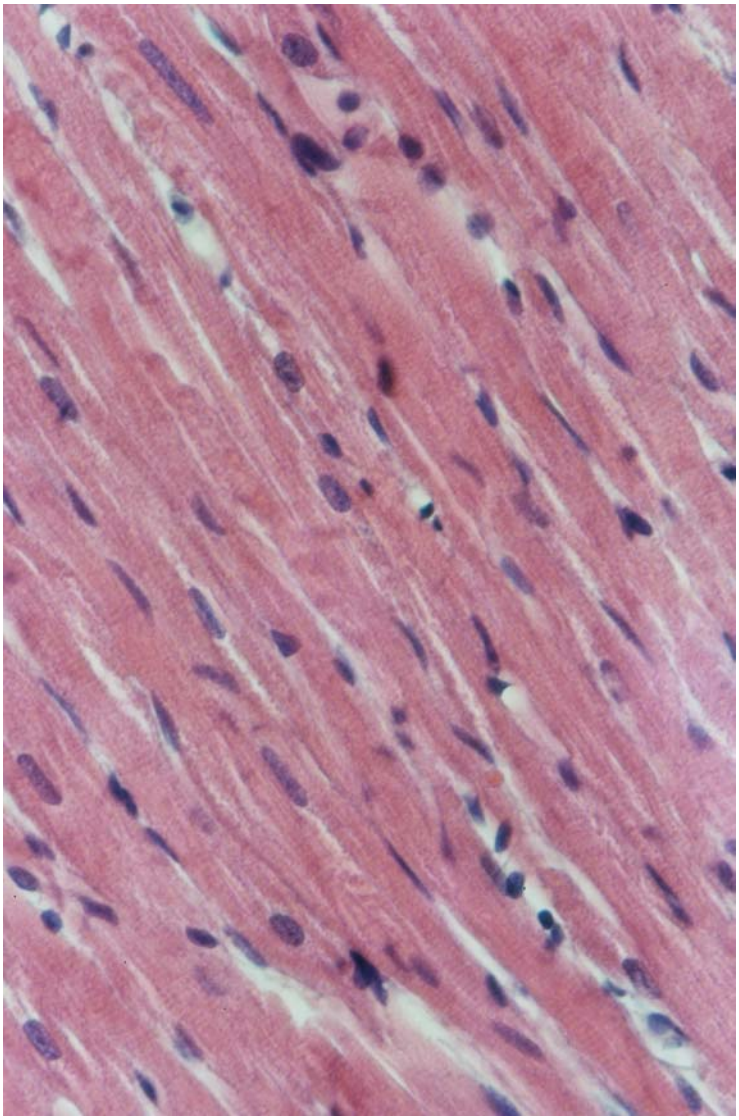


Fig. 12a. Heart biopsy of herbimycin A treated rat.

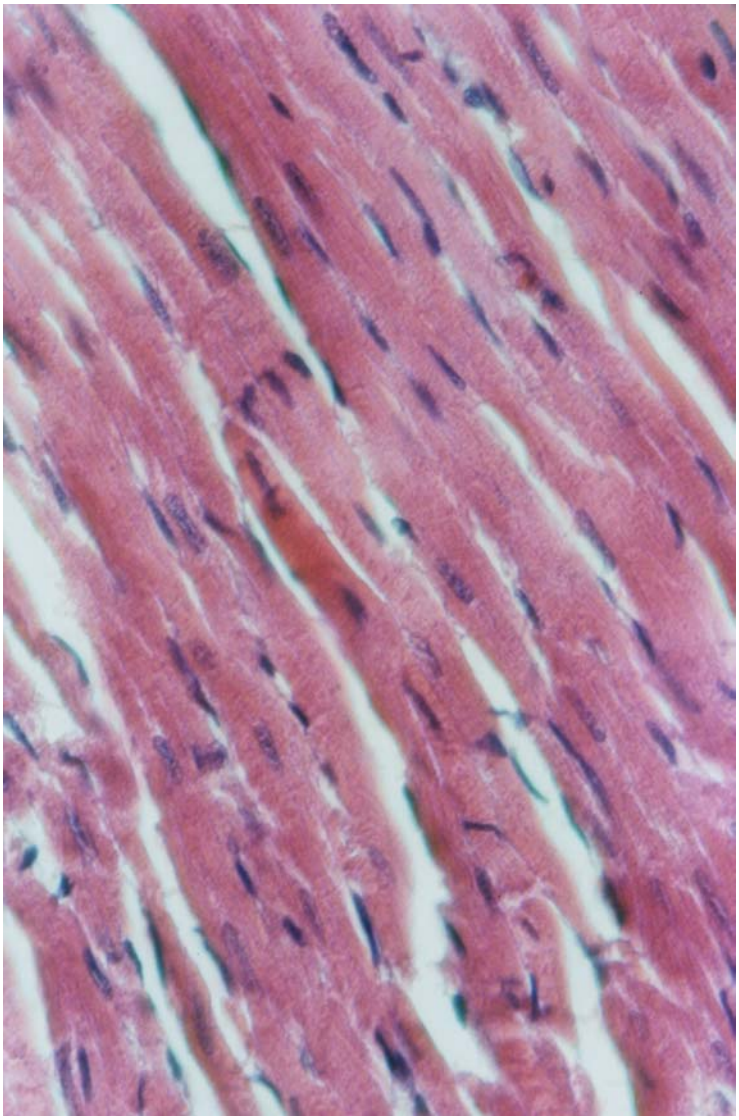


Fig. 12b. Heart biopsy of vehicle treated rat.

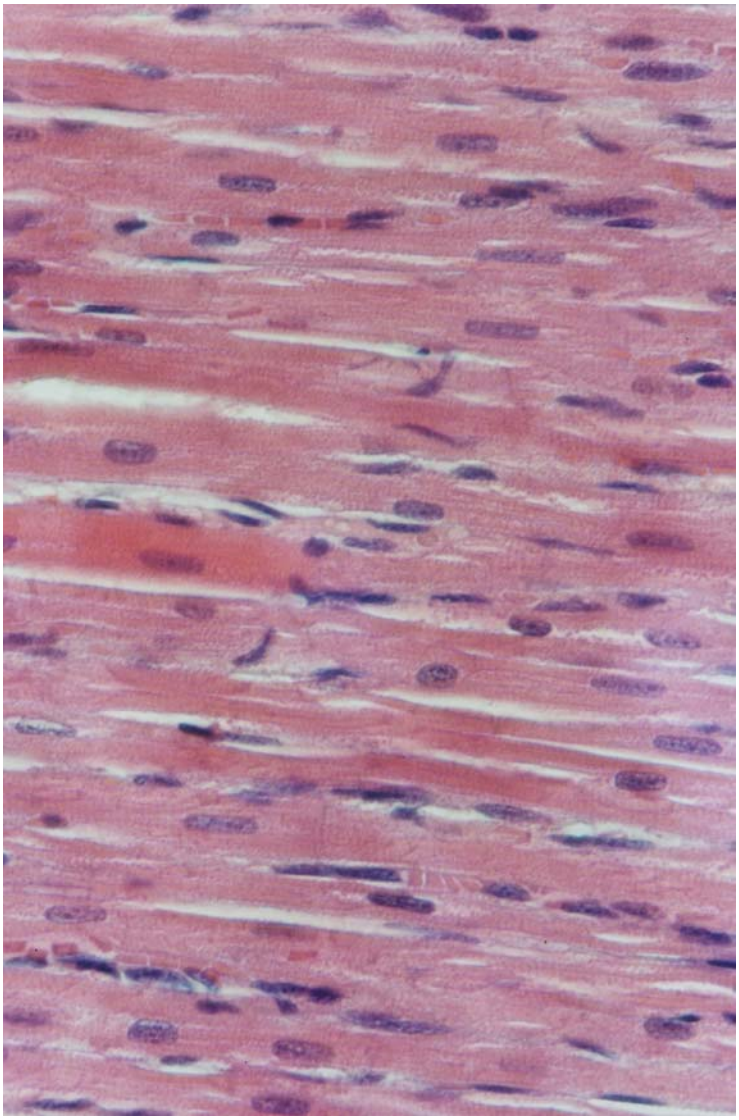


Fig. 12c. Heart biopsy of saline treated rat.

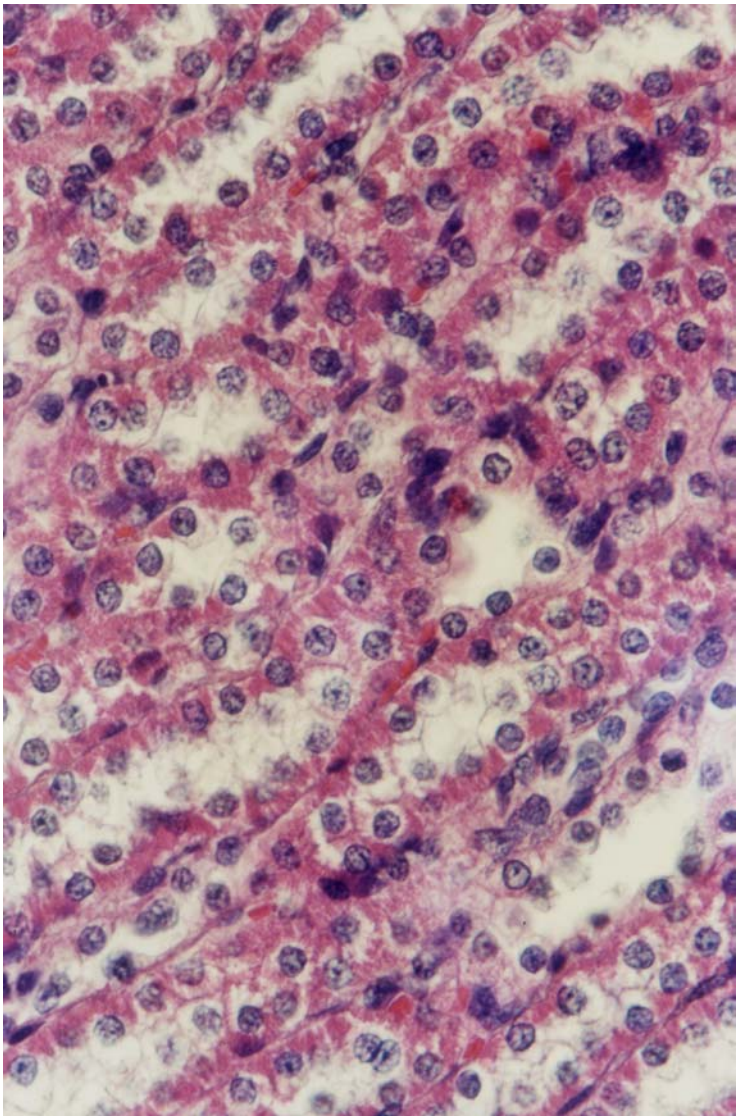


Fig. 13a. Kidney biopsy of herbimycin A treated rat.

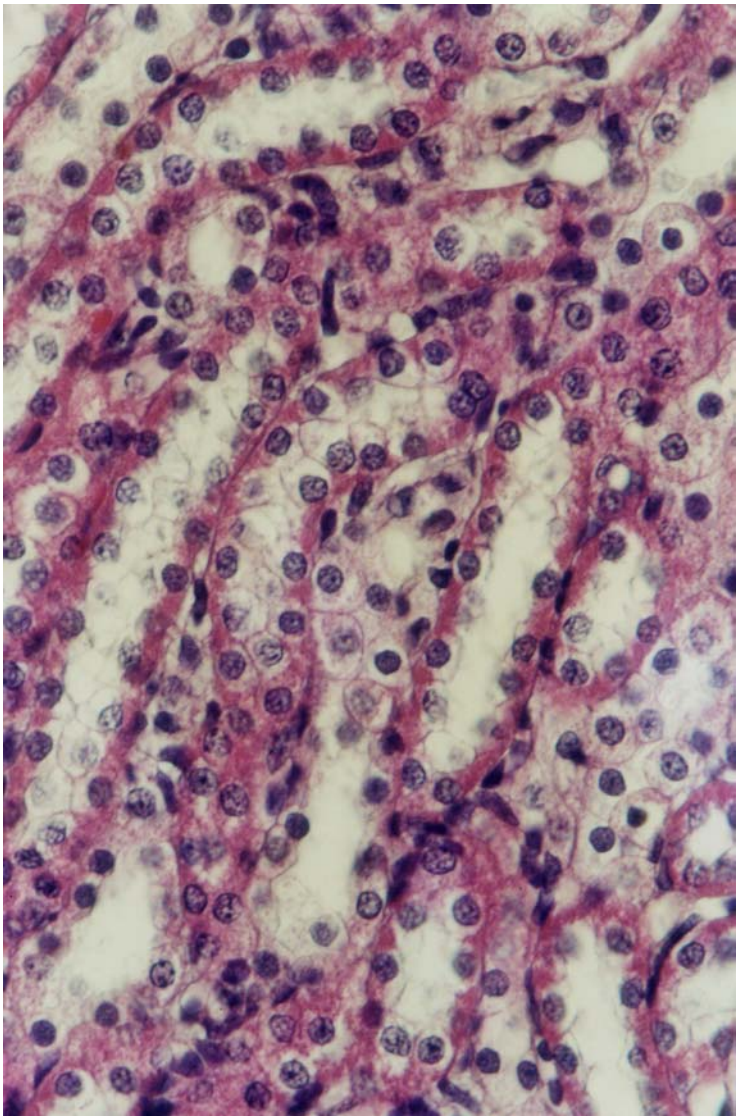


Fig. 13b. Kidney biopsy of vehicle treated rat.

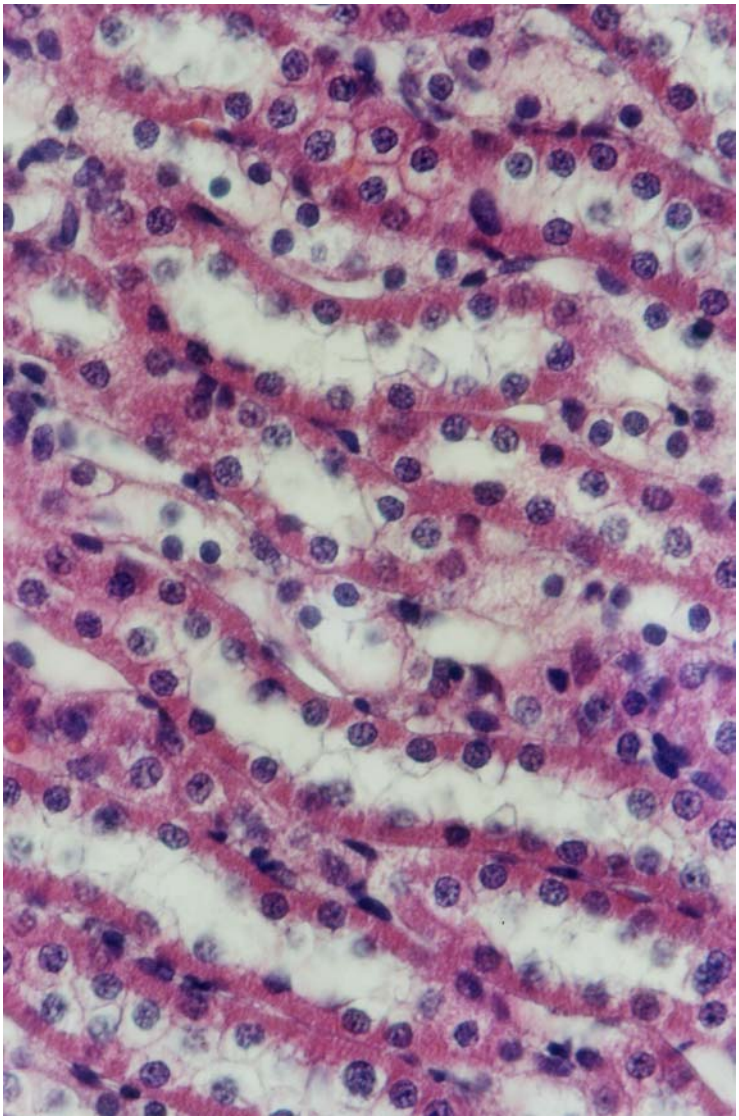


Fig. 13c. Kidney biopsy of saline treated rat.

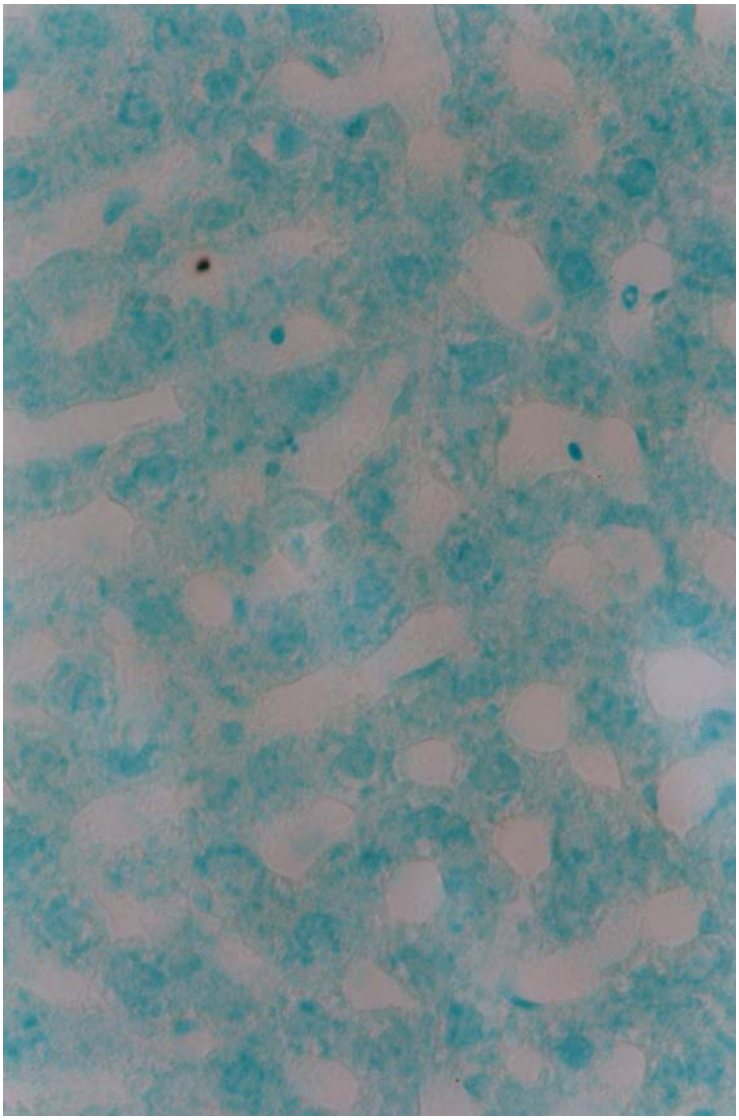


Fig. 14a. Liver TUNEL stain of herbimycin A treated rat.

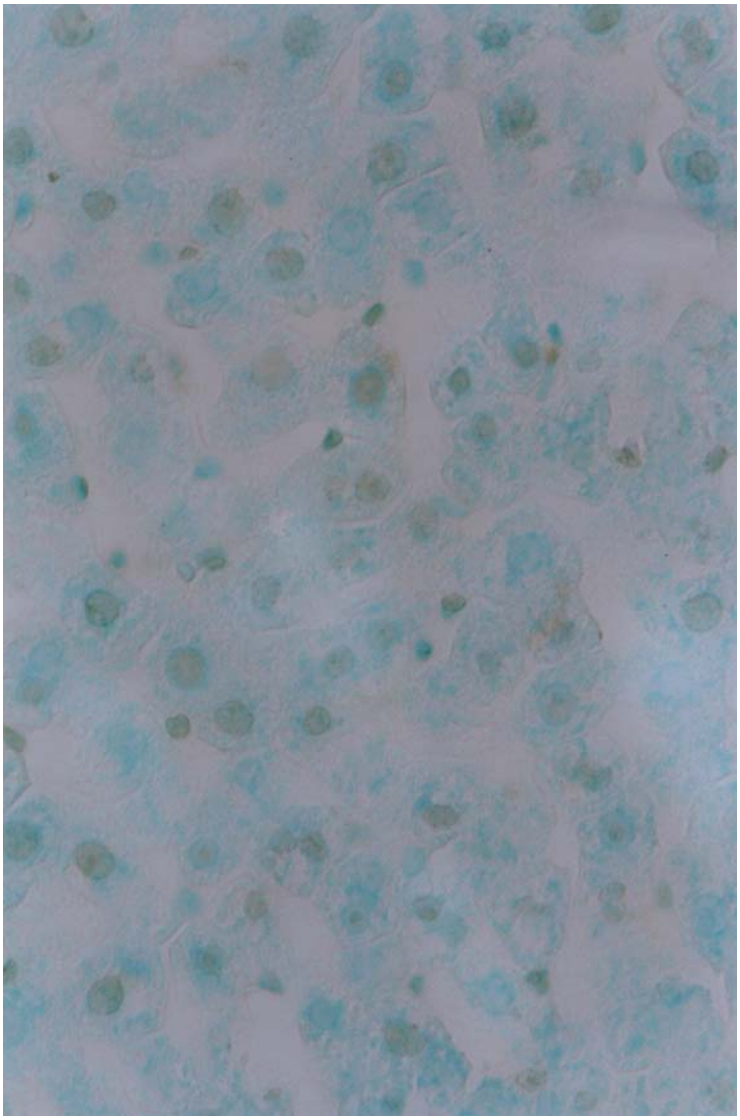


Fig. 14b. Liver TUNEL stain of vehicle treated rat.

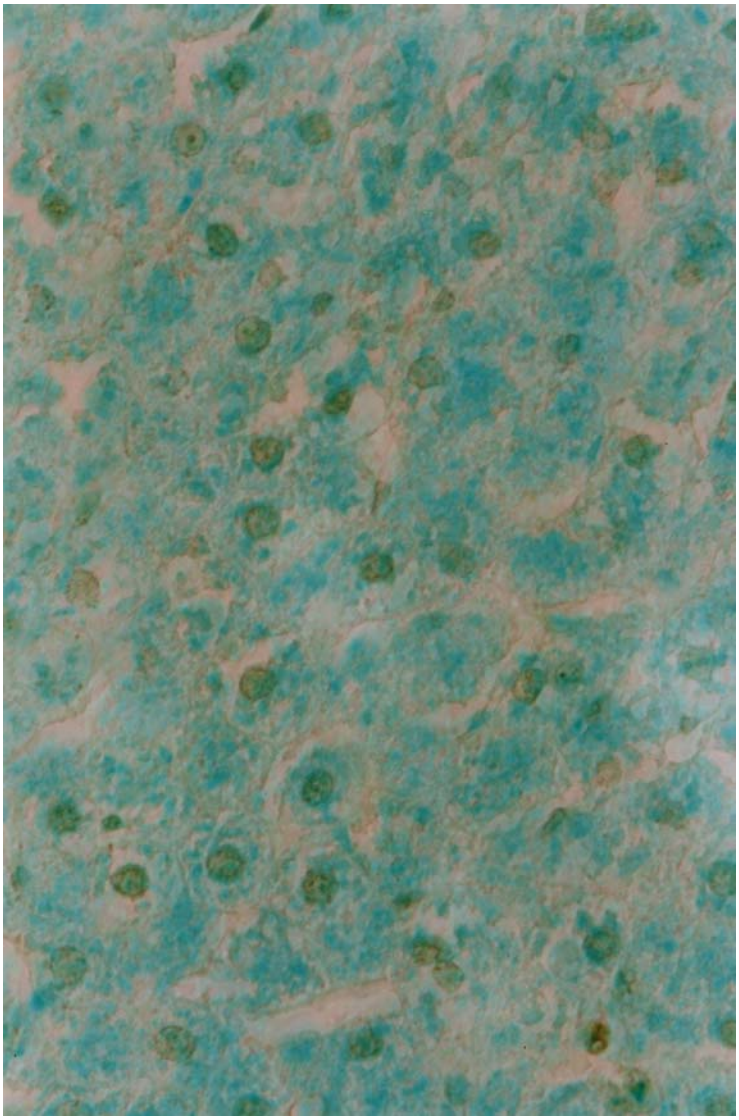


Fig. 14c. Liver TUNEL stain of saline treated rat.

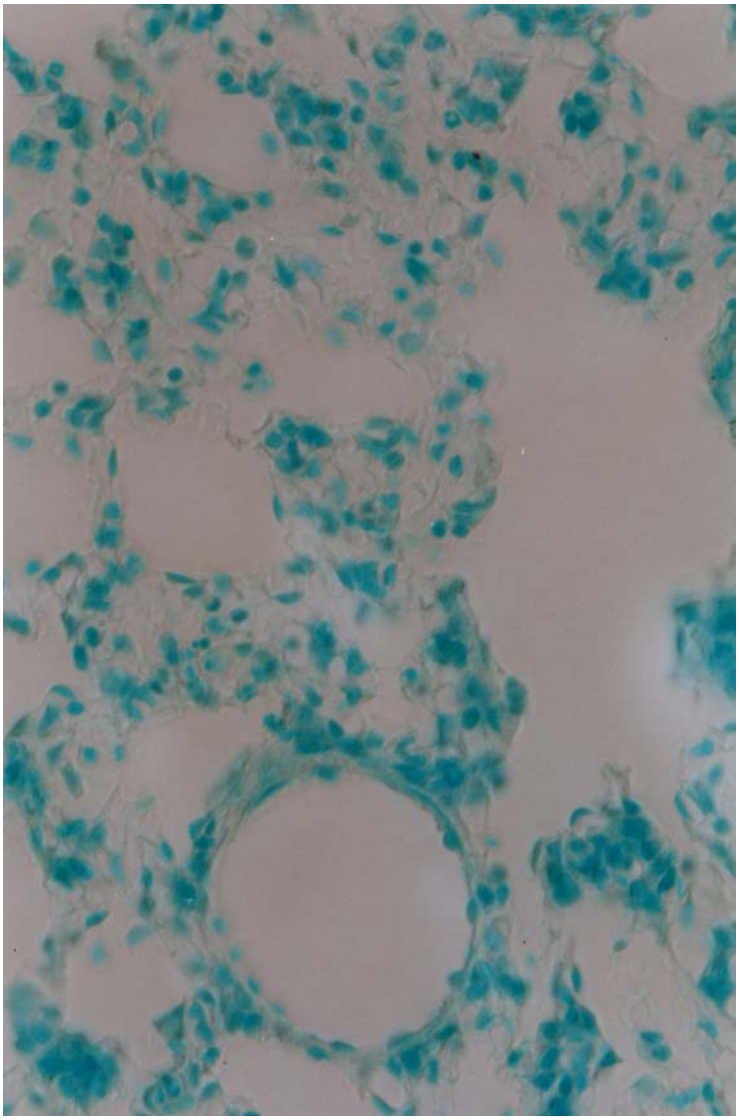


Fig. 15a. Lung TUNEL stain of herbimycin A treated rat.

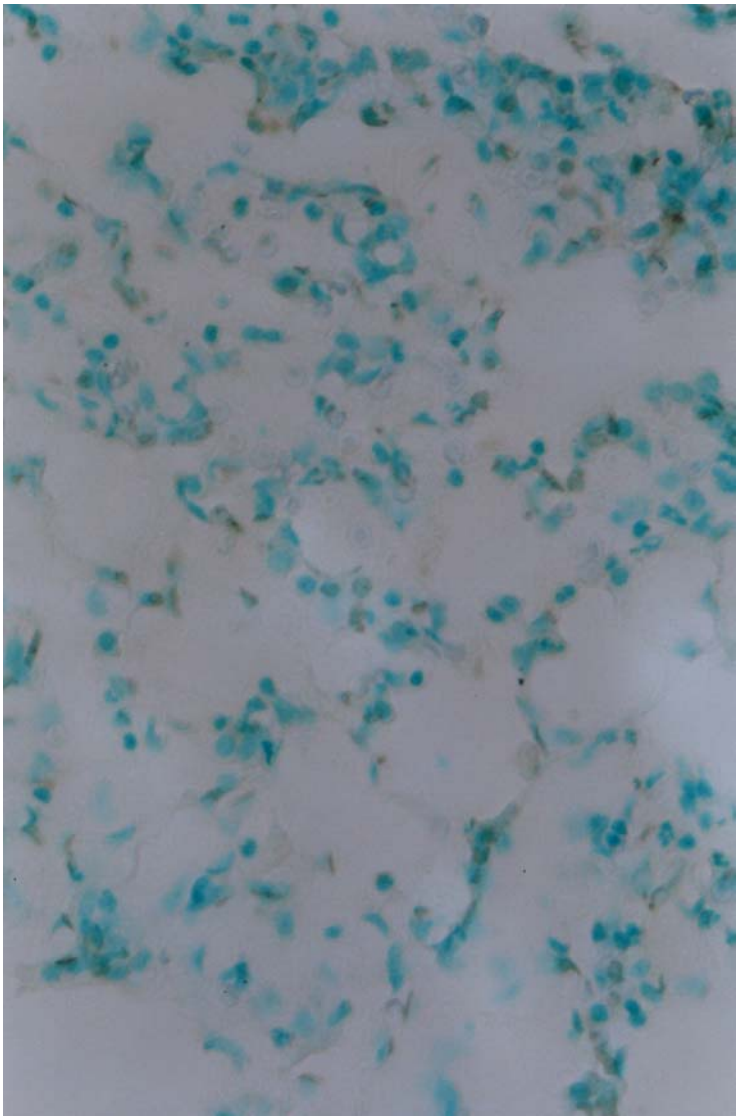


Fig. 15b. Lung TUNEL stain of vehicle treated rat.

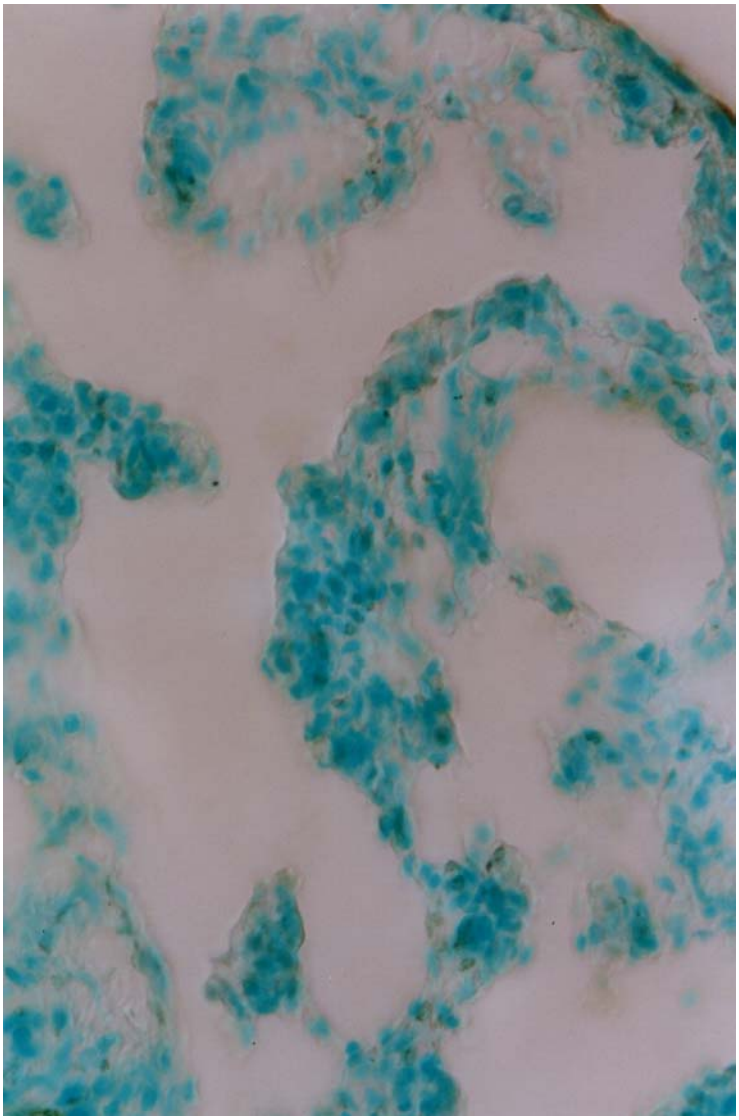


Fig. 15c. Lung TUNEL stain of saline treated rat.

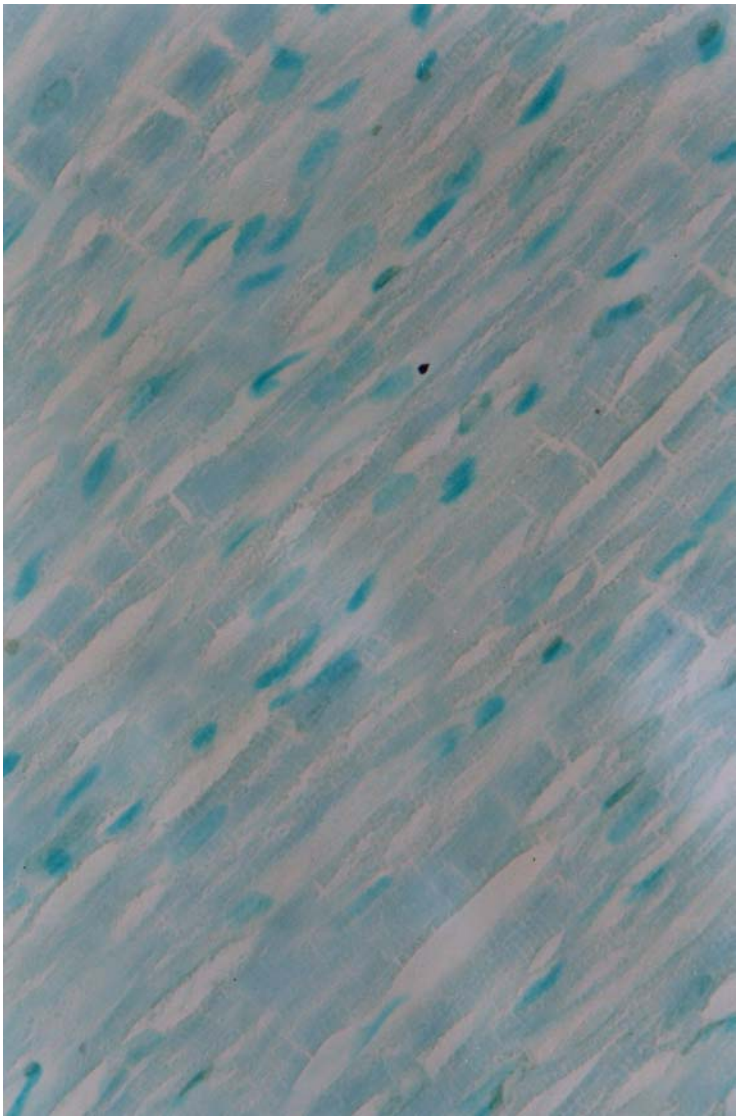


Fig. 16a. Heart TUNEL stain of herbimycin A treated rat.

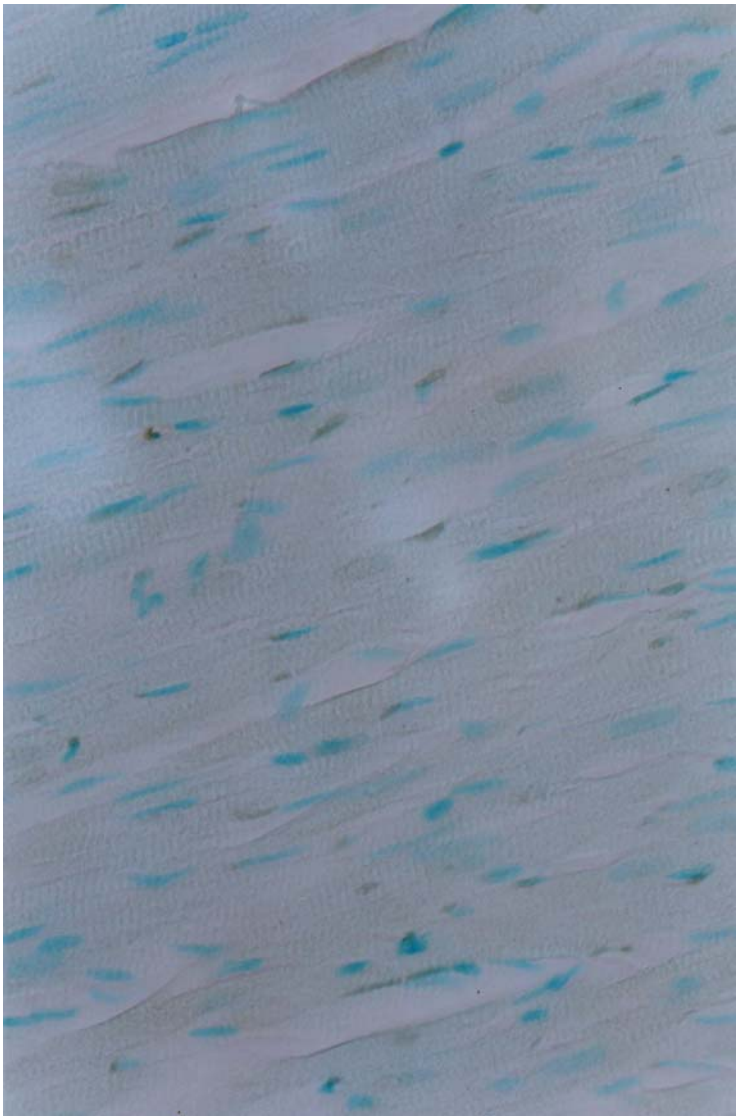


Fig. 16b. Heart TUNEL stain of vehicle treated rat.

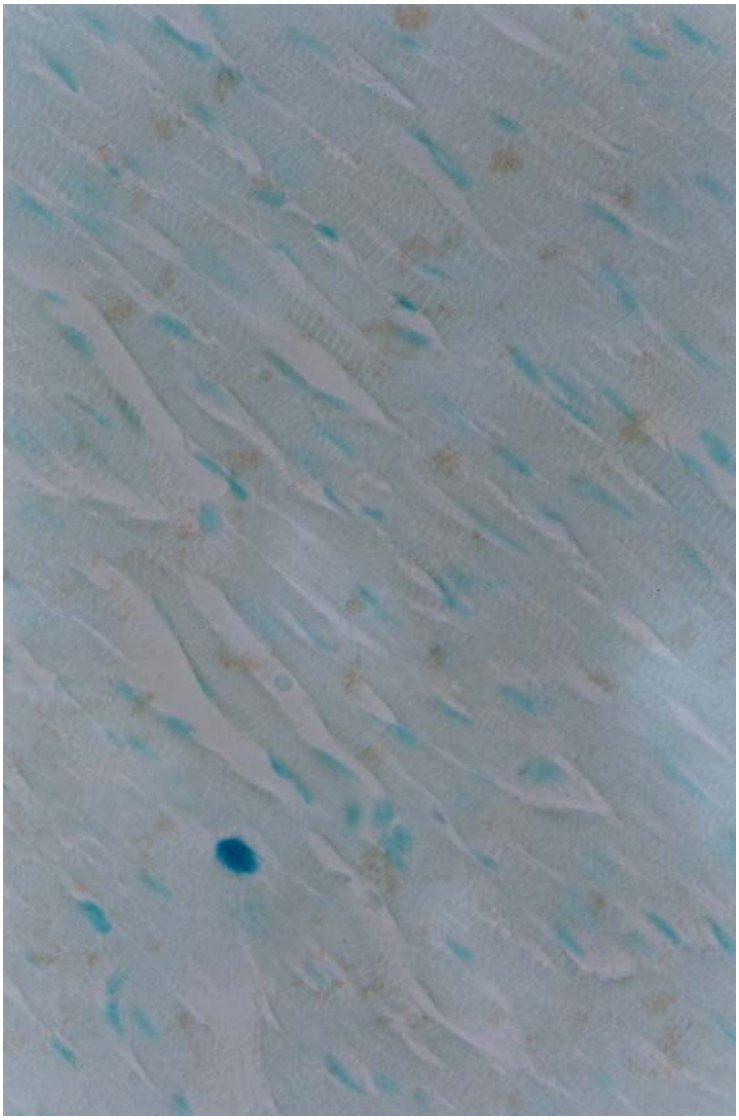


Fig. 16c. Heart TUNEL stain of saline treated rat.

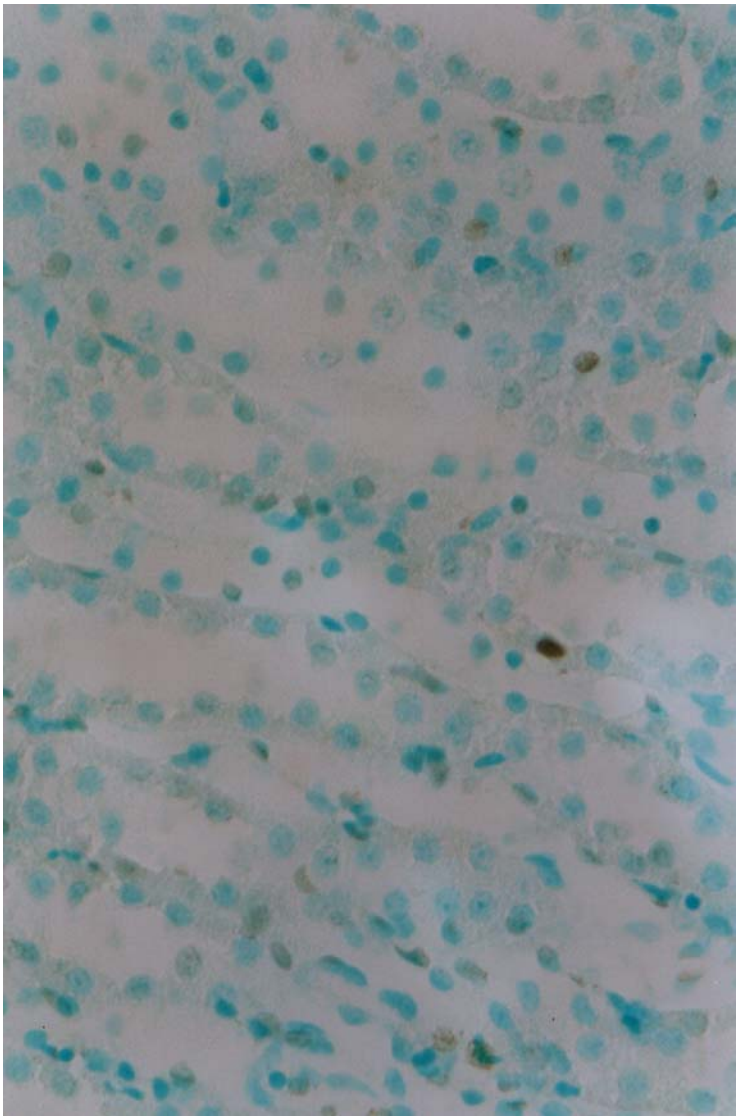


Fig. 17a. Kidney TUNEL stain of herbimycin A treated rat.

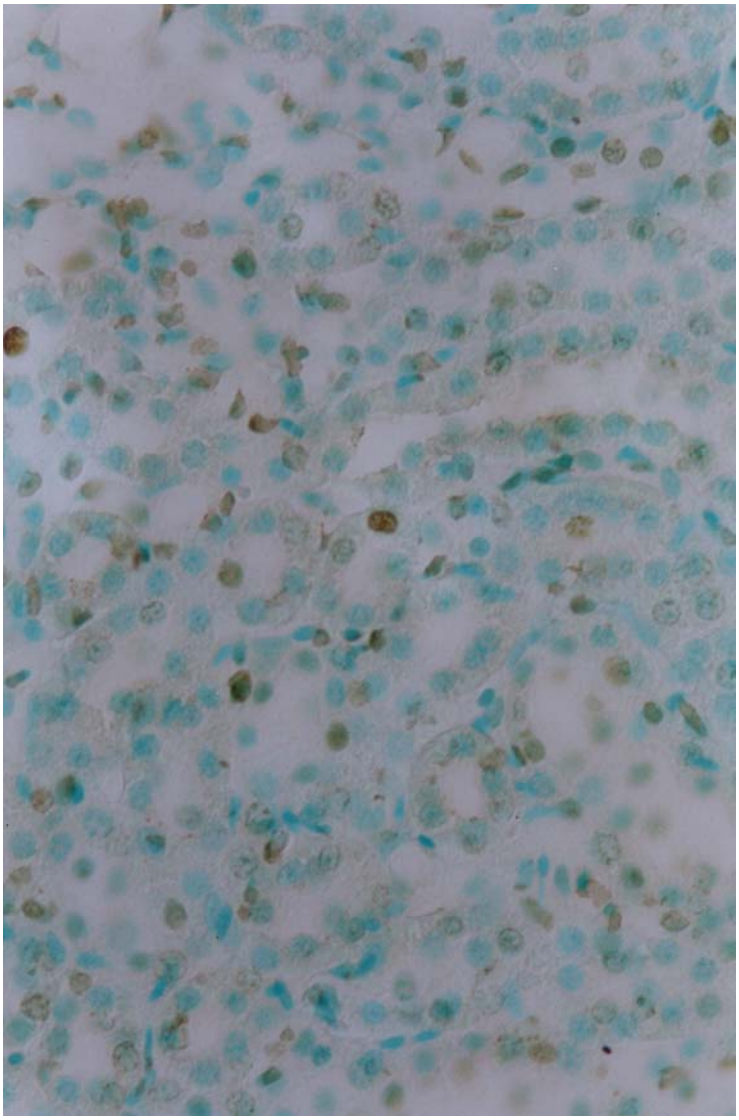


Fig. 17b. Kidney TUNEL stain of vehicle treated rat.

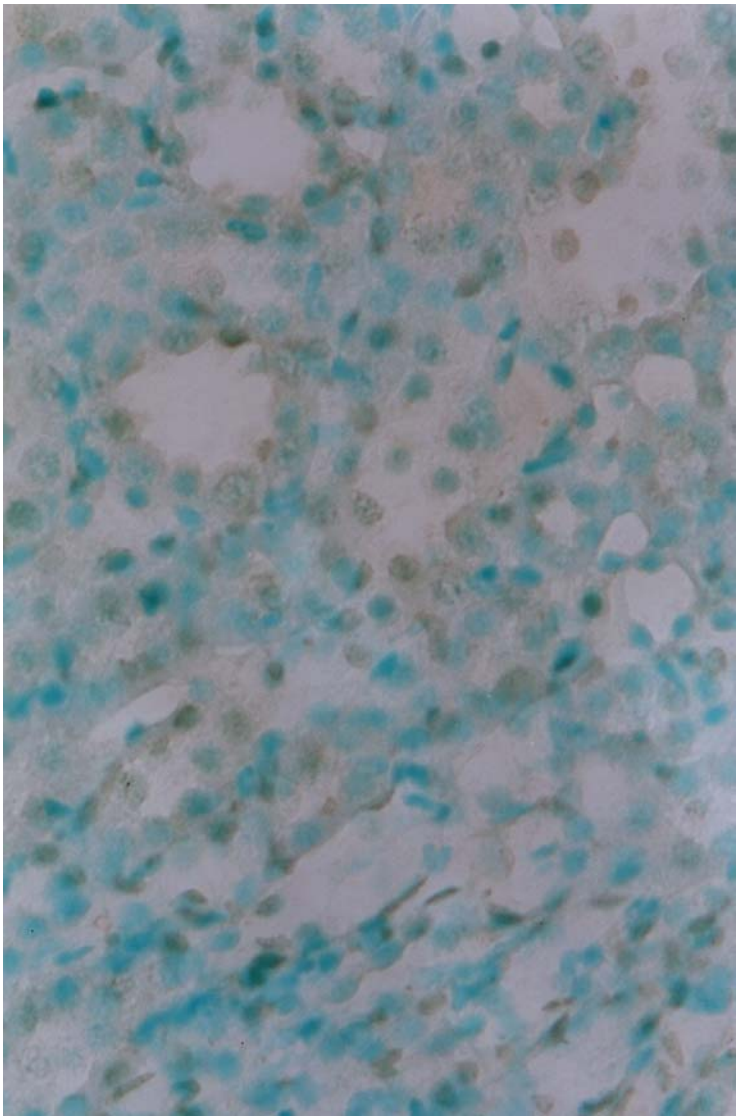


Fig. 17c. Kidney TUNEL stain of saline treated rat.

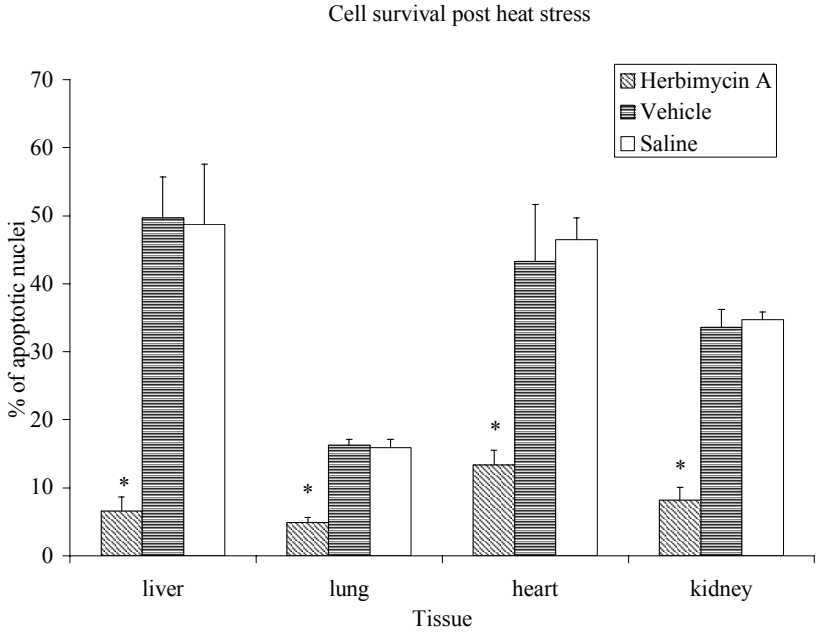
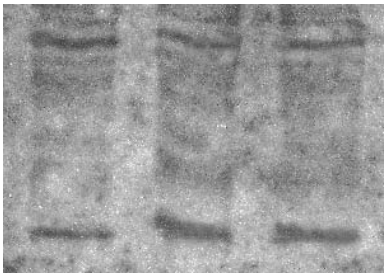


Fig. 18. Amount of apoptotic nuclei in each of the four tissues analyzed, from herbimycin A, vehicle and saline treated rats, based on the TUNEL results. The values are expressed as a percentage of the total number of cells in the respective fields. Each bar is the mean ± S.E.M. (* denotes $P < 0.05$ when compared against the corresponding tissue type of vehicle and saline treated rats).

3.7. Caspase 3 western blots

Figures 19a to 19d show the western blot data for caspase 3 activation from the liver, lung, heart and kidney respectively, for herbimycin A, vehicle and saline treated rats, 24 hours after heat stress. From the western blots, two distinct bands, at 32 kD and 20 kD were observed for all four tissues, obtained from the three treatment groups. The 32 kD band represents procaspase 3, and the 20 kD band represents active caspase 3 (Liu et al, 1996). It was observed that the procaspase 3 bands from all four tissues types of the herbimycin A treated rats were more intense, compared to the respective bands from the tissues of the vehicle and saline treated rats. Conversely, the caspase 3 bands from the tissues of the herbimycin A treated rats were less intense, compared to the respective bands from the tissues of the vehicle and saline treated rats. Figures 20a to 20b show the densitometric data of caspase 3 activation, based on the results from figures 19a to 19d. Based on the densitometric data, it can be observed that procaspase 3 was present in significantly larger amounts ($P < 0.05$) in the liver, lung, heart and kidney tissues of herbimycin A treated rats, as compared to the respective tissues from vehicle and saline treated rats. Furthermore, caspase 3 was present in lower amounts in the tissues of the herbimycin A treated rats, when compared against the respective tissues of the vehicle and saline treated rats. No statistically significant difference was observed in the densitometric data, between the tissues of the vehicle and saline treated rats.

Herb A Vehicle Saline

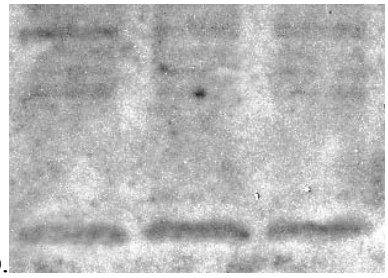


32kD

20kD

Fig. 19a.

Herb A Vehicle Saline

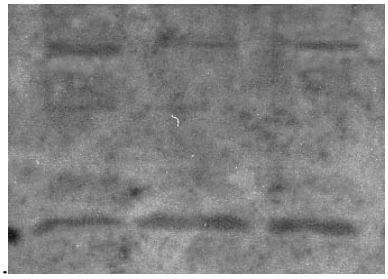


32kD

20kD

Fig. 19b.

Herb A Vehicle Saline

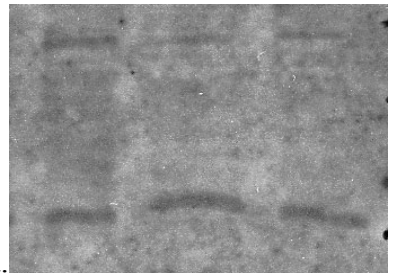


32kD

20kD

Fig. 19c.

Herb A Vehicle Saline



32kD

20kD

Fig.19d.

Fig. 19. Western blots of caspase3 from the liver (Fig. 19a.), lung (Fig. 19b.), heart (Fig. 19c.) and kidney (Fig. 19d.) of herbimycin A (Herb A), vehicle and saline treated rats. 30 μg of protein was loaded per lane.

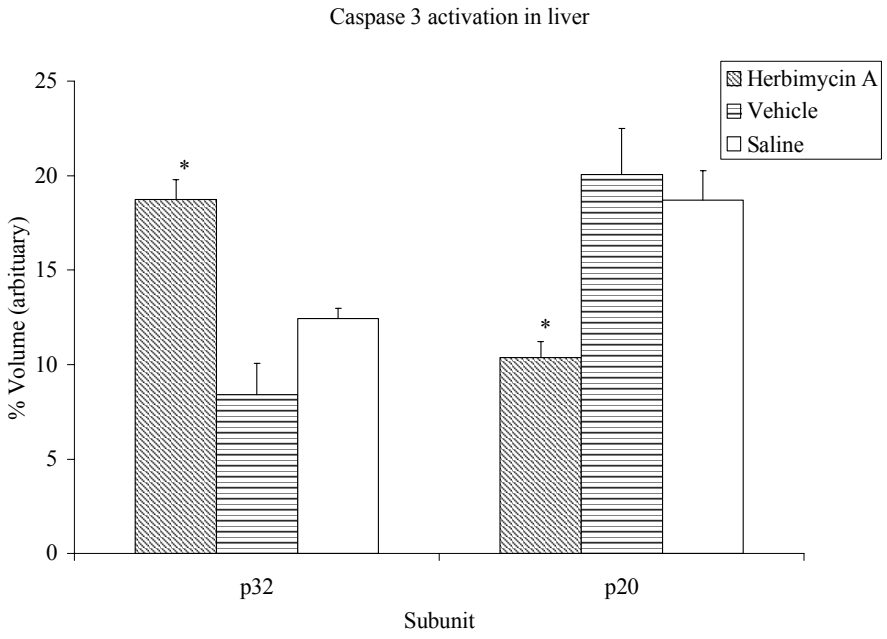


Fig. 20a.

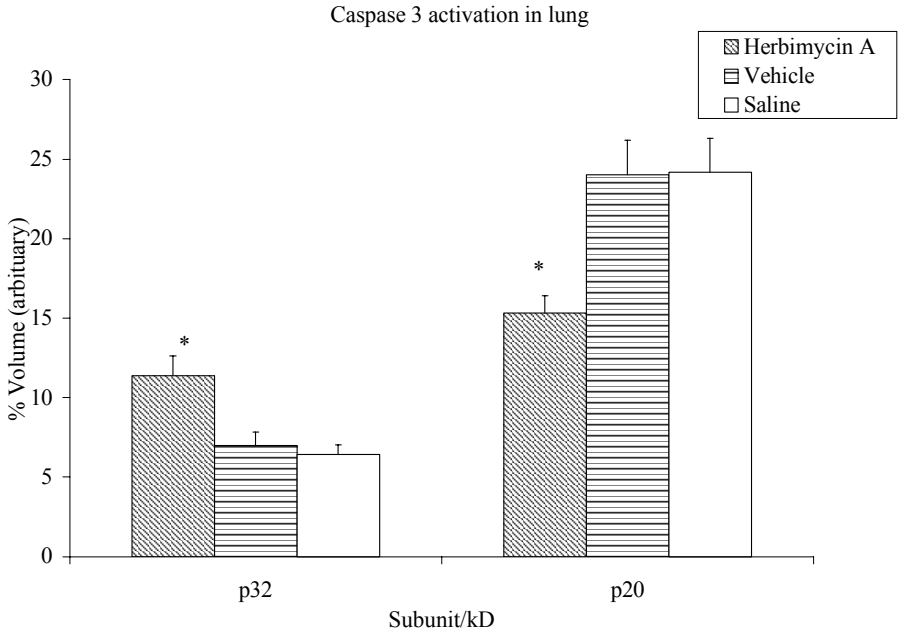


Fig. 20b.

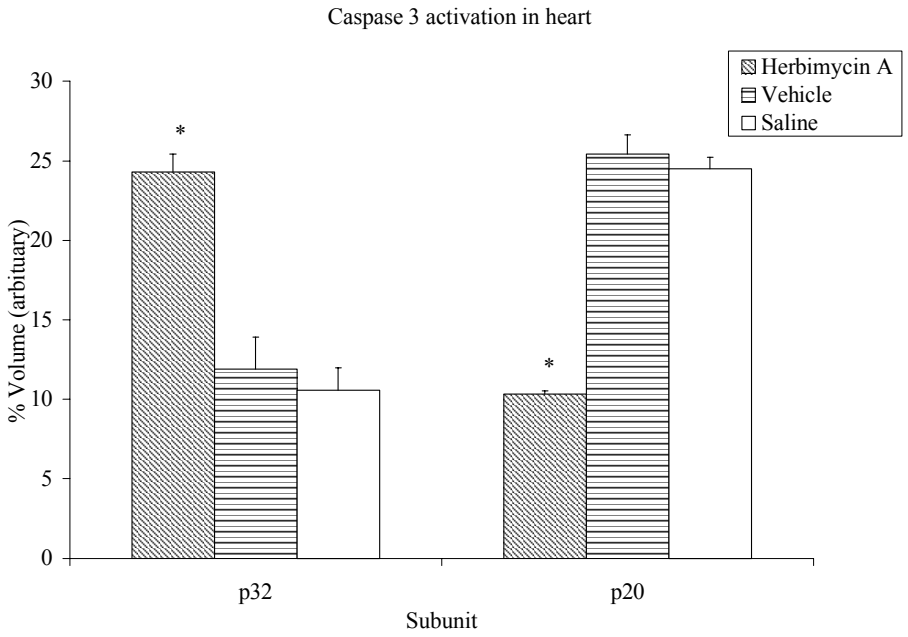


Fig. 20c.

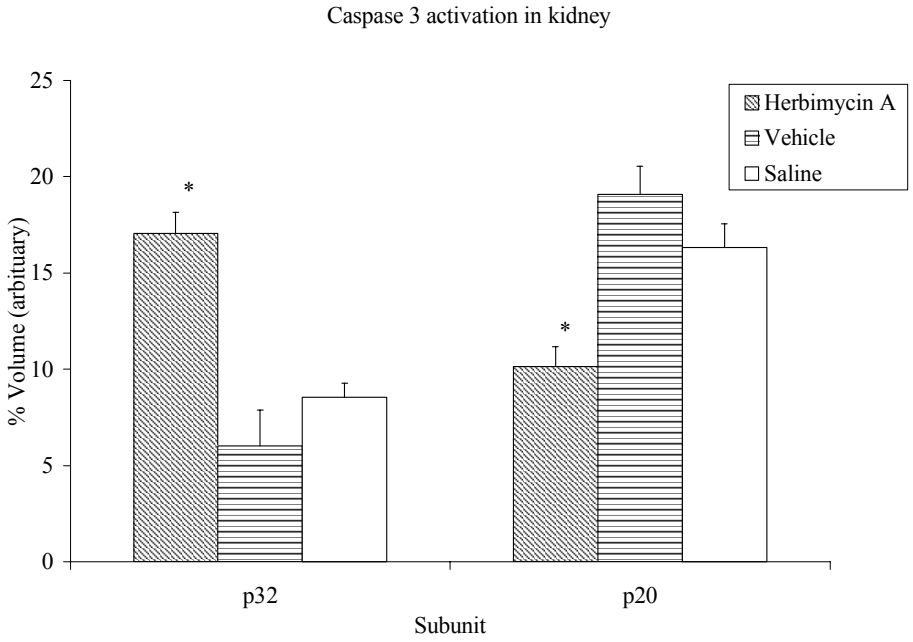


Fig. 20d.

Fig. 20. Densitometric analysis of caspase 3 activation 24 hours after heat stress, in the liver (Fig. 20a.), lung (Fig. 20b.), heart (Fig. 20c.) and kidney (Fig. 20d.), of herbimycin A, vehicle and saline treated rats. Each bar is the mean ± S.E.M. (* denotes $P < 0.05$ when compared against vehicle and saline treated rats).

Chapter 4

Discussion

4.1 Herbimycin A and hsp70

Early studies by Murakami et al (1991) reported that the benzoquinoid ansamycin antibiotic, herbimycin A, was capable of increasing the expression of hsp70, in a range of cells, including human A431 epidermoid carcinoma cells, HeLa S3 cells and chick embryo fibroblasts. Originally isolated as a potential herbicide (Omura et al, 1979), herbimycin A has been suggested to act as an inhibitor of tyrosine specific protein kinases (Uehara et al, 1989), via the inactivation of p-$60^{v\text{-}src}$ tyrosine kinase. Javadpour et al (1998), demonstrated that, besides being active in cell cultures, herbimycin A was capable of inducing the expression of hsp70 in rat tissues, namely at the gut, mesentery, pulmonary and liver tissue. They further demonstrated that herbimycin A attenuated ischemia reperfusion induced pulmonary neutrophil infiltration, suggesting that hsp70 induced by herbimycin A was responsible for the reduced tissue injury. In this study, the hypothesis that herbimycin A was able to induce hsp70 in rats, and subsequently protect the animals from heat stress, was tested. Western Blotting was performed on tissues isolated from the liver, lung, kidney and heart of the rats. These organs were selected based on the findings from previous studies, which demonstrated that hsp70 rapidly accumulates in them, in response to stress exposure and hyperthermia (Flanagan et al, 1995; Skidmore et al, 1995).

A pilot study using the IP route of administration of herbimycin A to rats was used to determine the optimum time for hsp70 expression. Based on the results obtained, it was determined that maximum hsp70 expression occurred between 12 and 18 hours post administration, in the liver, lung, heart and kidney (Fig. 4). Hence 12 hours was chosen as the point of peak hsp70 expression and the subsequent experiments were timed to allow the collection of tissues to coincide with that point. Results from the western blotting data of rats that were treated with saline and vehicle, showed no up regulation of hsp70

expression following sacrifice, 12 hours post administration, when compared to herbimycin A treated rats. Densitometry results showed that the expression of hsp70 in the vehicle and saline treated rats were similar (Fig. 7), indicating that neither of them was able to increase the expression of hsp70. Hence, the results clearly indicate that the increased expression of hsp70 observed in the rat tissues was due to the action of herbimycin A. The expression of hsp70 was enhanced in all four tissues, with the greatest increase observed in the liver and lung tissues. This might be accounted for by the differing levels at which each organ responds to stress exposure, where some organs are more sensitive to the heat shock response than others (Flanagan et al, 1995). Certain components in the hsp70 induction mechanism in response to stress and following herbimycin A treatment might be similar. Hence herbimycin A could have induced hsp70 to a greater extent in both the liver and lung, as compared to the heart and kidney. These findings from the western bolts support our hypothesis that herbimycin A can induce the expression of hsp70 in the liver, lung, heart and kidney of rats, when administered through the IP route.

The precise mechanism by which herbimycin A induces the expression of hsp70 is not known, but several postulations have been put forward to date. Murakami et al (1991) suggested that herbimycin A may interact with the sulphydryl groups of newly synthesized immature endothelial growth factor (EGF) receptors in the A431 cells, causing heat shock proteins to be induced in order to degrade the aberrant EGF receptor intermediates in the cells. Thus the induction of heat shock proteins may be the stress response of the cells to herbimycin A, which acts as a sulphydryl reagent. They further suggested that certain cellular proteins in these cells might have been damaged as a result of the drug treatment. This claim was supported by the fact that the observed subcellular location of hsp70 was

not in the nucleus, but rather in the cytoplasm of the A431 cells. The cytoplasmic localization of the stress proteins was similar to that of hsp70 induced by chemical stressors such as heavy metals, arsenite, and ethanol, in contrast to the nuclear distribution of hsp70 following heat exposure and anoxia (Welch & Suhan, 1986), thus indicating the presence of potentially denatured or aberrant proteins.

It is well accepted that the primary signal by which the stress response is initiated is through the intracellular accumulation of abnormally folded proteins (Hightower, 1980). Such an accumulation is thought to overburden the activities of molecular chaperones in the cell, many of which themselves are stress proteins. In response to the abnormally folded proteins the cell redirects its pattern of gene expression, leading to the preferential expression of heat shock proteins, many of which, through their action as molecular chaperones, facilitate the repair and replacement of essential cellular machineries damaged as a consequence of the stressful event. Support for the 'abnormal protein' hypothesis is strong and most of the potent inducers of the heat shock response, such as heavy metals, heat, and amino acid analogs, fall into the category of being protein denaturants. This view was further supported by the fact that simply injecting cells with a collection of already denatured proteins was sufficient to elicit the induction of the heat shock response (Ananthan et al, 1986). Surprisingly, Hedge et al (1995) were unable to find any evidence for the presence of any adverse effects on protein maturation or any accumulation of abnormally folded proteins in cells treated with herbimycin A. Neither did they find any changes in the integrity of the intermediate cytoskeleton, or any obvious alterations in the nucleolus, and no redistribution of hsc70 into the nucleus/nucleolus, all of which are obvious indications of a typical stress response. Hence, it can be concluded that the

induction of hsp70 by herbimycin A occurs through another mechanism, other then protein damage.

Herbimycin A did not induce hsp90, 60 or 25 in rat neonatal cardiac cells, unlike in thermal preconditioning, where the various HSPs were also induced (Morris et al, 1996). This led Morris et al (1996) to propose that the mechanism of hsp70 induction by herbimycin A may not occur via the activation of the HSF, unlike the agents or mechanisms that induce the heat shock response (Minowada & Welch, 1995). Instead, herbimycin A may act via a distinct and possibly less "stressful" pathway for hsp70 induction. This proposal was further supported by the fact that herbimycin A appeared to induce both hsp70 and hsc70 strongly, and this contrasts with the pattern of Hsp70 induction evoked by the stress response. To determine if the hsp70 induction by herbimycin A was related to its tyrosine kinase inhibitory properties, Morris et al (1996) employed another tyrosine kinase inhibitor, genistein, under similar conditions. Despite utilizing a range of doses that were adequate for tyrosine kinase inhibition, genistein was unable to induce any HSPs and was similarly unable to protect the cardiomyocytes from lethal stress, unlike herbimycin A. Hence, the tyrosine kinase inhibitory activity of herbimycin A is unlikely to be responsible for its action with regard to hsp70 induction and enhanced tolerance against lethal stress. Although genistein and herbimycin A are both tyrosine kinase inhibitors, their modes of action are quite dissimilar. Herbimycin A has a benzoquinone moiety and is thought to modify thiol groups on its target kinase covalently (Uehara et al, 1989), and therefore it may have other actions related to this thiol reactivity.

Studies have suggested that herbimycin A may directly modify the transcription factor, nuclear factor kappa b (NFκB) (Nishiya et al, 1995). NFκB is an inducible

eukaryotic transcription factor that normally exists in an inert cytoplasmic complex, bound to the inhibitory proteins of the IκB family, and it is induced by a range of stimuli, including viral and bacterial infection, ultraviolet radiation and inflammatory cytokines (Thanos & Maniatis, 1995). It was determined that the activation of NFκB requires the phosphorylation and degradation of the inhibitory protein IκBα, which permits the translocation of NFκB to the nucleus where it binds to specific κB sites in a variety of genes encoding for signaling proteins (Thanos & Maniatis, 1995). NFκB is an immediate early mediator of immune and inflammatory responses that control the replication of several viruses. It is also involved in several pathological events such as inflammation and the progression of the acquired immunodeficiency disease syndrome (AIDS) (Lenardo & Baltimore, 1989). HSF1 activation and NFκB inhibition were thought to be linked, as a number of chemical inducers of HSF1, in addition to hyperthermia, are potent inhibitors of NFκB activity (Rossi et al, 1997, 1998). It has also been shown that NFκB activation is inhibited by sodium salicylate which prevents the degradation of IκB (Kopp & Gosh, 1994). It has been further demonstrated that sodium salicylate induces the heat shock responsive chromosomal puffs in *Drosophila* salivary glands and induces HSF DNA binding activity in cultured *Drosophila* cells, as well as activating DNA binding by HSF in cultured human cells (Jurvich et al, 1992). In contrast, a recent report demonstrated that the heat shock response was able to inhibit the activation of NFκB in the absence of HSF1 (Malhotra et al, 2002). This supports the potential link between NFκB inhibition and herbimycin A, with the latter inducing the heat shock response via a pathway independent of HSF1 activation, as proposed by Morris et al (1996).

4.2 Animal model of heat stress

A free moving rat model of heat stress was developed for the purpose of evaluating the effectiveness of herbimycin A in attenuating the effects of heat stress exposure. Heat stress was achieved by exposing the animals to an elevated environmental temperature of 45 ^{o}C, for 25 minutes in a climatic chamber. A heat stress protocol was designed to raise the core temperature of the animals to 41.5 ^{o}C or greater, a temperature widely accepted as the temperature marker for heat stress (Hall et al., 2000; Kregel and Moseley, 1996). Exposure to environmental temperature of 45 ^{o}C for 25 minutes caused the animals' core temperatures to rise rapidly to values greater than 41.5 ^{o}C. Several animal studies have shown that heat stroke develops following a drastic drop in mean arterial blood pressure (Kregel et al., 1988; Lin, 1999). These studies also indicate that the mean arterial blood pressure falls rapidly when the core temperature exceeds 41.5 ^{o}C.

Based on the results obtained from the use of temperature sensing pills and rectal probes, it can be concluded that both methods are equally effective in recording the temperature profile of the rats, during heat stress exposure (Fig. 8). It was noted that during the recovery period after heat stress, the rats in the free moving model cooled at relatively faster rates, compared to the rats under restraint. This could be explained by the behavioral responses of the rats to heat stress. During the recovery period, it was observed that the rats in the free moving model constantly spread saliva over themselves, in an attempt to cool themselves, in addition to lying prostate, which is regarded as a behavioral response to heat stress (Yanase et al, 1991). The rats under restraint were not able to facilitate their cooling by similar behavioral responses. Thus the free moving model of heat stress proved to be an effective and "natural" means of heat stress exposure. Furthermore, the free moving model eliminated the possibility of hsp70 induction due to

stress caused by restraint (Udelsman et al., 1993), hence it was used to test the effectiveness of herbimycin A in thermal protection.

4.3. Herbimycin A and temperature

Temperature data obtained from the free moving heat stress model, during the evaluation of herbimycin A, showed that herbimycin A treated rats achieved significantly lower ($P < 0.05$) peak core temperatures, compared to the saline and vehicle treated rats (Fig. 9). As both vehicle and saline treated rats showed no statistically significant differences in their temperature profiles during and after heat stress, it can be concluded that herbimycin A was responsible for the lower peak core temperatures that were observed in the herbimycin A treated rats.

The observed lower temperatures may be related to the release of endotoxins from the gastrointestinal tract and cytokine production, following exposure to heat stress, as proposed by Mosely (1997). Several studies have demonstrated the presence of systemic endotoxemia (Bosenberg et al, 1988; Bouchama et al, 1991b) and elevations in circulating levels of cytokines with heat exhaustion and heat stroke. Strenuous exercise has also been demonstrated to elicit a cytokine response (Cannon & Kluger, 1983; Villar et al, 1994). It was postulated that the increases in cytokine levels could take place in response to circulating endotoxins, which could have translocated across the gut barrier, due to heat related alterations in gut permeability (Hall et al, 2001). This postulation was supported by the fact that, prophylactic gut sterilization (Gathiram et al, 1987a) and the administration of anti-endotoxin antibodies (Gathiram et al, 1987b) allowed animals to tolerate higher core temperatures. Thus the improved tolerance to heat stress could be related to the organism's improved ability to dissipate heat more efficiently, as well as the organism's

ability to either block or tolerate gut associated endotoxin translocation, down regulate cytokine production, or develop an increased tolerance to cytokine exposure. Interestingly, elevations in cellular hsp70 were found to be associated with attenuation in heat induced permeability of the epithelial monolayer (Moseley et al, 1994).

It was noted that the heat induced increase in epithelial permeability was a reversible phenomenon that occurred at temperatures that were not lethal to the individual cells (Moseley et al, 1994). Thus the association of hsp70 accumulation with the maintenance of the epithelial barrier integrity suggests a means to confer heat tolerance in a multi cellular system. The preservation of the epithelial barrier through an HSP-associated mechanism, possibly through the stabilization of the cytoskeleton or through the preservation of important cell to cell contacts, may be an important factor in preventing heat associated endotoxin translocation across the gut. HSP associated heat adaptation may also be related to endotoxin tolerance, where conditioning stresses that result in HSP accumulation or the over expression of the hsp70 gene in cells may confer tolerance to endotoxins in animals (Hotchkiss et al, 1993; Ryan et al, 1992; Saklatvala et al, 1991) and cells (Chi & Mestril, 1996). This endotoxin resistance may reflect an HSP-associated change in cytokine production and resistance. In support of this proposal, macrophages that were stimulated to accumulate HSPs showed both transcriptional inhibition and decreased secretion of inflammatory cytokines TNF-α and interleukin 1 (IL-1) (Ensor et al, 1994; Synder et al, 1992). In a similar fashion, animals that were made to undergo a conditioning stress sufficient to cause hsp70 accumulation showed a decrease in circulating TNF-α after endotoxin exposure (Kluger et al, 1997). It was further demonstrated that the cellular accumulation of HSP made the cells resistant to the cytotoxic effects of TNF-α (Jaattela et al, 1992; Landry et al, 1989). It should be also noted

that both TNF-α and IL-1 are capable of up regulating the expression of HSPs (Freshney et al, 1994; Saklatvala et al, 1991). Hence the ability of herbimycin A to attenuate the peak temperatures attained after heat stress exposure could be related to the effects of increased expression of hsp70, as described above.

4.4. Herbimycin A and tissue injury

The rats were allowed to recover for duration of 24 hours, before sacrifice and tissue harvesting for histology analysis. From the H&E staining, no obvious changes in morphology were observed between the herbimycin A treated rats, and the vehicle and saline treated rats, in the liver, lung, heart and kidney. Gross morphological features of necrosis were absent. It is possible that a period of 24 hours post-heat stress was too short a time frame in which to observe any morphological changes that could be attributed to tissue necrosis. However, it is well known that heat stress causes apoptosis, or programmed cell death. Apoptosis plays an important role in the removal of unwanted cells during the development of organisms, as well as in adult homeostasis (Jacobson et al, 1997). It has been shown that apoptotic nuclei in tissues can be detected as early as 12 hours post stress exposure, and can decline to basal levels within 48 hours (Sakaguchi et al, 1995). Thus the TUNEL staining was able to pick out the presence of apoptotic nuclei in the tissues of the heat stressed rats.

Essentially, there are two pathways that can lead to apoptosis, namely the mitochondrial (intrinsic) pathway and the death receptor (extrinsic) pathway. In the intrinsic pathway, the mitochondria play two roles as an integrator of multiple proapoptotic signaling cascades and as a coordinator of the catabolic reactions that lead to apoptosis. As a result of the multiple apoptotic signals of various sources (Ferri et al,

2001), the outer membrane of the mitochondria becomes permeable resulting in the release of molecules that are normally confined to the mitochondrial intermembrane space. These molecules translocate from the mitochondria to the cytosol, via Bcl-2 and Bcl-2-related protein controlled reactions (Kroemer & Reed, 2000). Several molecular mechanisms have been proposed for this permeabilization of the outer mitochondrial membrane. They include the physical disruption of the outer membrane as a result of mitochondrial matrix swelling due to non-specific pore formation in the inner membrane and increased net influx of ions and water, and pore formation in the external mitochondrial membrane by proteins such as Bax (Zamzami et al, 1995; Antonsson et al, 1997; Schlesinger et al, 1997; Marzo et al, 1998; Zamzami & Kroemer, 2001)

Cytochrome *c* is one of these released intermembrane proteins, and once in the cytosol, it interacts with apoptotic protease activation factor 1 (Apaf-1), hence initiating the ATP-dependent oligomerization of Apaf-1, simultaneously exposing its caspase recruitment domain (CARD domain) (Hu et al, 1999; Li et al, 1997). Oligomerized Apaf-1 then binds to the cytosolic procaspase-9 in a homotopic interaction involving the CARD domain of caspase-9, hence leading to the formation of the caspase-9 activation complex, the apoptosome. Activated caspase-9 then causes the proteolytic activation of caspase-3, hence initiating the caspase cascade (Li et al, 1997), culminating in apoptotic cell death. Besides cytochrome *c*, the flavoprotein apoptosis inducing factor (AIF) and second mitochondria derived activator of caspase (Smac) are also released from the mitochondria. Smac activates apoptosis through the neutralization of inhibitory activity of inhibitory apoptotic proteins (IAPs) that associate with and inhibit the activity of caspases (Du et al, 2000). AIF on the other hand directly translocates to the nucleus, where it triggers caspase-independent nuclear changes (Susin et al, 1999; Joza et al, 2001; Daugas et al, 2000).

The extrinsic pathway of apoptosis involves the recruitment of plasma membrane death receptors. These receptors (TNF-R1, CD95/APO-1/Fas, TRAIL-R1 etc) are members of the TNF receptor superfamily, and they play a role in inflammatory and immune responses. These death receptors have an intra-cytoplasmic domain, the death domain (DD), which in turn interact with the DD of the cytosolic TNF receptor death domain protein (TRADD) or Fas associated death domain protein (FADD). These proteins contain death effector domains (DED) that interact with the DED of procaspase 8, thereby initiating their auto proteolytic activation to form active caspase 8. Apoptosis is then initiated by either the direct activation of the other caspases downstream of caspase 8 or via the mitochondrial apoptotic pathway (Scaffidi et al, 1999), triggering the permeabilization of the mitochondrial outer membrane.

Based on the results of the TUNEL staining, it is clearly evident that herbimycin A was able to reduce the percentage of apoptotic nuclei following heat stress, as compared to vehicle and saline treated rats, which showed no statistically significant differences in their scores (Fig. 18). The western blot results based on caspase 3 activation (Fig. 19a to19b, and Fig. 20) show that procaspase 3 was cleaved to active caspase 3 to a greater extent in the liver, lung, heart and kidney tissues of the vehicle and saline treated rats, compared to the respective tissues of the herbimycin A treated rats. This could account for the higher level of apoptotic nuclei observed in the tissues of the vehicle and saline treated rats post heat stress. The presence of the over expression of hsp70 in the tissues of herbimycin A treated rats could have been responsible for the inhibition of apoptosis in these tissues.

Indeed it is interesting to note that hsp70 is capable of inhibiting apoptosis, hence increasing the survival of cells exposed to a variety of lethal stimuli (Jaattela et al, 1992; Mosser et al, 1997). A summary of how hsp70 can inhibit apoptosis is shown on Fig. 21.

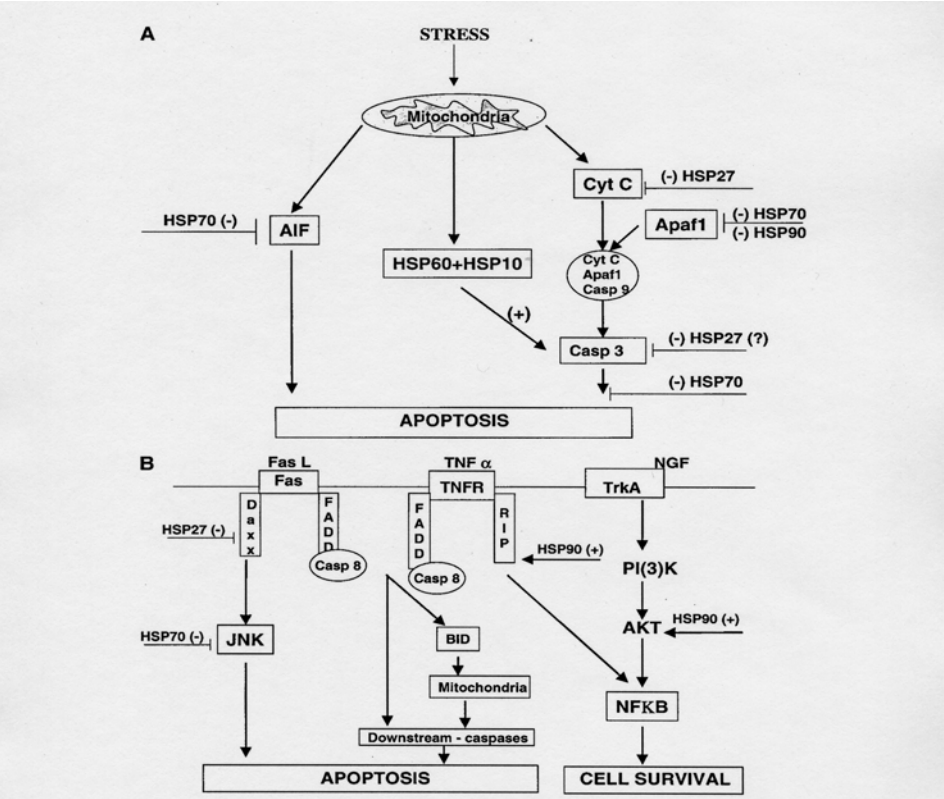


FIG. 1. Proposed apoptosis regulatory function of HSPs. (A) Modulation of the intrinsic pathway of apoptosis by HSPs. HSP27 binds to cytochrome *c* (53), HSP70 and HSP90 bind to Apaf 1 (67, 68, 83) in all cases resulting in the inhibition of apoptosome formation and thereby prevention of caspase-9 maturation. Additional negative influence of HSP27 and HSP70 on events further downstream have also been described (54, 72). In contrast, HSP60 and HSP10 can directly promote the proteolytic maturation of caspase-3 (88, 89). (B) Modulation of the extrinsic pathway of apoptosis by HSPs. HSP27 interacts with and inhibits Daxx apoptotic pathway (56) while HSP70 binds to JNK1 resulting in inhibition of JNK activation (74). HSP90 interacts with RIP-1 kinase and Akt resulting in both cases in promotion of NF-κB-mediated inhibition of apoptosis (84, 87). A minus sign denotes negative regulation a plus sign indicates positive regulation, and a question mark indicates putative effects.

Fig. 21. Hsp70 and Apoptosis (adapted from Garrido et al, 2001).

It was proposed that hsp70 could offer protection to cells from energy deprivation and/or ATP depletion associated with cell death (Wong et al, 1998). Through the generation of transient or stable transfections, it was shown that elevated levels of hsp70 reduced or blocked caspase activation and hence suppressed mitochondrial damage and nuclear fragmentation (Buzzard et al, 1998). These findings were reinforced by the discovery that hsp70 inhibited apoptosis downstream of the release of cytochrome *c* and upstream of caspase 3 activation (Li et al, 2000). It was proposed that this inhibition occured via a hsp70 mediated modulation of the apoptosome. Furthermore, hsp70 was shown to directly bind to Apaf-1, hence preventing the recruitment of procaspase 9 to the apoptosome (Saleh et al, 2000; Beere et al, 2000). One study demonstrated that the over expression of hsp70 protects Apaf-$1^{-/-}$ cells from apoptotic cell death induced by serum withdrawal (Ravagnan et al, 2001), indicating that Apaf-1 is not the only target of hsp70. It was suggested that hsp70 directly binds to AIF and inhibits AIF induced chromatin condensation. This was based on the fact that hsp70 inhibited apoptosis induced by the over expression of both full length AIF, which have to leave the mitochondria to become apoptogenic (Susin et al, 1999; Loeffler et al, 2001), and AIF lacking the mitochondrial localization sequence (AIF Δ1-100). Moreover, endogenous levels of hsp70 appeared to be sufficiently high to control AIF mediated apoptosis, as down regulation of hsp70 by an antisense construct sensitized the cells to serum withdrawal and AIF (Ravagnan et al, 2001). Also, hsp70 was shown to prevent morphological changes downstream of caspase 3 activation that are characteristic of dying cells (Jaattela et al, 1998). It was also proposed that hsp70 interacted with the apoptotic pathway at early steps, such as by preventing JNK activation (Meriin et al, 1999). Indeed it was shown that hsp70 binds to and functions as a natural inhibitory protein of c-Jun N-terminal Kinase (JNK1) (Park et al, 2001). Hence our hypothesis that

herbimycin A is able to attenuate the injury incurred to cells as a result of heat stress exposure is valid.

4.5 Conclusion

Through our work, it is clearly evident that herbimycin A is capable of inducing hsp70 in vivo, namely in the rat. Though we did not directly show that hsp70 was responsible, it can be seen that hsp70 was a likely mediator of the thermoprotective properties of herbimycin A. Rats with high levels of hsp70 in their tissues were able to maintain lower peak core temperatures following heat stress, and also demonstrated a lower percentage of apoptotic nuclei in the tissues under investigation. This is in contrast to rats with lower levels of hsp70, where the reverse was observed. This study has confirmed that herbimycin A can be used effectively to protect rats exposed to heat stress. However, the mechanisms of action of herbimycin A remain elusive. Future work concerning the study of the effect of NFκB inhibition (refer to "Herbimycin A and hsp70" under section 4.1 of discussion) could throw more light onto the possible mechanisms of the action of herbimycin A. Whether it can perform a similar function in humans is yet to be determined, but if so, it would provide a tremendous opportunity to exploit the potentially beneficial effects of hsp70 upregulation in the prevention and treatment of disease conditions.

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