Effect of heat shock factor inhibitor, KNK437, on stress-induced *hsp30* gene expression in *Xenopus laevis* A6 cells

by

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I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners. I understand that my thesis may be made electronically available to the public.

Abstract

Prokaryotic and eukaryotic organisms respond to various stresses with the production of heat shock proteins (HSPs). HSPs are molecular chaperones that bind to unfolded proteins and inhibit their aggregation as well as maintaining their solubility until they can be refolded to their original conformation. Stress-inducible *hsp* gene transcription is mediated by the heat shock element (HSE), which interacts with heat shock transcription factor (HSF). In this study, we examined the effect of KNK437 (N-formyl-3,4-methylenedioxy-benzylidene-g-butyrolactam), a benzylidene lactam compound, on heat shock, sodium arsenite, cadmium chloride and herbimycin A-induced *hsp* gene expression in *Xenopus laevis* A6 kidney epithelial cells. In studies limited to mammalian cultured cells, KNK437 has been shown to inhibit HSE-HSF1 binding activity and stress-induced *hsp* gene expression. In the present study, western and northern blot analysis revealed that exposure of A6 cells to heat shock, sodium arsenite, cadmium chloride and herbimycin A induced the accumulation of HSP30 protein and *hsp30* mRNA, respectively. Western blot analysis also determined that exposure of A6 cells to heat shock, sodium arsenite, cadmium chloride and herbimycin A induced the accumulation of HSP70 protein. Pre-treatment of A6 cells with 100 µM KNK437 inhibited stress-induced *hsp30* mRNA as well as HSP30 and HSP70 protein accumulation. Immunocytochemistry and confocal microscopy were used to confirm the results gained from western blot analysis as well as determine the localization of HSP30 accumulation in A6 cells. A 2 h heat shock at 33°C resulted in the accumulation of HSP30 in the mostly in the cytoplasm with a small amount in the nucleus. Heat shock at 35°C resulted in substantial HSP30 accumulation in the nucleus. This is in contrast with A6 cells treated for 14 h with 10 µM sodium arsenite, 100 µM cadmium chloride and 1 µg/mL herbimycin A, where HSP30 accumulation was found only in the cytoplasm and

not in the nucleus. A 6 h pre-treatment with 100 µM KNK437 completely inhibited the

accumulation of HSP30 in A6 cells heat shocked at 33 or 35°C as well as cells treated with 1

µg/mL herbimycin A. The same pre-treatment with KNK437 resulted in a 97-100% decrease in

HSP30 accumulation in A6 cells treated with 10 µM sodium arsenite or 100 µM cadmium

chloride. These results show that KNK437 is effective at inhibiting both heat shock and

chemical induced *hsp* gene expression in amphibian cells.

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1 Introduction

Throughout evolution, it has been imperative that organisms, both prokaryotic and eukaryotic, have the ability to survive and adapt to thermal stress. Thermal stress leads to a complex regulation of gene expression and biochemical adaptive responses (Lindquist, 1986). Within the category of altered gene expression, there is a variation in the pattern of expression of stress inducible proteins known as the heat shock proteins (HSPs) (Lindquist & Craig, 1988; Feige *et al*., 1996).

1.1 Heat Shock Proteins

Ritossa and colleagues were the first to discover HSPs in the salivary glands of *Drosophila* in 1962. The heat shock family is composed of six major families, HSP100, HSP90, HSP70, HSP60, HSP40 and the small HSP family (Katschinski, 2004). Certain HSPs including HSP60, HSC70 and HSP90 are present constitutively in the cell and function in protein folding, assembly and transport (Georgopoulos and Welch, 1993), while others such as HSP70 are stress inducible (Lisowska *et al*., 1994; Feige *et al*., 1996). HSPs act as molecular chaperones, which bind to damaged and denatured proteins in order to prevent their aggregation and misfolding during cellular stress. The exact method that cells use to sense stress is largely unknown, however it is thought that damaged protein is likely involved in the sensing mechanism (Agashe & Hartl, 2000).

1.2 The heat shock response

In addition to heat, HSPs are synthesized in response to many other stressors. Environmental stress including heavy metals, arsenites, ethanol and hydrogen peroxide can induce HSP accumulation as well as pathophysiological states such as aging, damage by oxygen

radicals and bacterial and viral infection. It has also been shown that non-stressful conditions such as development and the presence of growth factors and can induce *hsp* gene expression. The quantity of HSPs expressed varies with the inducer or environment that the cells are subjected to (Lindquist and Craig, 1988; Parsell and Lindquist, 1993; Morimoto *et al.*, 1994; Feige *et al.*, 1996).

HSP gene regulation occurs primarily at the transcriptional level, although control at the posttranscriptional and translational levels has been described (Lis and Wu, 1993; Morimoto, 1993). Heat shock transcription factors (HSFs) are involved in the activation of stress-inducible *hsp* gene expression (Morimoto *et al.*, 1994). Only one *hsf* gene has been isolated from *Saccharomyces cerevisiae* (Sorger & Pelham, 1988; Wiederrecht *et al.*, 1988) and from *Drosophila melanogaster* (Clos *et al.*, 1990). However, multiple members of the HSF family have been found in vertebrates (HSF1-4) (Wu, 1995; Nover *et al.*, 1996; Morano & Thiele, 1999; Nakai, 1999). HSF1 is stress inducible and is homologous to the single HSF gene found in yeast and fly. HSF2 does not appear to be stress inducible, but instead is activated during developmental processes. HSF3 and HSF4 have only been characterized in the avian and mammalian systems, respectively (Nakai, 1999). The expression of HSF3 in chicken is limited to undifferentiated cells and embryonic tissue. HSF1 and HSF3 seem to have redundant roles in undifferentiated cells, however, HSF3 is expressed under heat shock conditions in the blood cells of chicken embryos, where HSF1 is lacking (Shabtay & Arad, 2006). HSF4 is constitutively active as a trimer as it lacks a leucine zipper-like domain (HR-C) which suppresses trimer formation. Information gained from HSF4 mouse knockouts determined that HSF4 is required for cell growth and differentiation of the eye lens during development (Fujimoto *et al.*, 2004).

All HSF proteins have very divergent amino acid sequences with the exception of two well-conserved domains; the N-terminal DNA binding domain and the trimerization or activation domain. Adjacent to the DNA binding domain there are hydrophobic heptad repeats (HR-A/B) that act as leucine zippers. There is also an additional hydrophobic heptad region (HR-C) located near the C-terminus (Lis and Wu, 1993; Morimoto, 1993) (Figure 1). Both HSF1 and HSF2 genes have been found in *Xenopus laevis* (Stump *et al*., 1995; Hilgarth *et al*., 2004).

With the exception of yeast, HSFs are found in the cell in a monomeric state and have no DNA binding ability until the cell is stressed. There are several steps in the stress inducible HSF1 regulatory pathway. The first is the stress-induced conversion of HSF monomers to homotrimers, which allows the HSF1 trimers to bind to a conserved 15 bp region of the heat shock element (HSE) found upstream of heat shock protein genes (Figure 2). The second step involves a conformational change to the transcriptional activation domain (Westwood *et al*., 1991; Westwood and Wu, 1993). Removal of the stressor leads to the final stage of the HSF1 regulatory pathway called deactivation. HSF1 trimers return to a monomeric state and no longer activate transcription of *hsp* genes. If the cells are exposed to the stressor for a long period of time, HSF1 may lose its binding and transcriptional abilities before removal of the stressor. This process is called attenuation (Clos *et al*., 1990; Rabindran *et al*., 1991; Nakai & Morimoto, 1993).

Figure 1. Functional organization of human HSF1. N and C represent the amino and carboxy

terminals respectively. Adapted from Locke and Noble (2002).

DNA Binding Domain

Heptad Repeats A/B

Heptad Repeat C

Activation Domain

N

C

Negative Regulation Domains
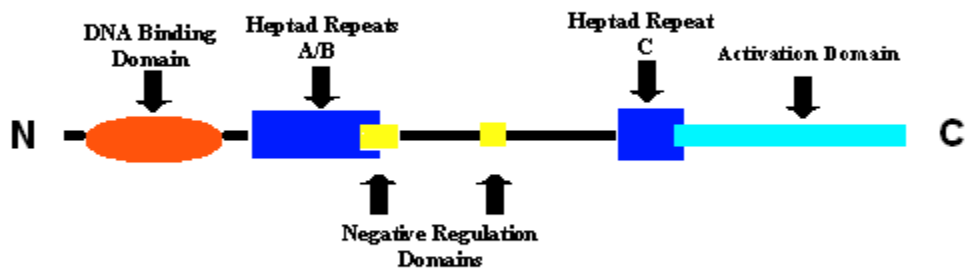
Figure 2. HSF Activation Model. Adapted from Locke and Noble (2002).

Inactive HSF monomer in unstressed cells
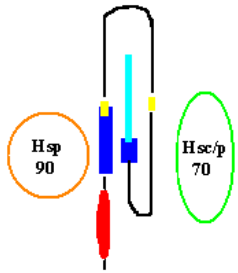
Inactive HSF monomer in stressed cells

Active HSF trimer in stressed cells

Hsp 90

Hsc/p 70
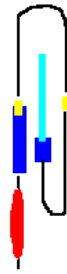
STRESS

RECOVERY

STRESS

RECOVERY

HSE
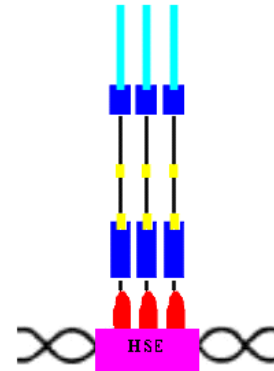
1.3 Chemical inducers of *hsp* gene expression

In addition to heat shock, chemical stressors such as sodium arsenite, cadmium chloride and herbimycin A can induce the expression of *hsp* genes. Arsenic is a known human carcinogen that is most commonly encountered in drinking water but is also found in the metal smelting and glass making industries. Arsenic exposure in humans results in increased risk for diabetes, spontaneous abortions, gangrene, atherosclerosis and ischemic heart disease (Chen *et al.*, 1996; Nickson *et al.*, 1998; Gebel, 2001; Hughes, 2002; Tseng *et al.*, 2002). Examination of cells exposed to arsenic has revealed many side effects including apoptosis, malignant cell transformation, cell cycle arrest, induction of the stress response, inhibition of cell proliferation and cytoskeletal injury (Chou 1989, Li & Chou, 1992; Liu *et al.*, 2001; Bode & Dong, 2002). While many side effects of arsenic exposure are known, the exact mechanism of toxicity to the cells is not. It has been suggested that changes to DNA repair and methylation or the induction of reactive oxygen species may be involved in this mechanism which may be involved in the induction of heat shock proteins (Harris & Shi, 2003; Shi *et al.*, 2004). Sodium arsenite is a well known inducer of the heat shock response (Darasch *et al.*, 1998; Kim *et al.*, 2001).

The kidney is the organ that is most strongly affected by cadmium toxicity (Faurskov & Bjerregaard, 2002). There is also evidence that cadmium increases the levels of reactive oxygen species in certain cell types (Thevenod & Friedman, 1999; Yang *et al.*, 2004; Kim *et al.*, 2005). An increase in reactive oxygen species has been shown to induce a heat shock response as proteins that are oxidatively damaged will bind HSPs and therefore release HSF1 (McDuffee *et al.*, 1997). Cadmium exposure was also found to lead to apoptosis through the unfolded protein response (Yokouchi *et al.*, 2007). Treatments with cadmium chloride have been shown to cause

the induction of heat-shock proteins in mammalian and amphibian cells (Kim *et al*., 2001; Gauley & Heikkila, 2006).

Herbimycin A is a benzoquinoid ansamycin antibiotic that was originally isolated as a potential herbicide and is now known to inhibit protein tyrosine kinase activity (Uehara *et al*., 1989; Fukazawa *et al*., 1990). Herbimycin A has been shown to induce *hsp* gene expression in mammalian, avian and amphibian cells (Caltabiano *et al*., 1986, Murakami *et al*., 1991; Levinson *et al*., 1980, Briant *et al*., 1997). Interestingly, treatment of mammalian cells with herbimycin A did not result in any detectable negative effects to the cells such as protein insolubility, changes to the intermediate filament cytoskeleton or interference with protein folding and assembly as seen previously with heat shock and chemical treatments (Hegde *et al*., 1995).

1.4 Small heat shock proteins

1.4.1 Structure of sHSPs

The sHSP family of proteins is not well conserved with the exception of an 80-100 amino acid domain that shares sequence homology with the eye lens protein α-crystallin. This domain is therefore called the α-crystallin domain and is located at the C-terminal end (Ingolia *et al*., 1982). The sequence similarity of this region ranges from 20% in distantly related members of the sHSP family to 60% in closely related mammalian family members (Berengian *et al*., 1999). Although the sequence of the α-crystallin domain is variable, the domain forms a compact β-sheet sandwich which is well conserved (Kim *et al*., 1998; van Montfort *et al*., 2001 a,b). Following the C-terminal, there is a short, flexible, highly polar tail called the C-terminal tail/ extension (Carver *et al*., 1992; 1995; Ehrnsperger *et al*., 1997), which is though to be involved in maintaining the solubility of the sHSP (Fernando & Heikkila, 2000) as well as the formation of oligomeric complexes (Fernando & Heikkila, 2002). The N-terminal end is hydrophobic and is

not well conserved in sequence or length (Arrigo & Landry, 1994; MacRae, 2000), however a WDPF domain is found at the very N-terminal end of some sHSPs. The WDPF domain has been shown to maintain the oligomer structure and chaperone activity of HSP27 (Theriault *et al*., 2004). The different molecular sizes of the sHSP family members can be explained by the differing lengths of variable sites between the WDPF domain and the α-crystallin domain as well as the variable length of the C-terminal tail (Gusev *et al*., 2002). SHSP monomers range in size from 12 to 43 kDa (MacRae, 2000; van Montfort, 2001a) and form highly polymeric structures which have been shown to be necessary for substrate binding and chaperone function (Arrigo and Landry, 1994). The size of sHSP polymers range from 100kDa to 10Ma when cells are undergoing stress (Arrigo & Landry, 1994; Fernando *et al*., 2003; Azzoni *et al*., 2004). The crystal structure of many sHSP polymers appears to be globular or ring-like structures with a central cavity (Sun & MacRae, 2005).

1.4.2 Expression of sHSPs

SHSPs have been described in all eukaryotes (de Jong *et al*., 1993, 1998; Kappe *et al*., 2001) and prokaryotes (Kappe *et al*., 2002) examined. The number of genes that encode sHSPs depends on the organism. There have been ten discovered in human; four in *Drosophila*, more than sixteen in *Xenopus laevis* and over thirty in plants (Parsell and Lindquist, 1993; Arrigo and Landry, 1994; Tam and Heikkila, 1995; Ohan and Heikkila, 1995; Helbing *et al*., 1996; Heikkila *et al*., 1997b, Michaud *et al*., 2002). Expression of sHSPs depends on growth conditions, developmental state as well as the oncogenic status of the cell (Haslbeck, 2002). Comparing the expression of sHSP to other HSPs in the cell in response to heat shock and other stressors has demonstrated that sHSPs are some of the most strongly induced HSPs (Arrigo & Landry, 1994; Haslbeck, 2002).

1.4.3 Function of sHSPs

1.4.3.1 Chaperone activity

It has been well documented that sHSPs act as molecular chaperones *in vitro* since they can bind to non-native or unfolded proteins and prevent them from aggregating (Haslbeck *et al*., 1999; Horwitz, 1992; Jakob *et al*., 1993; Ehrnsperger *et al*., 1998; Derham & Harding, 1999; Lee & Vierling, 2000; Haslbeck, 2002). *In vitro* sHSP chaperone activity depends on oligomerization and it has been hypothesized that the same is true for sHSPs *in vivo* (MacRae, 2000; Narberhaus, 2002). It is thought that when the sHSP chaperones are exposed to stress, they dissociate into smaller subunits thereby exposing their hydrophobic surface (Raman *et al*., 1995; Lee *et al*., 1997). This hydrophobic surface is then accessible to the substrate (van Montfort *et al*., 2001a; Zantema *et al*., 1992; Haslbeck *et al*., 1999).

While sHSPs are able to bind non-native proteins and keep them in a soluble, inactive state, they are not directly involved in the refolding process. When the stress has been removed, the sHSPs bring the unfolded proteins to ATP dependant HSPs such as HSP70, which are responsible for the refolding process (Fernando and Heikkila, 2000). Phosphorylation of serine residues of animal sHSPs causes oligomer dissociation (MacRae, 2000; Kato *et al*., 1994, 2002; Fernando *et al*., 2003), lowering *in vitro* chaperone activity (Rogalla *et al*., 1999; Ito *et al*., 2001; Fernando *et al*., 2003).

1.4.3.2 Actin capping/decapping

Cellular stress leads to modifications of the cytoskeleton as well as the rapid production of heat shock proteins. The cytoskeleton is comprised of three types of protein fibres: microtubules, microfilaments and intermediate filaments. SHSPs preserve the condition of microfilaments and intermediate filaments (Liang and MacRae, 1997). Microfilaments are

involved in maintaining the shape of the cell, motility, endocytosis, exocytosis, cytokinesis, cell-cell/ cell-substrate interactions and signal transduction. G-actin refers to the soluble pool of globular monomeric actin, which polymerizes into F-actin, also known as microfilaments. It is unknown whether the interaction between F-actin and sHSPs is direct or indirect. However, it has been proposed that phosphorylated sHSPs in a group of small oligomers coat the microfilaments promoting their recovery and preventing actin severing proteins activated during the stress response from attacking the microfilaments. Non-phosphorylated sHSP monomers may cap the growing end of the actin filament and play a role in regulating microfilament assembly (Mournier and Arrigo, 2002).

1.4.3.3 Apoptosis

SHSPs are overexpressed in malignant tumours and have been implicated in the growth of tumour cells and are able to confer cytostatic resistance (Kang *et al*., 2004; Rocchi *et al*., 2004). The protective effect of sHSPs is partly due to chaperone activity whereby proteins are protected from aggregation (Ehrnsperger *et al*., 1997; Lee *et al*., 1997). However, sHSPs are known to bind directly to certain components of the mitochondrial apoptosis pathway both upstream and downstream and therefore can prevent apoptosis in response to cell stress (Arrigo, 2005; Kamradt *et al*., 2005). Protection of the actin cytoskeleton is also a proposed method of sHSP mediated inhibition of apoptosis (Lavoie *et al*., 1993; Huot *et al*., 1996).

HSP27 and αB-crystallins are well studied members of the human sHSP family and have been shown to have an anti-apoptotic effect in response to various stressors. They are also involved in regulating metastatic potential and tumour progression (Arrigo, 2005; Sun & MacRae 2005). HSP27 in rat olfactory neurons determine the balance between differentiation and apoptosis. Overexpression of HSP27 leads to an increase in the rate of differentiation and a

decrease in the rate of cell death, whereas underexpression leads to a complete loss of differentiation and a huge increase of apoptotic cells (Mehlen *et al*., 1999).

1.4.3.4 Cellular differentiation

Since the discovery of the involvement of increased *Drosophila* HSP27 protein expression in ecdysterone mediated differentiation of imaginal disks (Pauli *et al*., 1990), many studies have examined the involvement of various sHSPs in differentiation. In general it has been found that at least one sHSP was induced during the process of differentiation of a particular type of cell (Arrigo, 2005). Enhanced expression of HSP27 has been implicated in the differentiation processes of muscle cells (Benjamin *et al*., 1997); neurons (Mehlen *et al*., 1999), chondrocytes (Favet *et al*., 2001) and cardiomyocytes (Davidson & Morange, 2000). Mehlen and colleagues (1997) have also found that increased expression of HSP27 during differentiation is usually concurrent with an increase in phosphorylation. Interestingly, *in vivo* studies have suggested that HSP27 is probably not involved in all differentiation processes (Duverger *et al*., 2004).

1.4.3.5 Redox reactions

Reactive oxygen species (ROS) are produced under normal physiological conditions in all aerobic organisms. ROS can lead to oxidative damage without the activity of various important enzymes and antioxidants naturally present in the cell (Papp *et al*., 2003). Various sHSPs and HSP70 act as cytoplasmic antioxidants to protect proteins from oxidative damage by covering the sites sensitive to damage by ROS. In the case that the oxidative damage is too strong for the HSPs to handle, they hold the denatured proteins until they are refolded or denatured (Papp *et al*., 2003). Oxidative stress leads to induction of HSPs which is largely mediated by the

oxidatively damaged proteins themselves (McDuffee *et al*., 1997) which activates HSF1 (Morimoto, 1999).

1.4.3.6 Heat shock proteins and disease

Several common neurodegenerative conditions such as Alzheimer's, Parkinson's and Huntington's are associated with the aggregation of improperly folded proteins forming high-ordered insoluble fibrils (Reviewed in Selkoe, 2004; Landles & Bates, 2004). The above mentioned diseases have mutations in one or more proteins causing changes in protein conformation and leading to improperly functioning proteins that are prone to aggregation because of their exposed hydrophobic surfaces. Chaperones have evolved to block these hydrophobic surfaces as well as maintaining the cellular pool of proteins in a properly folded state (Hartl & Hayer-Hartl, 2002). The ubiquitin-proteasome system is also present to target improperly folded proteins for degradation (Weissman, 2001).

1.5 *Xenopus laevis* as a model organism

The South African clawed frog, *Xenopus laevis* has been used extensively as a model for amphibian development. This aquatic frog is inexpensively and easily cared for in the laboratory. The eggs are large and suitable for microinjection and the embryos develop quickly (Sive *et al*., 2000). *Xenopus laevis* is also an excellent bioindicator for genotoxic stress. Metallic pollution is an escalating problem in the aquatic environment and amphibians have been used to investigate the effects various chemicals and heavy metals have on the viability and development (Zoll *et al*., 1990; Djomo *et al*., 2000; Mouchet *et al*., 2005a,; 2006).

A *Xenopus laevis* A6 kidney epithelial cultured cell line, which originated in renal uniferous tubules of adult *Xenopus* (Rafferty & Sherwin, 1969), has been used in numerous studies. The

A6 cell line is hardy and quick growing as the doubling time averages at 22 hours. The rate of cell division remains logarithmic before confluency and cells continue to divide after confluency is reached. This is in contrast with a decreased growth rate caused by contact inhibition that is sometimes seen in other cell lines (Rafferty, 1968). *Hsp30* gene expression has been previously described in *Xenopus laevis* A6 kidney epithelial cells and embryos (Ali *et al*., 1993; Tam and Heikkila, 1995; Lang *et al*., 1999; Heikkila, 2003; Heikkila, 2004; Gellalchew and Heikkila, 2005).

1.6 SHSP family members

1.6.1 HSP27

HSP27 is a heat shock inducible sHSP that is present constitutively in many cell types and tissues (Arrigo & Landry, 1994). HSP27 is involved in cell growth/differentiation (Mehlen *et al*., 1997; Arrigo & Préville, 1999), increased resistance to cellular oxidation (Arrigo, 2001) and is a predictor of a negative response to treatment in patients with breast cancer (Love & King, 1994), and prostate cancer (Rocchi *et al*., 2004). Also, HSP27 is a negative regulator of apoptosis and is most likely important for general development as HSP27 knockout mice do not survive (Brar *et al*., 1999, Garrido, 2002).

1.6.1.1 HSP27 in *Xenopus laevis*

HSP27 has recently been discovered in *Xenopus laevis* and is a homolog of human HSP27 (Tuttle *et al*., 2007). Interestingly, *Xenopus* HSP27 has only a 19% identity with *Xenopus* HSP30C, with the highest similarity in the alpha crystallin domain. In the desert topminnow, HSP27 is similar to mammalian and avian HSP27 rather than to HSP30 (Norris *et al*., 1997). Similarly, *Xenopus* HSP27 grouped with HSP27 family members in different species such as

chicken HSP24, human HSP27, mouse HSP25 and topminnow HSP27 rather than with *Xenopus*

HSP30 (Tuttle *et al.*, 2007). Constitutive levels of *hsp27* mRNA were found in *Xenopus laevis*

developing embryos beginning at the early tailbud. Following heat shock, increased *hsp27*

mRNA levels continued to increase. This is in contrast with *hsp30* mRNA which is heat

inducible but not constitutively expressed. Recombinant HSP27 forms large multimeric

complexes which inhibit thermal aggregation (Tuttle *et al.*, 2007).

1.6.2 HSP30

In addition to *Xenopus*, *hsp30* genes have also been reported in other amphibians, fish and

chicken (Mulligan-Tuttle & Heikkila, 2007; Norris *et al.*, 1997; Katoh *et al.*, 2004). Other than

in *Xenopus laevis*, *hsp27* and *hsp30* genes have been only found in the same organism in

topminnow and chicken (Norris *et al.*, 1997; Katoh *et al.*, 2004).

1.6.2.1 Structure of HSP30 in *Xenopus laevis*

In *Xenopus laevis*, the HSP30 family is composed of multiple members. Five acidic

hsp30 genes have been cloned and sequenced; *hsp30* A-E. The *hsp30*A gene contains an

insertional mutation in the coding region and therefore only encodes a 10kDa protein. The

*hsp30*B gene was found to contain a frameshift mutation resulting in a TGA stop codon close to

the C terminus and is therefore considered a pseudogene. The *hsp30*B gene does not contain an

HSE sequence and is not heat inducible in micro-injected oocytes. Krone *et al.*, (1992) were

responsible for the complete isolation of the *hsp30*C and D genes as well as the promoter and N-

terminal coding region of *hsp30*E. *Hsp30* C and D both encode 24 kDa proteins. *Hsp30* genes A

and B are found in one cluster of genes and *Hsp30* C-D are found in another cluster. The genes

in their respective clusters are arranged in a head-to-tail formation. These two clusters are

closely related as there is a 99% sequence similarity between both the coding and flanking regions of *hsp30*A and C as well as *hsp30* B and E (Krone *et al*., 1992). It is not known whether the clusters arose either from a single duplication or several independent events. The clone that Bienz (1984) reported to contain *hsp30* A and B did not include any upstream sequences and therefore it is unknown whether a gene is present that is similar to the *hsp30*D gene. The coding region of *hsp30*D has a higher degree of similarity to the coding regions of *hsp30*A and C than to *hsp30*B and E (Krone *et al*., 1992). All five *hsp30* genes were found to contain no introns (Bienz, 1984; Krone *et al*., 1992). In addition to *hsp30*A-E, two *hsp30* cDNAs have been identified; X4 and X5 (Bienz, 1984).

1.6.2.2 Stress induced HSP30 synthesis

Heat shock induction of HSP30 accumulation is followed by the assembly of HSP30 high molecular weight complexes, which bind to denatured proteins. These aggregates include different isoforms of HSP30 as well as other proteins such as HSP70 (Ohan *et al*., 1998). HSP30 is phosphorylated by mitogen-activated kinase-activated protein kinase-2 (MAPKAPK-2) during recovery from stress (Fernando *et al*., 2003) and forms smaller multimeric HSP30 complexes, which have a reduced binding activity to denatured proteins (Heikkila, 2003). The proteins that are released from the HSP30 aggregates are refolded and reactivated by an ATP-dependent molecular chaperone folding system that may include proteins such as HSP/HSC70 (Heikkila *et al*., 2004). Also, phosphorylation was found to destabilize the HSP30 multimeric complex and inhibit its chaperone activity (Fernando *et al*., 2003).

All members of the acidic HSP30 family members in *Xenopus laevis* are stress inducible. Heat shock and other stressors including sodium arsenite, hydrogen peroxide and herbimycin A have been shown to induce *hsp30* gene expression in A6 cells (Darasch *et al*., 1988; Heikkila *et*

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al, 1987; Briant *et al*, 1997; Muller *et al*., 2004). A synergistic effect of *hsp30* gene expression was observed when a combination of stressors was used (Heikkila *et al*., 1987; Briant *et al*, 1997).

1.6.2.3 Function of HSP30

During heat shock, HSP30C is able bind citrate synthase (CS) and luciferase (LUC), inhibit their aggregation and maintain them in a soluble state (Fernando & Heikkila, 2000). Experiments using HSP30 terminal deletion mutants determined that the C-terminal was necessary for molecular chaperone activity, while the N-terminal was not. It is thought that the multiple polar amino acids found at the C-terminal end of HSP30C may be involved in preserving the solubility of HSP30C itself as well as that of the target protein. It is also possible that the C-terminal is required for the binding of HSP30C to the target protein (Fernando & Heikkila, 2000; Abdulle *et al*., 2002). HSP30D also acts as a molecular chaperone, however it differs from HSP30C in 19 amino acids and is less effective as a molecular chaperone than HSP30C (Abdulle *et al*., 2002).

1.7 Inhibition of the Heat shock Response

Hyperthermic treatment of cancer cells leads to damage of proteins and membrane structures (Dewey, 1989; Yatvin and Cramp, 1993; Lepock, 2004) and eventual cell death either through apoptosis or necrosis (Brown and Attardi, 2005). Thermal therapy is currently used in cancer treatment, however, it does have some important drawbacks that limit its effectiveness. A stress response is initiated in response to hyperthermia, which leads to the induction of HSPs (Coss, 2005). As mentioned earlier, various HSPs act as molecular chaperones, which protect cellular proteins and direct denatured proteins for proteolysis. HSPs also act as inhibitors of

apoptosis, which block apoptotic signalling, inhibit apoptosis and improve the survival of the cells (Jäättelä, 1999; Jolly and Morimoto, 2000; Parcellier *et al*, 2003; Beere, 2004). Therefore a method to inhibit heat shock protein induction prior to thermal therapy of cancer cells would augment the success of this treatment.

1.7.1 Quercetin

The flavonoid quercetin (3,3',4',5,7-pentahydroflavon) is a specific *hsp* synthesis inhibitor in several cell lines including colon carcinoma (COLO 320M, HT-29 (Hosokawa *et al*., 1990; Lee *et al*., 1994), HeLa (Hosokawa *et al*., 1990; Hansen *et al*., 1997), erythroleukemia (K562) (Elia & Santoro, 1994) and breast carcinoma cells (MDA-MB-231) (Hansen *et al*., 1997). Quercetin also inhibits the activity of several protein kinases (Gschwendt *et al*., 1983; Levy *et al*., 1984; Matter *et al*., 1992) as well as the growth of cells *in vitro* (Larocca *et al*., 1990; Scambia *et al*, 1991; Ranelletti *et al*., 1992; Avila *et al*., 1994).

Experiments by Hansen and colleagues, (1997), comparing the inhibition of hsp synthesis in HeLa and MDA-MB-231 cells, demonstrated that quercetin acts in a cell type specific manner. A gel shift assay established that HeLa cells treated with quercetin had decreased binding of HSF to HSE. This is consistent with other experiments performed on HeLa and COLO 320M cell lines (Nagai *et al*., 1995; Hosokawa *et al*., 1992). Interestingly, the same experiment performed with MDA-MB-231 cells determined that there was a decrease in hsp synthesis but no decrease in binding of HSF to HSE. This is similar to results found with the HT-29 and K562 cell lines (Lee *et al*., 1994, Elia *et al*., 1996). There was only small decrease in HSF1 levels when MDA-MB-231 cells were treated with quercetin. Hansen and colleagues, (1997) therefore concluded that the decrease of *hsp* expression seen in quercetin treated MDA-MB-231 cells following heat shock was due to a lack of activation of HSF and/or a lack of initiation of *hsp* gene transcription.

1.7.2 KNK437

Recently, a benzylidene lactam compound, KNK437, was created by the Kaneka Corporation (Takasago, Japan) and found to be a potent *hsp* gene expression inhibitor (Figure 2). Much less is known about KNK437 than quercetin. However, it is less toxic to cells as well as more effective in inhibiting thermotolerance (Yokota *et al*., 2000). It has been suggested that KNK437 may inhibit HSF1 activation or inhibit the interaction between HSF1 and HSE (Hosokawa *et al.*, 1992). In support of the latter possibility, it was determined by a gel shift assay using heat shocked human squamous carcinoma cells that KNK437 reduced heat-induced binding of HSF1 to HSE. The reduced binding of HSF1 to HSE did not result from a decrease of HSF1 as the level of HSF1 was not affected by KNK437 (Ohnishi *et al.*, 2004). In support of these results, it was reported that exposure of the human esophageal cancer cell line, TE-2, did not result in a decrease in the amount of HSF1 granules nor did it result in a change in appearance of the granules (Nonaka *et al*., 2003). HSF1 granules were observed when cells were exposed to heavy metals or proteasome inhibition. A possible explanation for decreased binding of HSE to HSF1 was that KNK437 may inhibit the trimerization of HSF1 which is required for binding to HSE. Another possibility was that KNK437 may have inhibited the phosphorylation of HSF1 after heating, thereby inhibiting its activation. Unphosphorylated HSF1 is able to bind to HSE but is unable to acquire transcriptional activity (Cotto *et al.*, 1996). Previous literature has indicated that KNK437 also inhibited the induction of HSPs *in vivo* (Koishi *et al*., 2001). While it was reported that KNK437 did not have any anti-tumorigenic activity on its own, KNK437 did not interfere with the activities of protein kinase A or C or tyrosine kinase (Yokota *et al*., 2000).

Figure 3. Structure of the heat shock inhibitor KNK437.
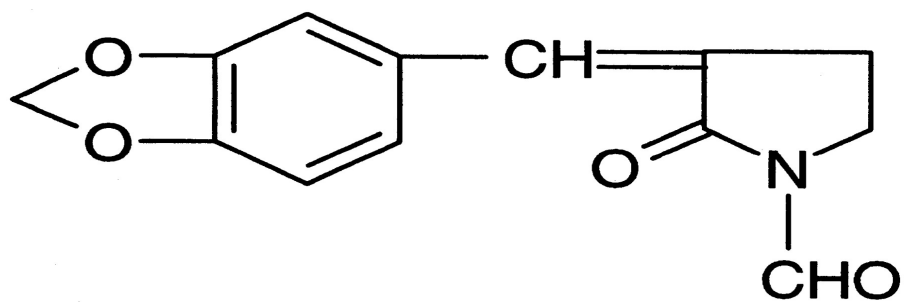
1.7.3 Heat shock protein gene expression inhibition in *Xenopus* A6 cells

Manwell and Heikkila (2007) found that both quercetin and KNK437 inhibited the expression of *hsp30, hsp47* and *hsp70* genes in heat shocked A6 cells. However KNK437 was found to be more effective in inhibiting *hsp* gene expression than quercetin. Finally, KNK437 was capable of inhibiting the acquisition of thermotolerance in A6 cells (Manwell & Heikkila, 2007). A similar finding was made with COLO 320DM and human esophageal cancer cell lines (Yokota *et al*., 2000; Nonaka *et al*., 2003).

1.8 Objectives

There are many known inducers of the heat shock response, however there are few known specific inhibitors. In our laboratory and in others, KNK437 has proven to be a very effective inhibitor of heat shock factor (Yokota *et al*., 2000; Manwell and Heikkila, 2007). KNK437 was effective at inhibiting heat shock-induced *hsp30* gene expression in *Xenopus laevis* A6 cells (Manwell and Heikkila, 2007). However, it was not known whether KNK437 could inhibit *Xenopus hsp30* gene expression induced by non-heat shock stressors. The main objectives of this study are listed below:

- To characterize the accumulation of *hsp30* mRNA as well as HSP30 and HSP70 protein in *Xenopus* A6 cells treated with sodium arsenite, cadmium chloride or herbimycin A using northern and western blot analyses.

- To determine the ability of the heat shock factor inhibitor, KNK437, to inhibit the accumulation of *hsp30* mRNA, HSP30 and HSP70 in *Xenopus* A6 cells treated with sodium arsenite, cadmium chloride, or herbimycin A treatments by means of northern and western blot analyses.

- To observe the intracellular localization of sodium arsenite-, cadmium chloride- and herbimycin A-induced accumulation of HSP30 in *Xenopus* A6 cells incubated either in the absence or presence of KNK437 using immunofluorescence and confocal laser scanning microscopy.

2 Experimental Procedures

2.1 *Xenopus laevis* A6 Cell Culture and Treatment

Xenopus laevis kidney A6 epithelial cells were acquired from ATCC (American Type Culture Collection, Rockville, Maryland). The cells were cultured in Leibovitz (L)-15 media (Sigma, Oakville, Ontario) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (100 U/mL and 100 μg/mL, respectively; Sigma) and were grown at 22°C in T75 cm^2 flasks. Upon confluency, cells were washed with 2 mL versene [0.02% (w/v) KCl, 0.8% (w/v) NaCl, 0.02% (w/v) KH_2PO_4, 0.115% (w/v) Na_2HPO_4, 0.02% (w/v) sodium ethylenediaminetetraacetic acid (Na_2EDTA)] and then with 1 mL of 1X trypsin (Sigma) diluted in 100% Hank's balanced salt solution (HBSS, Sigma) until cells began to detach from the flask. Ten mL of fresh media was then added to the detached cells, which were divided into additional flasks. Cell treatments were performed 2 or 3 days after cell splitting to allow the cells to reach 90-100% confluence. Cell treatments with HSF inhibitor KNK437 (*N*-formyl-3,4-methylenedioxy-benzylidene-γ-butyrolactam; Calbiochem, Gibbstown, New Jersey) were performed either 2 or 6 h prior to chemical or heat treatments with 100 uM KNK437 dissolved in dimethylsulphoxide (DMSO; Sigma). The heat shock treatments were at 33 or 35ºC followed by a 2 h recovery period at 22°C for protein isolation and without recovery for the isolation of RNA. The chemical treatments included various concentrations of cadmium chloride or sodium arsenite dissolved in distilled water or herbimycin A dissolved in DMSO. Cells were harvested immediately following treatment by washing the cells in 2 mL of 65% HBSS, and then removed in 1mL of 100% HBSS by scraping. The cells were pelleted at 14,000 rpm for 1 min and pellets were stored at -80°C until protein or RNA isolation.

2.2 *Hsp30C* antisense riboprobe production and Northern blotting

2.2.1 *Hsp30* template construction

The entire open reading frame of the *hsp30C* gene was previously inserted into the pRSET expression vector (Invitrogen, Carlsbad, California) by Pasan Fernando (Fernando & Heikkila, 2000). Plasmids containing the *hsp30C* insert were transformed into *Escherichia coli* DH5α cells. Individual colonies were inoculated into 15 mL Falcon tubes and grown overnight in 5 mL of LB broth [1% (w/v) tryptone-peptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, pH 7.5] containing 100 μg/mL ampicillin at 37°C.

2.2.2 Plasmid isolation

Cells were pelleted in a centrifuge (Eppendorf Centrifuge 5810R, Brinkmann Instruments Ltd., Mississauga, Ontario) at 5,000 rpm for 10 min at 4°C. Plasmid DNA was isolated by resuspending the pelleted bacterial cells in 200 μL of an ice-cold solution made of 50 mM glucose, 25 mM Tris (pH 8.0) and 1 mM EDTA (pH 8.0) and transferring the mixture into a microcentrifuge tube where they were lysed by mixing gently with 200 μL of a solution made with 0.2 M NaOH and 1% (w/v) SDS (sodium dodecyl sulfate). The tubes were kept on ice for the remainder of the plasmid isolation experiment. An ice-cold solution of 3 M potassium acetate and 5 M glacial acetic acid was added and the tubes were again mixed gently and placed on ice for 5 min. Following centrifugation at 14,000 rpm for 5 min at 4°C, the supernatant was transferred to a new tube. A 2 h RNase A treatment (10 μg/mL; BioShop, Burlington, Ontario) was performed at 37 °C to eliminate RNA from the sample. Next, 600 μL of phenol:chloroform (1:1) was added and the sample was vortexed to mix. The tube was centrifuged at 14,000 rpm for 3 min at 4°C and the upper aqueous layer was transferred to a new tube. The phenol/chloroform step was repeated. A 600 μL solution of isoamyl alcohol:chloroform (1:24)

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was added, the tube was vortexed and centrifuged at the settings mentioned above. Again the upper aqueous layer was transferred to a new tube. To precipitate the nucleic acids, 600 µL of isopropanol was added. The samples were vortexed and incubated at room temperature for 2 min. They were then centrifuged at 14,000 rpm for 5 min at room temperature. The supernatant was removed and 500 µL of 70% ethanol was added to wash the pellet. The sample was centrifuged at 14,000 rpm for 2 min at 4°C. The ethanol was removed and the ethanol wash step was repeated. The pellets were allowed to air dry and were then resuspended in 50 µL distilled water and stored at -20°C.

2.2.3 *In vitro* transcription

The plasmid containing the *hsp30C* insert as mentioned above was linearized using the *Pvu*II restriction enzyme (Roche Molecular Biochemicals, Laval, Quebec). The cut plasmid was then run on a 1% (w/v) agarose gel in 1x TAE. The *hsp30C* insert was cut out using a razor blade and extracted from the gel using a DNA extraction tube (Millipore Corp., Bedford, Massachusetts) which was centrifuged at 5,000 rpm for 10 min. The DNA therefore passed through the filter in TAE buffer. The filter was removed and the DNA was precipitated by adding 100% ice-cold ethanol (2x volume) and 3 M sodium acetate (pH 5.2, 1/10 of volume). The tube was placed at -80°C for 30 min and then centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant was discarded and the pellet was washed in 1 mL of 70% ice-cold ethanol and centrifuged at the settings mentioned above. The ethanol was removed and the ethanol wash step was repeated. The pellet was air dried and resuspended in 20 µL of sterile water and kept at -20°C.

In vitro transcription was used to generate digoxygenin (DIG)-labelled riboprobes. Each *in vitro* transcription reaction contained 4 µL of linearized DNA template, 4 µL of rNTP mix

[2.5 mM rGTP, 2.5 mM rATP, 2.5mM rCTP, 1.625 mM rUTP (Promega, Nepean, Ontario), 0.875 mM DIG-11-UTP (Roche Molecular Biochemicals), 1.5 µL diethylpyrocarbonate (DEPC)-treated water, 4 µL of 100 mM dithiothreitol to a final concentration of 20 mM (Promega), 4 µL of 5X transcription buffer to a final concentration of 1x (MBI Fermentas, Burlington, Ontario), 0.5 µl RNase inhibitor (MBI Fermentas) and 40 IU of SP6 RNA polymerase (Roche Molecular Biochemicals). The *in vitro* transcription reaction was performed for 1 h at 37°C. To remove any remaining DNA template, 2 µL of RNase-free DNase 1 (Roche Molecular Biochemicals) was added for 10 min at 37ºC. *In vitro* transcripts were then precipitated with the addition of 10 µL of 3 M sodium acetate (pH 5.2), 80 µL of TES [10 mM Tris-HCl (pH 7.4), 5 mM EDTA (pH 8.0), 1% (w/v) SDS] and 220 µL of ice-cold 100% filtered ethanol. The reaction was incubated at −80ºC for 30 min and then centrifuged at 14,000 rpm for 15 min at 4ºC. The supernatant was removed and the pellet was air dried and resuspended in 21 µL of DEPC-treated water. One µL was electrophoresed to verify the presence of the *in vitro* transcript. The remaining 20 µL was stored at −80ºC until northern blot analysis.

2.2.4 RNA isolation

RNA was isolated from A6 cell pellets using the Qiagen RNeasy Mini Kit (Qiagen, Mississauga, Ontario) according to the manufacturers' instructions in the RNeasy Mini Handbook Animal Cell Protocol (2006). RNA samples were quantified using a NanoDrop ND-1000 UV-Vis Spectrophotometer, Waltham, Massachusetts). Before the northern blot procedure, the integrity of the isolated RNA was determined by running 2 µg of each sample on a 1.2% formaldehyde agarose gel [1.2% (w/v) agarose, 10% (v/v) 10x MOPS (pH 7.0; 0.2 M 3-propane sulphonic acid, 50 mM anhydrous sodium acetate, 10 mM EDTA, pH 8.0) and 16% (v/v) formaldehyde]. The RNA samples were heat denatured for 10 min at 65ºC in a solution of 1 µL

10X MOPS, 1.6 µL formaldehyde, 2 µL loading dye [0.2% (w/v) bromophenol blue, 1 mM

EDTA (pH 8.0) and 50% (v/v) glycerol], 5 µL formamide and 0.5 µg /mL ethidium bromide.

The samples were then placed on ice for 5 min and electrophoresed at approximately 100V for 1

h.

2.2.5 Northern Hybridization

Ten µg of isolated RNA prepared as described above with the exception of ethidium bromide

and was electrophoresed for 2 h at 100V on a 1.2% formaldehyde agarose gel also described

above. The gel was then soaked in 0.05 M NaOH for 20 min and then rinsed in DEPC treated

water. The gel was soaked twice in 20X SSC buffer [3 M sodium chloride, 300 mM sodium

citrate (pH 7.0)] for 20 min and then transferred onto a positively charged nylon membrane

(Roche Molecular Biochemicals) overnight by capillary action. The blotting apparatus was set

up as follows. The gel was place upside down onto a piece of blotting paper soaked in 20X SSC

(VWR, Mississauga, Ontario) which acted as a wick, that was set on a glass support over a

Pyrex® dish filled with 500 mL 20X SSC. The nylon membrane which was cut to the same size

as the gel, was placed on top of the gel and covered with 2 pre-soaked pieces of blotting paper. A

4 inch stack of paper towels was placed on top of the blotting paper and a 500 g weight was

placed on top of the paper towels. The RNA was crosslinked to the membrane using a UV

Crosslinker (Ultra-lum Inc., Claremont, California) at 12,000 microJ/CM^2. The membrane was

soaked in 10% (v/v) glacial acetic acid for 5 min and then placed in 1X Blot Stain Blue

Reversible stain to determine if the transfer was successful and if the gel was loaded equally.

The membrane was then destained in DEPC water and photographed. The membrane was

transferred placed in RNA pre-hybridization buffer [50% (v/v) formamide, 5X SSC, 0.02% SDS,

0.01% N-lauryl sarcosine, 2% blocking reagent] in a hybridization bag (SealPAK pouches,

VWR) and placed in a Boekel Scientific Shake'N'Bake Hybridization Oven (VWR) at 65°C for 4 h. Hybridization was then performed with a Digoxygenin-labelled *hsp30C* RNA antisense riboprobe in RNA hybridization buffer overnight at 65°C.

To remove unbound probe from the membrane, SSC (saline sodium citrate) stringency solutions in decreasing concentrations were used as described below. First the membrane was washed twice in a solution of 2X SSC (with 0.1% (w/v) SDS) for 5 min at room temperature. Then a 15 min wash was performed in 0.5X SSC (with 0.1% (w/v) SDS) at 65°C followed by a 15 min wash in 0.1X SSC (with 0.1% (w/v) SDS) at 65°C. The rest of the experiment was performed at room temperature. Next the membrane was placed in washing buffer [100 mM maleic acid, 0.3% (v/v) Tween 20] for 1 min. The membrane was blocked for 30 min at room temperature in blocking solution [2% (w/v) blocking reagent, 10% (v/v) maleic acid buffer (pH 7.5)] and for another 30 min in blocking solution containing a 1:8,000 dilution of Anti-DIG-alkaline phosphatase-conjugated Fab fragment antibody (Roche Molecular Biochemicals). To remove any unbound secondary antibody, the membrane was washed twice for 20 min each in washing buffer and then placed for 2 min in detection buffer [0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl]. Finally the membrane was placed in a fresh hybridization bag and incubated for 10 min in an even coat of the chemiluminescent reagent, CDP-Star (Roche Molecular Biochemicals). The membrane was visualized using a DNP chemiluminescent imager (DNR Bio-Imaging Systems Ltd., Kirkland, Quebec) according to the manufacturer's instructions.

2.3 Western Blotting

2.3.1 Protein Isolation

Protein was isolated from A6 cell pellets using homogenization buffer at pH 7.4 [160 mM sucrose, 1.6 mM glycol tetraacetic acid (EGTA), 0.8 mM ethylenediaminetetraacetic acid

(EDTA), 32 mM NaCl, 24 mM N-Z-hydroxylethylpiperazine-N'-2 ethane sulfonic acid (HEPES), 1% (w/v) SDS, 100 µg/mL phenylmethyl-sufonyl fluoride (PMSF), 1 µg/mL aprotinin, 0.5 µg/mL leupeptin, pH 7.4]. The samples were sonicated (output 4.5, 65% duty cycle for 15 , 1 s bursts) using a Branson sonifier (Branson Sonic Power Co., Danbury, Connecticut) and then centrifuged at 14,000 rpm for 30 min at 4°C to pellet cellular debris. The protein contained in the supernatant was stored at -20 $^{\circ}$C.

2.3.2 Protein quantification

Protein was quantified using a bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, Illinois). A bovine serum albumin (BSA; Bioshop) protein standard was created by diluting BSA in distilled water at concentrations ranging from 0 to 2 mg/mL. Protein samples were diluted to a concentration of 1:2 in distilled water. Ten µL of BSA standards and protein samples were transferred in triplicate into a 96 well polystyrene plate. Then 80 µL of BCA reagent A and B at a ratio 50:1 were added to the BSA and protein samples (Pierce). The plate was tapped lightly on the side to mix and then incubated at 37°C for 30 min. The plate was then read at 562 nm using a Versamax Tunable microplate reader (Molecular Devices, Sunnyvale, California). A standard curve was created using the concentrations of the BSA protein standards which was used to determine the concentration of the protein samples.

2.3.3 Western Blotting

Twenty µg of protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a BioRad Mini Protean III system (BioRad, Mississauga, Ontario). Twenty µg of protein was diluted in distilled water to 20 µL. Five µL of 5x loading dye [0.0625 M Tris pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol,

0.00125% (w/v) bromophenol blue] was added to a final concentration of 1x. The protein was denatured by boiling for 10 min and pulse spun before loading onto the gel.

The separating gel [12% acrylamide, 0.32% n'n'-bis methylene acrylamide, 0.375 M Tris (pH 8.8), 1% SDS, 0.2% ammonium persulfate (APS) and 0.14% tetramethylethylenediamine (TEMED)], was poured and covered in 100% ethanol to create an even surface. The gels were allowed to polymerize for 25 min. The ethanol was removed and the stacking gel [4% acrylamide, 0.11% n'n'-bis methylene acrylamide, 0.125 M Tris (pH 6.8), 1% SDS, 0.4% APS and 0.21% TEMED] was poured and the comb was inserted. Twenty-five min later, the comb was removed and the protein was loaded in the wells. The gel was run at 90V through the stacking gel and at 170V through the separating gel.

Nitrocellulose membranes were soaked in transfer buffer [25 mM Tris, 192 mM glycine, 20% (v/v) methanol] for 30 min. After the gels were finished running, they were also soaked in transfer buffer for 10-15 min. The proteins on the gel were transferred onto the nitrocellulose membrane using a BioRad Trans-Blot Semi-Dry Transfer Cell (BioRad) at 20V for 20 min. Ponceau S stain [0.19% (w/v) Ponceau-S, 5% (v/v) acetic acid] was used to stain the membrane to determine whether the transfer was successful and the protein was loaded equally. The membrane was incubated for 1 h in Tris buffered saline solution (TBS) [2 mM Tris (pH 7.5), 0.1% Tween 20 (Sigma), 30 mM NaCl] with Tween 20 (TBS-T) containing 5% (w/v) Carnation skim milk powder creating a 5% blocking solution. The membrane was then incubated in fresh blocking solution with rabbit polyclonal anti-HSP30 antibody (1:3,000), HSP70 (1:200) or actin (1:200) for 1 h. The membrane was washed in TBS-T and then and incubated with alkaline phosphatase (AP)-conjugated goat anti-rabbit secondary antibody at a 1:3,000 dilution (BioRad). Again the membrane was washed and then was incubated in 51% alkaline phosphatase detection

buffer [100 mM Tris base, 100 mM NaCl, 50 mM MgCl$_2$] diluted in distilled water, 0.3% of 300

mg Nitro blue tetrazolium chloride (NBT), 0.17% of 150 mg 5-Bromo-4-chloro-3-indolyl

phosphate, toluidine salt (BCIP).

2.4 Immunocytochemistry and Laser Scanning Confocal Microscopy

The protocol followed for immunocytochemistry was as described by Spector *et al.*

(1998). *Xenopus laevis* A6 cells were grown on 22 x 22 mm, flame sterilized glass coverslips

which were previously treated in a basic solution [48% distilled water, 50% mL 100% filtered

ethanol and 2% 10M NaOH] to ensure that cells adhered to the coverslip. Once the cells were

confluent on the coverslips (approximately 48 h after cell seeding), heat shock or chemical

treatments were performed as described in section 2.1. Cells were first washed twice with

phosphate buffered saline (PBS; 8% NaCl, 0.2% KCl, 0.2% KH$_2$PO$_4$, 2.1% Na$_2$HPO$_4$·10H$_2$O, 1

mM MgCl$_2$, 1 mM CaCl$_2$) and fixed in 3.7% (w/v) paraformaldehyde in PBS. The cells were

washed in PBS and then permeabilized in 0.3% (v/v) Triton X-100 (Sigma) in PBS. The cells

were washed again in PBS and then incubated for 1 h in 3.7% (w/v) BSA fraction V (Sigma) in

PBS, filter sterilized using a 0.4 μm filter (Pall Filtration Corporation, Mississauga, Ontario).

Indirect labeling was performed using a 1:500 dilution of rabbit anti-*Xenopus* HSP30 polyclonal

antibody in 3.7% BSA fraction V for 1 h. PBS washes removed any unbound antibody. The

coverslips were then incubated in a secondary mouse anti-rabbit IgG antibody conjugated with

Alexa-488 (Invitrogen) at a 1:2000 dilution in 3.7% (w/v) BSA fraction V for 30 min. Again the

coverslips were washed to remove unbound secondary antibody. The cells were then incubated

in a 1:60 dilution of rhodamine-tetramethylrhodamine-5-isothiocyanate (TRITC; 300 U of

rhodamine phalloidin in 1.5 mL 100% methanol, Invitrogen) in 3.7% (w/v) BSA to directly label

F- actin. The coverslips were washed in PBS and then mounted on a microscope slide with

Vectashield (Vector laboratories Inc., Burlingame, California) containing 4,6-diamidino-2-phenylindole (DAPI), which stains nucleic acids. The coverslips were sealed onto the slides with clear nail polish and the slides were kept at 4ºC.

The slides were imaged with an inverted laser scanning Zeiss Axiovert 200 confocal microscope using LSM 510 META software (Carl Zeiss Canada Ltd., Mississauga, Ontario) according to the manufacturers' instructions. Both 40x and 63x oil objectives were used for imaging.

2.5 *Xenopus* HSP30C antibody purification

2.5.1 Expression of HSP30C recombinant protein

As mentioned earlier, the open reading frame of the *hsp30C* gene was previously inserted into the pRSET expression vector (Invitrogen) by Pasan Fernando and transformed into *Escherichia coli* DH5α cells. Although DH5α cells are good hosts for storing plasmids, they are not optimal for producing recombinant protein (Kroll *et al*, 1993). The pRSETB plasmid was therefore isolated from the DH5α cells and transformed into *E. coli* BL21 (DE3) cells. BL21 cells are much more efficient in producing recombinant protein.

Transformed DH5α cells were grown from a glycerol stock as mentioned in section 2.2.1 and pRSETB plasmids were isolated as in section 2.2.2. The pRSET plasmids were then digested as mentioned in section 2.23 and then both cut and uncut plasmids were electrophoresed in a 1% agarose gel to determine that the plasmid insert was the correct size (Figure 4).

BL21 cells, from a glycerol stock previously used in our laboratory, were grown overnight on LB plates [1% bacto-tryptone, 0.5% yeast extract, 1% NaCl, 1.5% agar, pH 7.0]. A single colony was used to inoculate 5 mL of ZB media [1% N-Z-Amine A, 0.5% NaCl] and was left to grow overnight at 37ºC. $CaCl_2$ was used to transform BL21 cells with the pRSETB

plasmid containing the HSP30C insert. Transformed cells were grown overnight on ZB plates

containing 100 μg/mL ampicillin. As the pRSETB plasmid contains an ampicillin resistance

gene, this ensures that only cells containing the plasmid would grow. A single colony of

transformed BL21 cells were grown overnight in a 10 mL culture of ZB + amp (100 μg/mL).

Ten mL of overnight culture was added to 500mL of M9ZB + amp [1% N-Z-Amine A, 0.5%

NaCl, 0.1% NH_4Cl, 0.3% KH_2PO_4, 0.6% Na_2HPO_4, 0.4% glucose, 1mM $MgSO_4$, 50 μg/mL

ampicillin]. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of

1.0 mM to induce the production of recombinant protein. After cells grew for 4-5 h, they were

homogenized in guanidium lysis buffer (6.0 M guanidine hydrochloride, 20 mM Na_2HPO_4, 500

mM NaCl, pH 7.8) and sonicated using a using a Branson sonifier (Branson Sonic Power Co.)

for 3 repetitions of 20, 1 s bursts (70% duty cycle and 5 output), then centrifuged at 5000 rpm in

a Sorval RC 5B Plus centrifuge using the SS-34 rotor for 30 min at 4^oC to pellet the cellular

debris. The lysate (supernatant) was removed and stored at -20ºC until purification.

2.5.2 Purification of HSP30C recombinant protein

HSP30C recombinant protein was purified using a Poly Prep chromatography column

(BioRad) using ProBond resin (Invitrogen) containing nickel under denaturing conditions. Three

mL of resin was added to the column and centrifuged at 100 rpm for 2 min. The overlaying

storage buffer was removed and the column was washed twice with 5 mL of distilled water and

then twice with 8 mL of denaturing buffer [8.0 M urea, 20 mM $NaPO_4$, 0.5 M NaCl, pH 7.8].

Five mL of bacterial lysate, pH adjusted to 7.8, was added to the column and bound for 45 min at

room temperature on an orbital nutator (Boekel Scientific, Feasterville, Pennsylvania) and then

centrifuged at 2,000 rpm to separate unbound lysate, which was removed. This process was

repeated several times with additional bacterial lysate. The washing process consisted of

agitation on the orbital nutator for 2 min, centrifugation at 2,000 rpm for 3 min and the removal of the supernatant. The column was washed 3 times with 5 mL denaturing buffer at pH 6, 5 times at pH 5.3 and 3 times at pH 5. The protein was then eluted off the column in 5 mL denaturing buffer at pH 4 in 1 mL fractions. The resin was packed by centrifugation at 2,000 rpm for 30 s and an additional 5 mL denaturing buffer at pH 4 was added to the column. The protein was again eluted in 1 mL aliquots. Twenty mL was removed from each of the aliquots and analyzed by SDS page as described in section 2.33 to determine which aliquots contained the recombinant protein. The aliquots containing recombinant protein were then dialyzed to remove the urea. The dialysis tubing was prepared by soaking in 500 mL distilled water for 15 min and then in 10 mM sodium bicarbonate at 80ºC for 30 min. The tubing was transferred to 500 mL of 100 mM EDTA for 30 min and then to 500 mL of distilled water at 80ºC for 30 min. Finally the tubing was washed inside and out with distilled water and soaked in TEN buffer [50 mM Tris, 25 mM NaCl, 0.2 mM EDTA, pH 8.0] for 15 min. The eluted protein was poured inside the tubing and clamped at both ends. The tubing was placed in a beaker containing 100 to 1,000 times the amount of eluted protein and stirred gently at 4°C for 3 h. The tubing was then transferred to a beaker containing fresh TEN buffer and stirred gently overnight at 4°C. The protein was concentrated using a MicroSep 3K Concentrator Column (Pall Filtration Corp.) by centrifugation at 5000 rpm at 4°C until the sample was concentrated to the desired volume. The protein was quantified with a BCA assay as described in section 2.3.2 and was stored at $-20\,^{\circ}$C. Twenty µL of purified recombinant protein was electrophoresed by SDS page as described in section 2.3.3 along with 20 µL samples of uninduced bacterial lysate and unpurified recombinant protein and was visualized using Coomassie Brilliant Blue R-250 staining (Figure 5).

2.5.3 Polyclonal antibody production

The rabbit anti-HSP30 serum had been previously prepared. HSP30 recombinant protein was diluted to 1.0 μg/μL in sterile 0.85% saline solution followed by the addition of an equal volume of Freund's Complete Adjuvant (Sigma) and injected into the rabbit using an an 18-gauge needle. Three additional protein injections were given at 3-week intervals with the Freund's Incomplete Adjuvant (Sigma) being substituted for Freund's Complete Adjuvant. At the end of the twelve week period, the rabbit was euthanised. The serum was separated from the rest of the blood and was stored at -20˚C.

In order to separate the HSP30 antibody from the crude rabbit serum, ProBond resin was added to a PolyPrep Chromatography Column and centrifuged. Unless otherwise mentioned, all wash steps were performed on an orbital nutator and followed by centrifugation at 1,000 rpm for 2 min and removal of the supernatant. Five mL of distilled water was used to wash the column 3 times. The column was then washed in 8 mL of equilibration buffer [50 mM Tris, 2 mM NaCl, pH 7.4] 3 times. Five mL of recombinant protein was diluted to a final volume of 8 mL in equilibration buffer. The column was agitated to 1 h on an orbital nutator at 4ºC. Eight mL of TBST [25 mM Tris, 150 mM NaCl, 2.5 mM KCl, 0.1% Tween-20] was added to wash the column. The rabbit anti-HSP30 serum was diluted 1:4 in TBST and bound to the column overnight at 4ºC and then washed in 5 mL of TBST 3 times. The column was washed 5 times with 5 mL of equilibration buffer, and then 5 times in 5 mL of wash buffer [50 mM Tris, 150 mM NaCl, pH 7.4]. The antibody was eluted from the column with the addition of 2 mL of 4 M $MgCl_2$ for 15 min at 4ºC. The supernatant was allowed to drain out the column and collected. Another 2 mL of $MgCl_2$ was added and again the flow through was collected. The flow through

was combined and the antibody was dialyzed and concentrated as described in section 2.5.2. The

antibody was then stored at -20ºC.

Figure 4. Confirmation of the HSP30C insert in pRSETB plasmids.

Plasmids containing *Xenopus hsp30C* cDNA were isolated from DH5α cells, as described in

Experimental Procedures. Both uncut pRSETB with the HSP30C insert and pRSETB plasmids

digested with PvuII containing the HSP30C insert were electrophoresed on a 1% agarose gel to

confirm the presence and size of the HSP30C insert. The ladder shown is a 1 Kb ladder and the

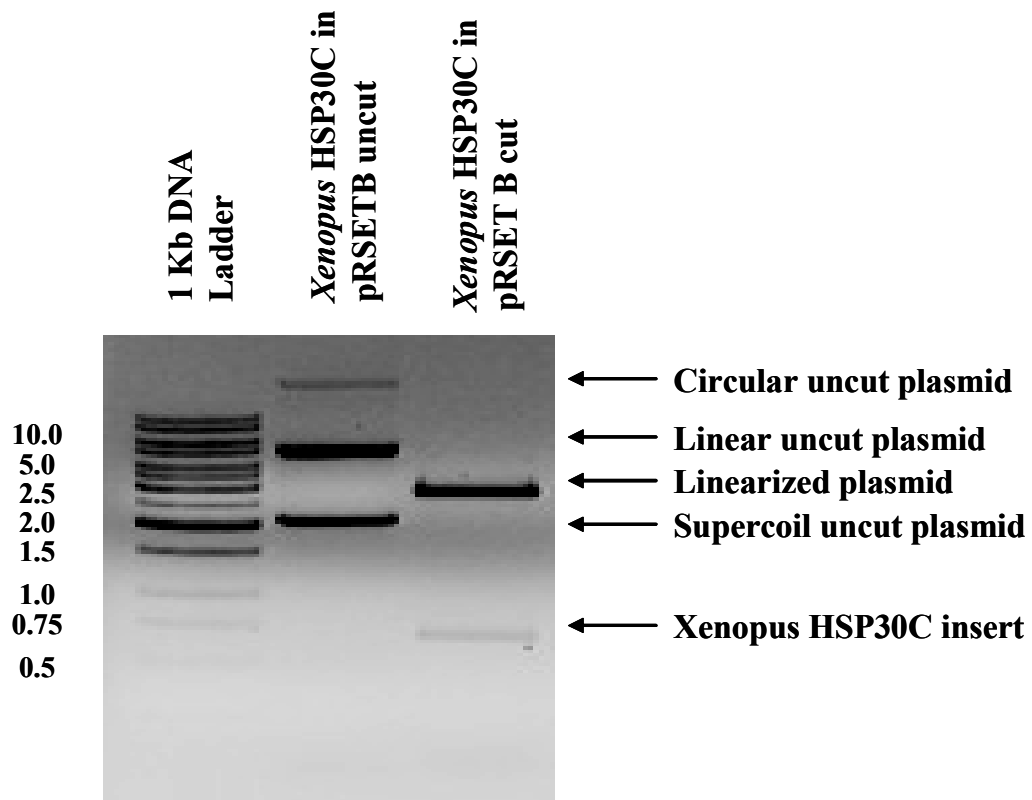molecular weights are given in kilobase pairs.

Figure 5. Purification of the *Xenopus* laevis HSP30C recombinant protein. *Xenopus laevis* HSP30C was overexpressed in BL21 cells by induction with IPTG. The BL21 cell lysates containing the recombinant protein bound a nickel affinity column through their histidine tag. The recombinant protein was then purified as described in Section 2.5.2 and analysed by SDS-PAGE and visualized by Coomassie Brilliant Blue R-250 staining. Lane 1 contains molecular weight markers shown in kDa. Uninduced bacterial lysate is seen in lane 2. Induced and unpurified bacterial lysate is seen in lane 3. Lane 4 contains purified recombinant HSP30C protein. HSP30C is indicated by the arrow.
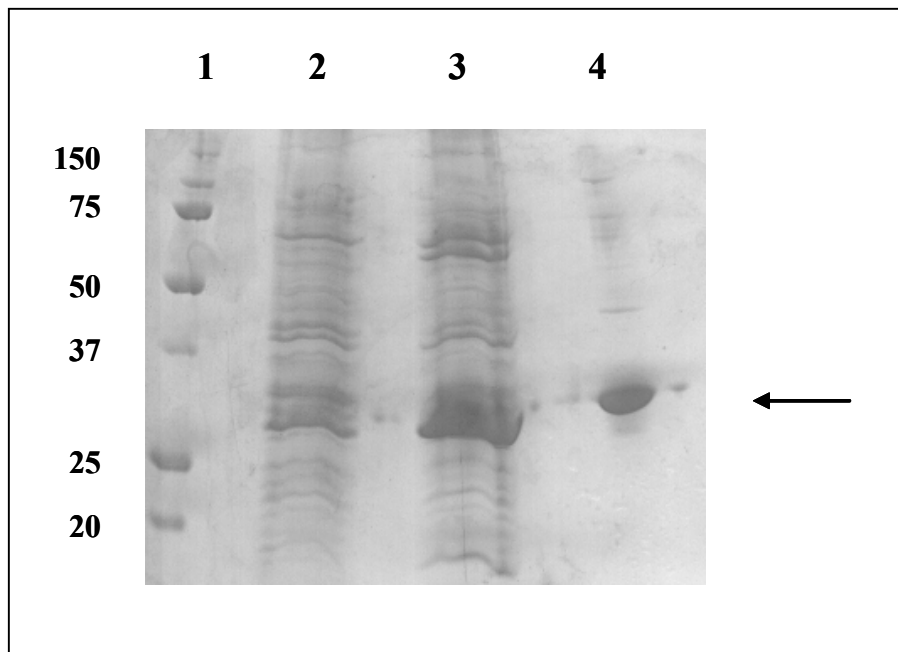
3 Results

3.1 Examination of the effect of KNK437 on the stress-induced accumulation of *hsp30* mRNA in *Xenopus* A6 cells

Initial studies, using Northern hybridization analysis, examined the effect of heat shock, sodium arsenite, cadmium chloride and herbimycin A on *hsp30* mRNA accumulation in *Xenopus laevis* A6 kidney epithelial cells. As shown in Figure 6, *hsp30* mRNA was not detectable in control A6 cells. However incubation of cells at either 33 or 35 ºC for 2 h induced the accumulation of this message. Treatment of A6 cells with 10 μM sodium arsenite for 4 h resulted in a small accumulation of *hsp30* mRNA whereas cells treated with 20 or 30 μM resulted in larger increases in the relative level of this mRNA. While incubation of cells in the presence of 100 μM cadmium chloride induced a small accumulation of message, treatment of A6 cells with 200 or 300 μM resulted in enhanced *hsp30* mRNA levels. Finally, a small amount of *hsp30* mRNA was noted in A6 cells treated with 0.25 μg/ml of herbimycin A. The relative level of this message increased with increased herbimycin A concentrations up to 2 μg/ml.

The next phase of this study evaluated the effect of the heat shock factor inhibitor, KNK437, a benzylidene lactam compound, on stress-induced *hsp30* mRNA accumulation. A 2 h pre-treatment with 100 μM KNK437 at 22°C reduced the accumulation of *hsp30* mRNA following treatment with the stressors mentioned above, however the relative amount of inhibition of *hsp30* mRNA accumulation differed depending on the stressor involved. Densitometric analysis using ImageJ software (Version 1.38) was performed on the blots to determine the difference in densities between samples treated with the stressor only, versus samples pre-treated with KNK437 before treatment with the stressor.   The difference was expressed as % inhibition and was averaged with the % inhibition of a total of 3 trials. Statistical

analysis was then performed using an unpaired T-test to determine whether the difference was significant. A 2 h pre-treatment with 100 μM KNK437 before heat shock at 33 or 35ºC resulted in the reduction of *hsp30* mRNA levels by 82% and 58%, respectively (Figure 7, 8). Reductions at both 33 and 35ºC were significantly different from heat shocked samples without a pre-treatment with KNK437. A 2 h pre-treatment with 100 μM KNK437 resulted in the inhibition of *hsp30* mRNA accumulation following a 20 μM sodium arsenite treatment for 2-12 h (Figure 9). Although KNK437 was less efficient in inhibiting relative levels of *hsp30* mRNA as the length of sodium arsenite treatment increased, the reductions were significant. At 2 h there was a 97% reduction in *hsp30* mRNA accumulation, whereas at 12 h there was only a 51% reduction (Figure 10). KNK437 also inhibited *hsp30* mRNA accumulation induced by exposure of A6 cells to 200 μM cadmium chloride for 4-12 h (Figure 11). At 4 h there was a 59% reduction in the relative level of *hsp30* mRNA accumulation, while at 12 h there was only a 33% reduction (Figure 12). KNK437 was most successful at inhibiting *hsp30* mRNA accumulation in A6 cells treated with 1 μg/mL herbimycin A for 2 –8 h (Figure 13, 14). For example, after 2 h of herbimycin A treatment, the extent of inhibition was 100%, whereas after 8 h, the reduction in *hsp30* mRNA levels was 88%.

Figure 6. The effect of various stressors on *hsp30* mRNA accumulation in *Xenopus* A6 cells. A6 cells were heat shocked (HS) at 33 or 35°C for 2 h or treated with various concentrations of either sodium arsenite (NaAs), cadmium chloride (Cd) or herbimycin A (HA) for 4 h. After treatments, the cells were harvested and total RNA was isolated. Ten μg of RNA was then analyzed by northern hybridization using an *hsp30* antisense riboprobe as described in Experimental Procedures. A representative reversible RNA blot stain is shown in the bottom panel, which was used to confirm equal loading and transfer.
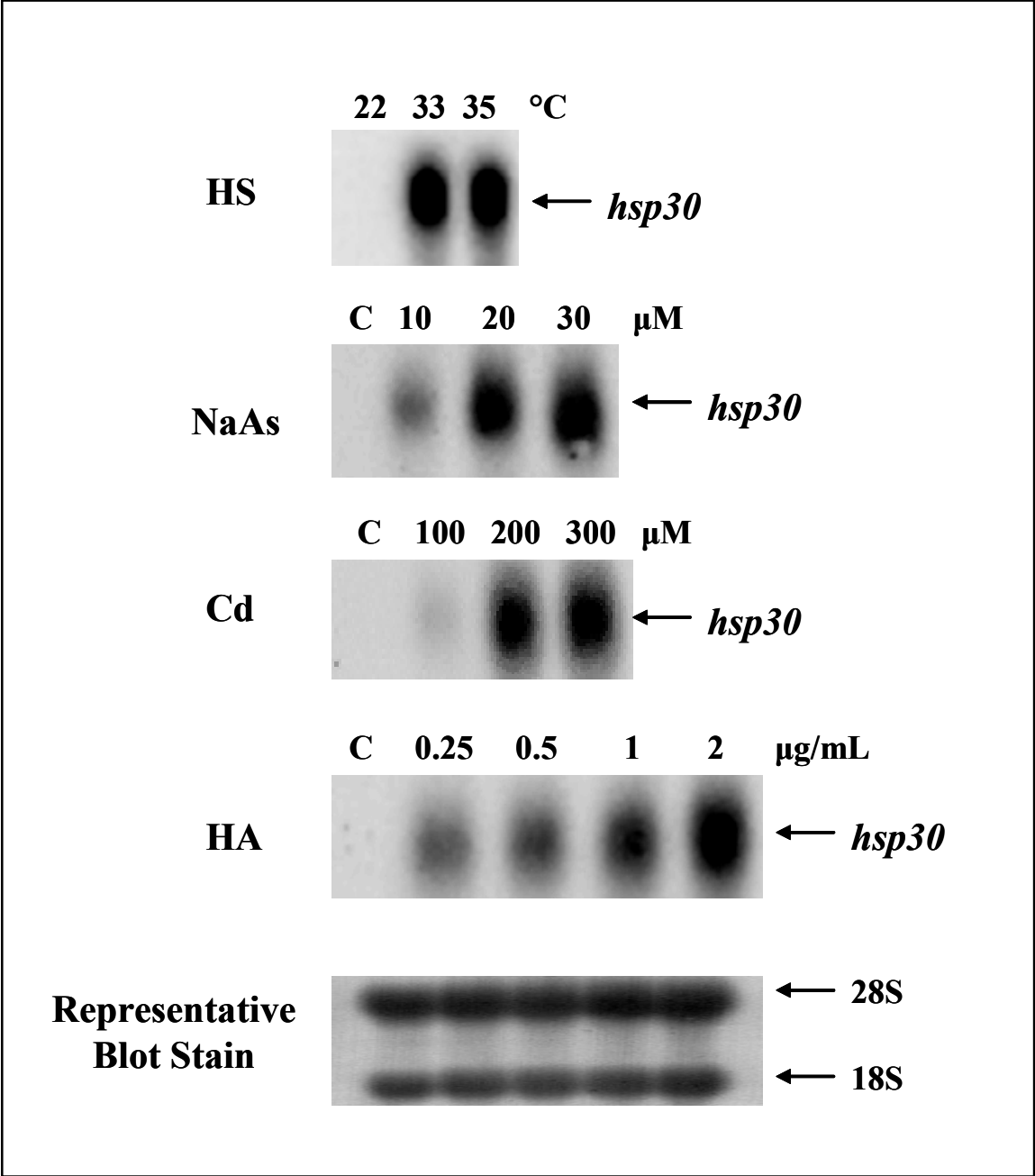
22 33 35 °C

HS ← *hsp30*

C 10 20 30 µM

NaAs ← *hsp30*

C 100 200 300 µM

Cd ← *hsp30*

C 0.25 0.5 1 2 µg/mL

HA ← *hsp30*

Representative Blot Stain ← 28S
← 18S

Figure 7. Effect of KNK437 on heat shock-induced accumulation of *hsp30* mRNA in A6 cells.   A6 cells were heat shocked at 33 or 35°C for 2 h with (+) or without (-) a 2 h pre-treatment with 100 μM KNK437.   After treatment, cells were harvested and total RNA was isolated.   Ten μg of RNA was then analyzed by northern hybridization using an *hsp30* antisense riboprobe.   A representative reversible RNA blot stain is shown in the bottom panel, which was used to confirm equal loading and transfer.
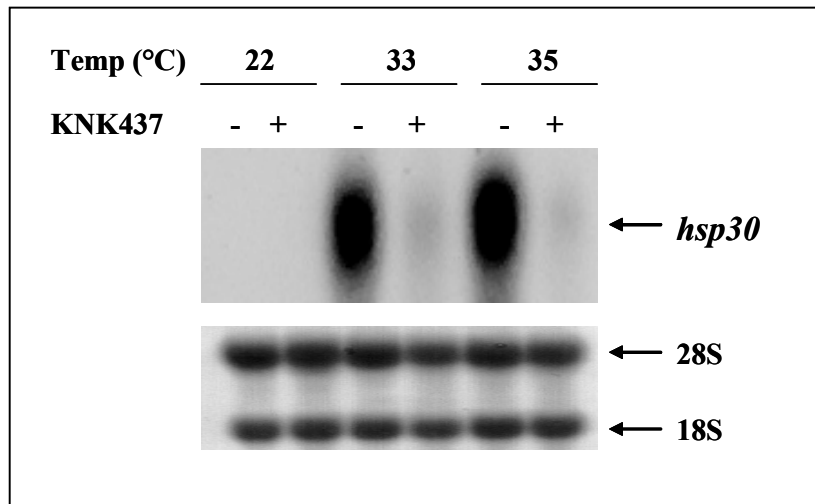
Figure 8. Inhibition of heat shock-induced accumulation of *hsp30* mRNA by KNK437. Image J software (1.38) was used for densitometric analysis of *hsp30* mRNA accumulation after heat shock at 33 or 35°C for 2 h with or without a 2 h pre-treatment with 100 μM KNK437. The ability of KNK437 to inhibit HSP30 protein accumulation at each timepoint is graphed as % inhibition. Error bars represent standard error. At each timepoint, significant differences are indicated, (* = P<0.001, ** = P<0.05).
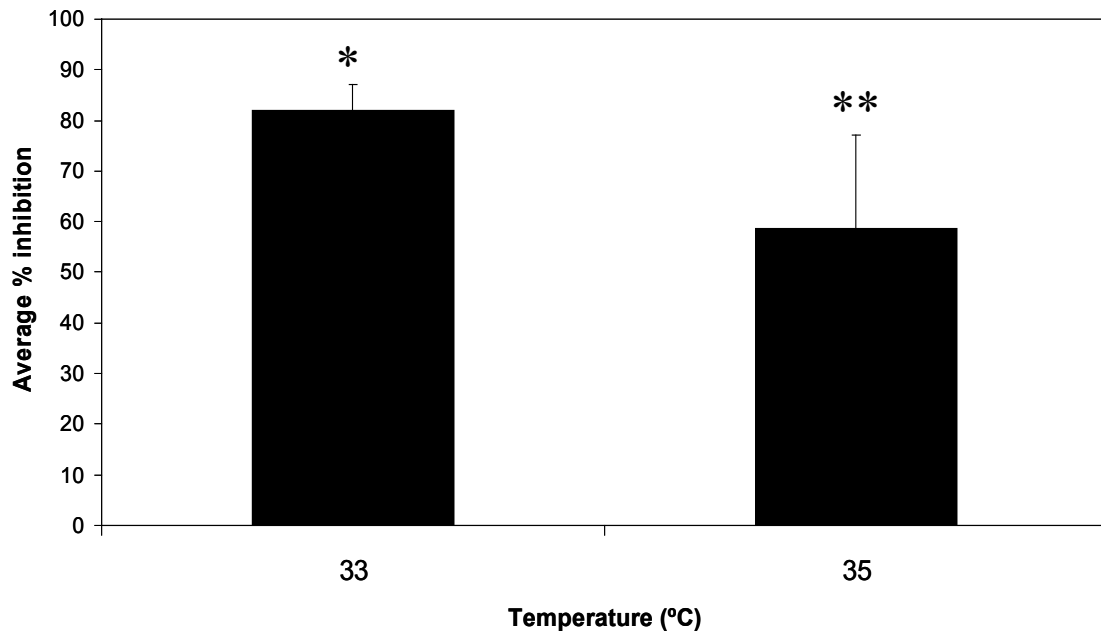
Figure 9. Effect of KNK437 on sodium arsenite-induced accumulation of *hsp30* mRNA in A6 cells. A6 cells were treated with 20 μM sodium arsenite for 2-12 h, with (+) or without (-) a 2 h pre-treatment with 100 μM KNK437. After treatment, cells were harvested and total RNA was isolated. Ten μg of RNA was then analyzed by northern hybridization using an *hsp30* antisense riboprobe. A representative reversible RNA blot stain is shown in the bottom panel which was used to confirm equal loading and transfer.
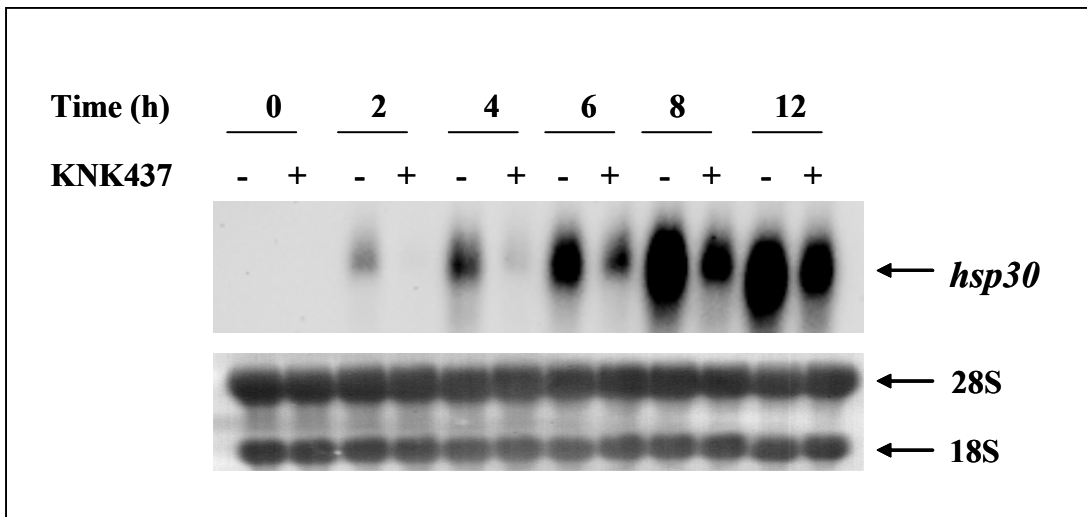
Figure 10. Inhibition of sodium arsenite-induced accumulation of *hsp30* mRNA by KNK437. Image J software (1.38) was used for densitometric analysis of *hsp30* mRNA accumulation after treatment with 20 μM sodium arsenite for 2-12 h with or without a 2 h pre-treatment with 100 μM KNK437. The ability of KNK437 to inhibit HSP30 protein accumulation at each timepoint is graphed as % inhibition. Error bars represent standard error. At each timepoint, significant differences are indicated, (* = P<0.001, *** = P<0.1).
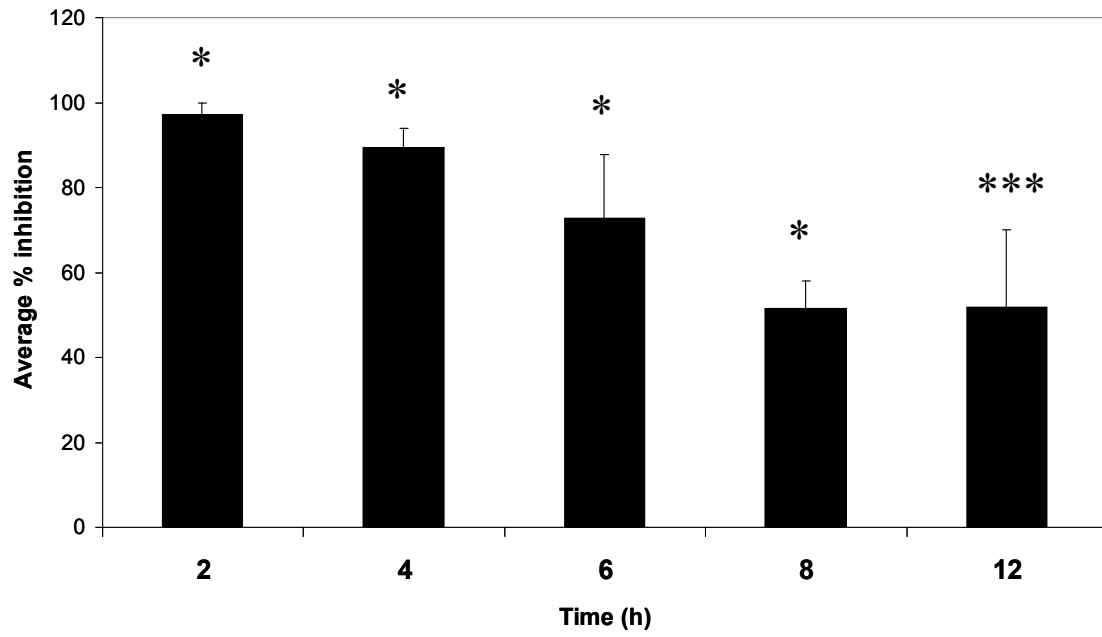
Figure 11. Effect of KNK437 on cadmium chloride-induced accumulation of *hsp30* mRNA in A6 cells.   A6 cells were treated with 200 μM cadmium chloride for 4-12 h, with (+) or without (-) a 2 h pre-treatment with 100 μM KNK437.   After treatment, cells were harvested and total RNA was isolated. Ten μg of RNA was then analyzed by northern hybridization using an *hsp30* antisense riboprobe. A representative RNA reversible blot stain is shown in the bottom panel which was used to confirm equal loading and transfer.
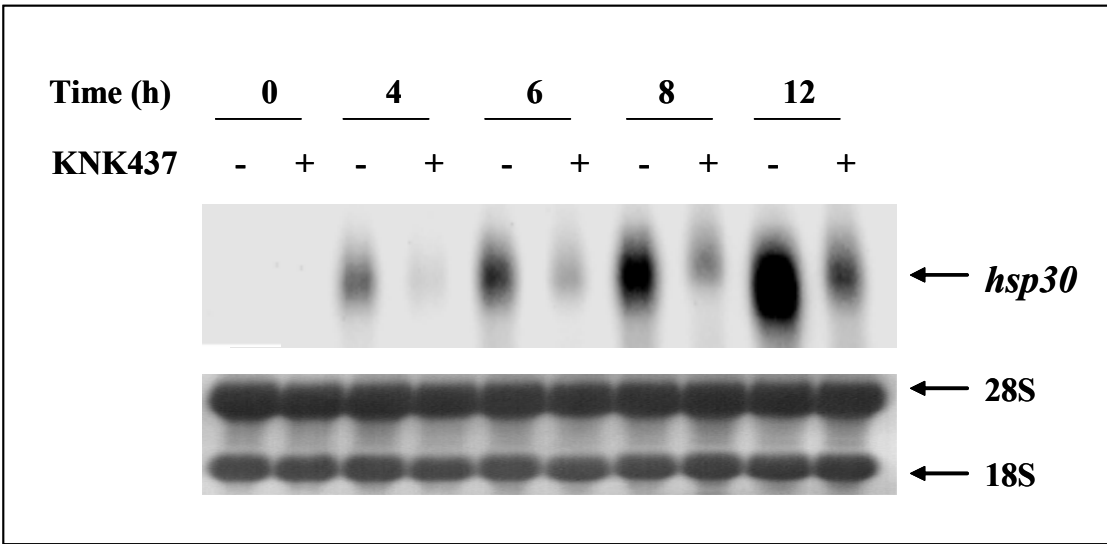
Figure 12. Inhibition of cadmium chloride-induced accumulation of *hsp30* mRNA by KNK437. Image J software (1.38) was used for densitometric analysis of *hsp30* mRNA accumulation after treatment with 200 μM cadmium chloride for 4-12 h with or without a 2 h pre-treatment with 100 μM KNK437. The ability of KNK437 to inhibit HSP30 protein accumulation at each timepoint is graphed as % inhibition. Error bars represent standard error. At each timepoint, significant differences are indicated, (* = P<0.001, *** = P<0.1).
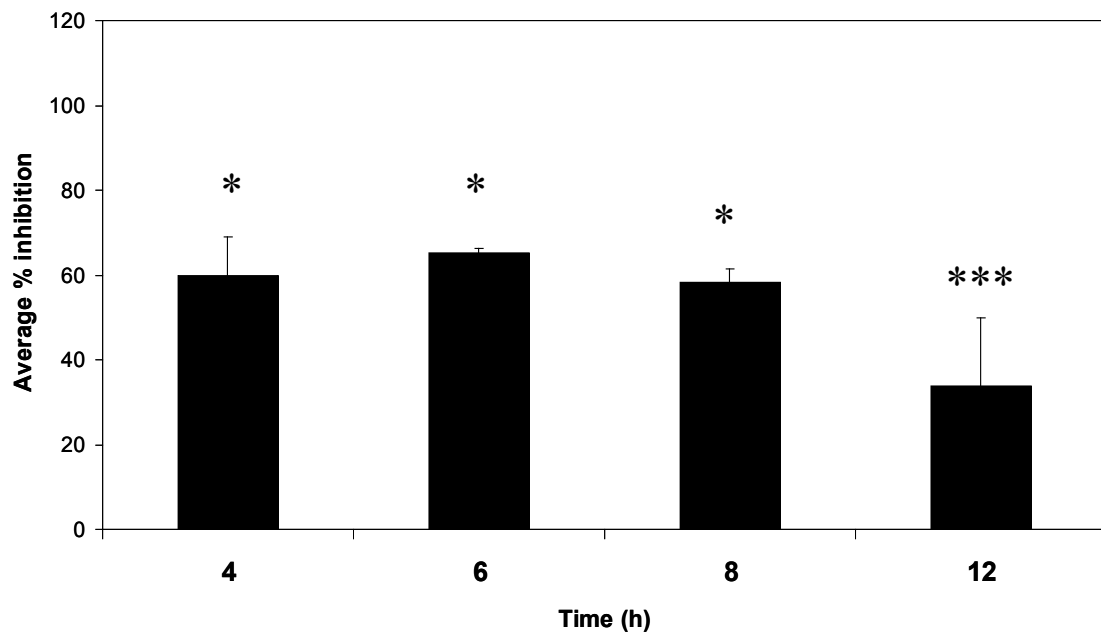
Figure 13. Effect of KNK437 on herbimycin A-induced accumulation of *hsp30* mRNA in A6 cells. A6 cells were treated with 1 µg/mL herbimycin A for 2-8 h, with (+) or without (-) a 2 h pre-treatment with 100 µM KNK437.   After treatment, cells were harvested and total RNA was isolated.   Ten µg of RNA was then analyzed by northern hybridization using an *hsp30* antisense riboprobe.   A representative RNA reversible blot stain is shown in the bottom panel which was used to confirm equal loading and transfer.

Time (h)   0   4   6   8   12

KNK437   -   +   -   +   -   +   -   +   -   +

← *hsp30*

← 28S

← 18S

Figure 14. Inhibition of herbimycin A -induced accumulation of *hsp30* mRNA by KNK437.

Image J software (1.38) was used for densitometric analysis of *hsp30* mRNA accumulation after treatment with 1 µg/ml herbimycin A for 2-8 h with or without a 2 h pre-treatment with 100 µM KNK437. The ability of KNK437 to inhibit HSP30 protein accumulation at each timepoint is graphed as % inhibition. Error bars represent standard error. At each timepoint, significant differences are indicated, (* = P<0.001, *** = P<0.1).
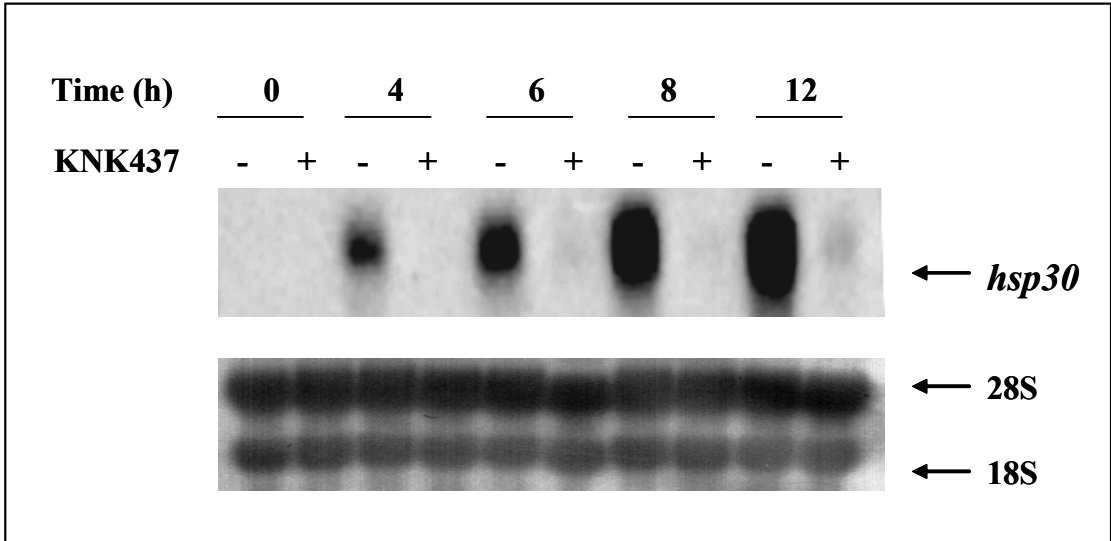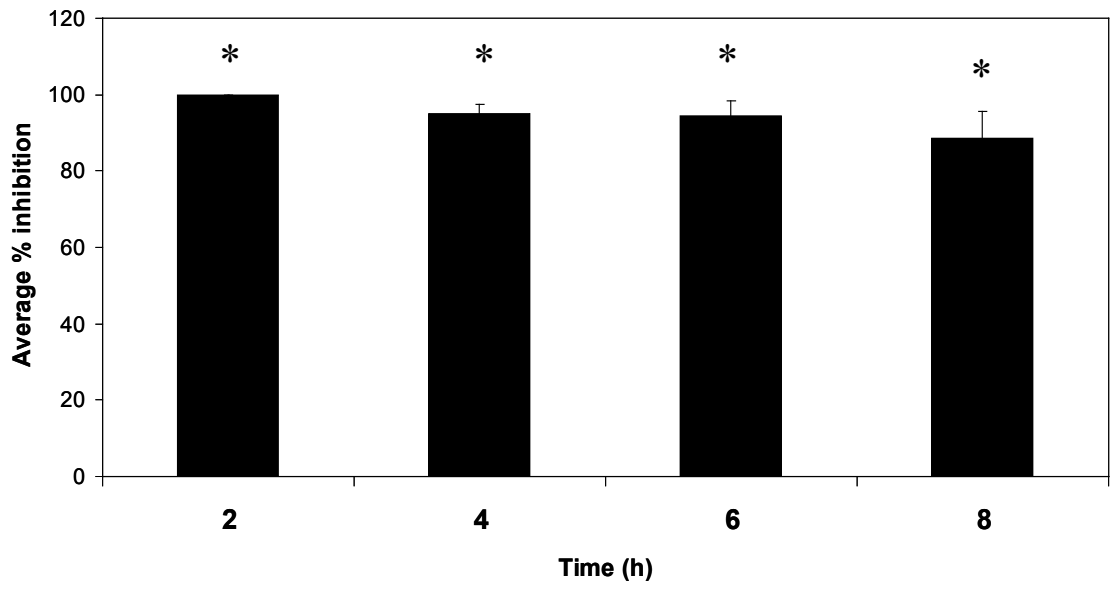
3.2 Examination of the effect of KNK437 on the accumulation of HSP30 protein in *Xenopus* A6 cells using western blot analysis

The next stage of this study investigated the effect of heat shock, sodium arsenite, cadmium chloride and herbimycin A on the accumulation of HSP30 protein in *Xenopus* A6 cells using western blot analysis. Again, densitometric analysis using ImageJ software (Version 1.38) was performed on the blots to determine the difference in densities between samples treated with the stressor only, versus samples pre-treated with KNK437 before treatment with the stressor.   The difference was expressed as % inhibition and was averaged with the % inhibition of a total of 3 trials. Statistical analysis was then performed using an unpaired T-test to determine whether the difference was significant.

HSP30 protein was not detected in control cells maintained at 22°C (Figure 15). Accumulation of members of the HSP30 family was observed when A6 cells were heat shocked at 33 or 35°C for 2 h. Treatment with 10 μM sodium arsenite for 12 h resulted in detectable of HSP30 protein. Increased accumulation of the relative levels of HSP30 protein was noted in cells treated with 20 – 40 μM sodium arsenite for 12 h. Detectable levels of HSP30 were also observed with A6 cells treated with 200-400 μM cadmium chloride. Slightly higher levels of HSP30 were observed with higher concentrations of cadmium chloride. Finally, a small amount of HSP30 protein accumulation was observed when A6 cells were treated 0.25 μg/ml of herbimycin A. The relative amount of HSP30 protein increased when A6 cells were treated with increasing herbimycin A concentrations ranging from 0.5 – 2 μg/ml (Figure 15).

The ability of KNK437 to inhibit the accumulation of HSP30 was determined by pre-treating A6 cells with 100 μM KNK437 before treatment with either heat shock, sodium arsenite, cadmium chloride or herbimycin A. It was determined that a 2 h KNK437 pre-treatment time

was required to inhibit 100% of heat shock-induced HSP30 protein (Figure 16). Also, a 2 h pre-treatment of A6 cells with 100 μM KNK437 was sufficient to completely inhibit the accumulation HSP30 protein induced by heat shock at 33°C for 2 or 4 h treatments (Figure 17, 18). When A6 cells were treated with 20 μM sodium arsenite, there was a complete inhibition of HSP30 after 6 and 8 h. However by 24 h, there was only a 59% inhibition (Figure 19, 20). Interestingly, there was almost a 100% inhibition of HSP70 protein by KNK437 up to 24 h (Figure 19). KNK437 also inhibited the accumulation of HSP30 protein induced by exposure of A6 cells to 200 μM cadmium chloride. There was a 100% reduction at 6 h and by 24 h, KNK437 was still able to inhibit HSP30 protein accumulation by 90% (Figure 21, 22). HSP70 protein was inhibited by KNK437 up to 24 h (Figure 21). Finally, KNK437 completely inhibited both HSP30 and HSP70 protein accumulation in A6 cells treated with 1 μg/mL herbimycin A (Figure 23, 24).

Figure 15. The effect of various stressors on HSP30 protein accumulation in *Xenopus* A6 cells. A6 cells were heat shocked (HS) at 33 or 35°C for 2 h or treated with various concentrations of sodium arsenite (NaAs), cadmium chloride (Cd) and herbimycin A (HA) for 12 h. After the stress treatments, cells were harvested and protein was isolated. Twenty μg of protein was then analyzed by western blot analysis using an anti-HSP30 antibody. A representative Ponceau S stain is shown in the bottom panel which was used to confirm equal loading and transfer.

Figure 16. **Effect of the length of KNK437 pre-treatment on heat shock-induced accumulation of HSP30 protein in A6 cells.** A6 cells were pre-treated for 0-6 h with 100 μM KNK437 (K) and then heat shocked at 33 °C for 2 h followed by a 2 h recovery. After treatment, cells were harvested and protein was isolated. Twenty μg of protein was then analyzed by western blot analysis using an HSP30 antibody. A representative Ponceau S stain is shown in the bottom panel which was used to confirm equal loading and transfer.

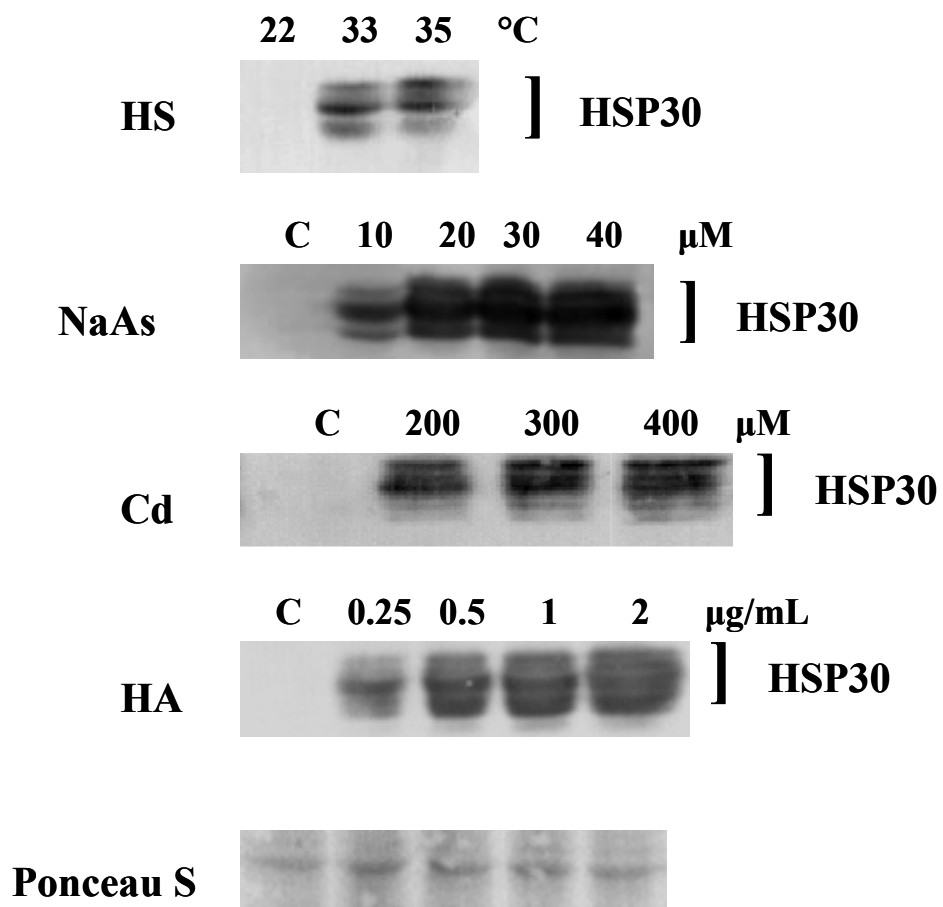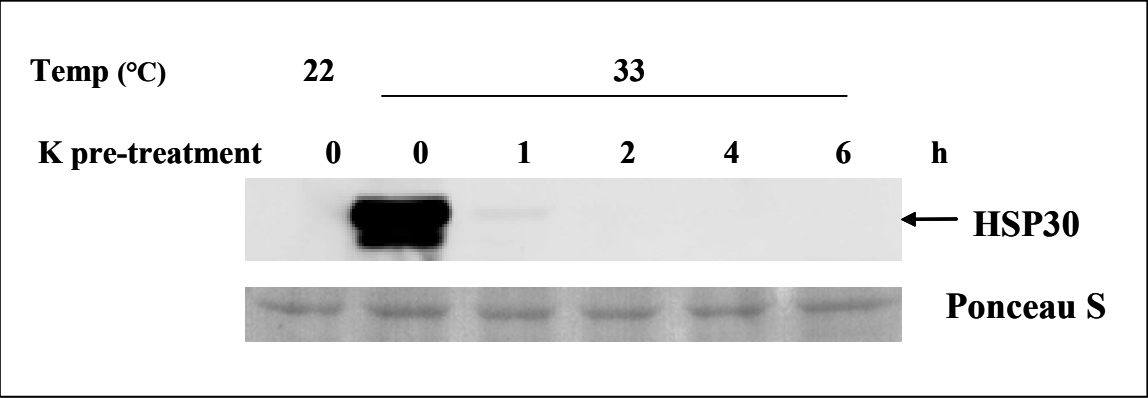| Temp (°C)	22	33					
K pre-treatment	0	0	1	2	4	6	h

← HSP30

Ponceau S

Figure 17. Effect of KNK437 on heat shock-induced accumulation of HSP30 and HSP70 protein in A6 cells. A6 cells were heat shocked at 33°C for 2 h followed by a 2 h recovery, with (+) or without (-) a 2 h pre-treatment with 100 μM KNK437. After treatment, cells were harvested and protein was isolated. Twenty or 40 μg of protein were then analyzed by western blot analysis using an HSP30 or HSP70 antibody, respectively. A representative Ponceau S stain is shown in the bottom panel which was used to confirm equal loading and transfer.

Figure 18. Inhibition of heat shock-induced accumulation of HSP30 protein by KNK437. Image J software (1.38) was used for densitometric analysis of HSP30 protein accumulation after heat shock at 33°C for 2-4 h, with or without a 2 h pre-treatment with 100 μM KNK437. The ability of KNK437 to inhibit HSP30 protein accumulation at each timepoint was graphed as % inhibition. Error bars represent standard error. At each timepoint, significant differences are indicated, (* = P<0.001).

Figure 19. Effect of KNK437 on sodium arsenite-induced accumulation of HSP30 and HSP70 protein in A6 cells.   A6 cells were treated with 20 μM sodium arsenite with (+) or without (-) a 2 h pre-treatment with 100 μM KNK437. After treatment, cells were harvested and protein was isolated. Twenty or 40 μg of protein was then analyzed by western blot analysis using an HSP30 or HSP70 antibody, respectively. A representative Ponceau S stain is shown in the bottom panel which was used to confirm equal loading and transfer.
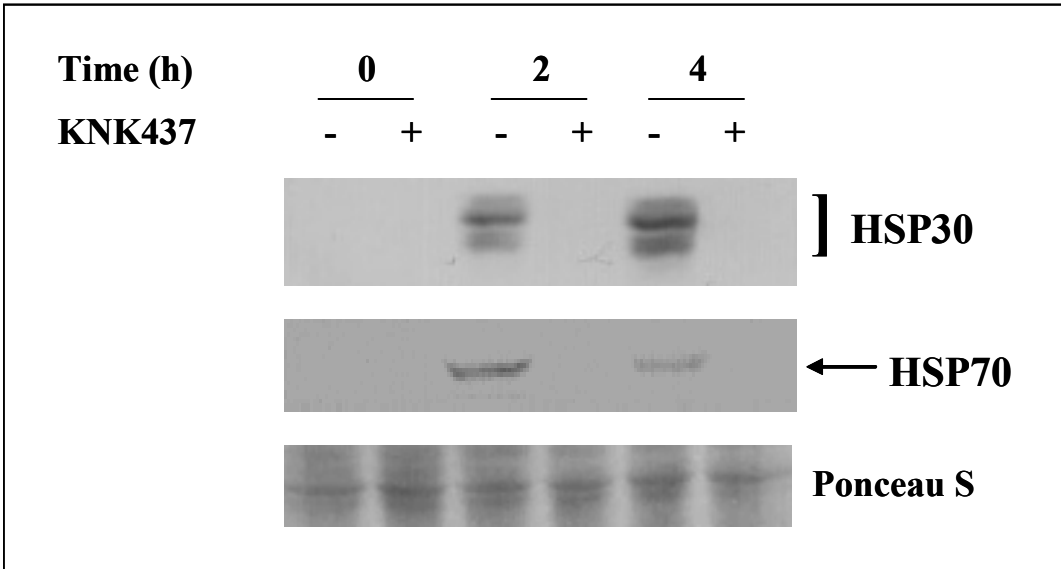
Figure 20. Inhibition of sodium arsenite-induced accumulation of HSP30 protein by KNK437. Image J software (1.38) was used for densitometric analysis of HSP30 protein accumulation after treatment with 20 µM sodium arsenite for 6-24 h, with or without a 2 h pre-treatment with 100 µM KNK437. The ability of KNK437 to inhibit HSP30 protein accumulation at each timepoint was graphed as % inhibition. Error bars represent standard error. At each timepoint, significant differences are indicated,(* = P<0.001).
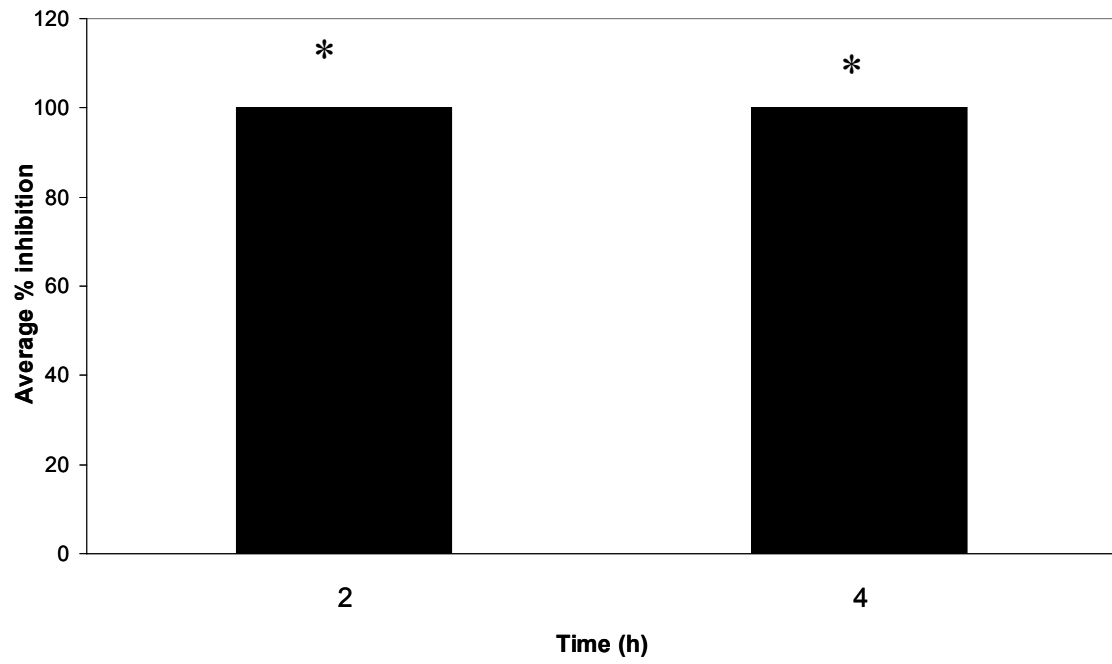
Figure 21. Effect of KNK437 on cadmium chloride-induced accumulation of HSP30 and HSP70 protein in A6 cells.   A6 cells were treated with 200 μM cadmium chloride with (+) or without (-) a 2 h pre-treatment with 100 μM KNK437. After treatment, cells were harvested and protein was isolated. Twenty or 40 μg of protein was then analyzed by western blot analysis using an HSP30 or HSP70 antibody, respectively. A representative Ponceau S stain is shown in the bottom panel which was used to confirm equal loading and transfer.
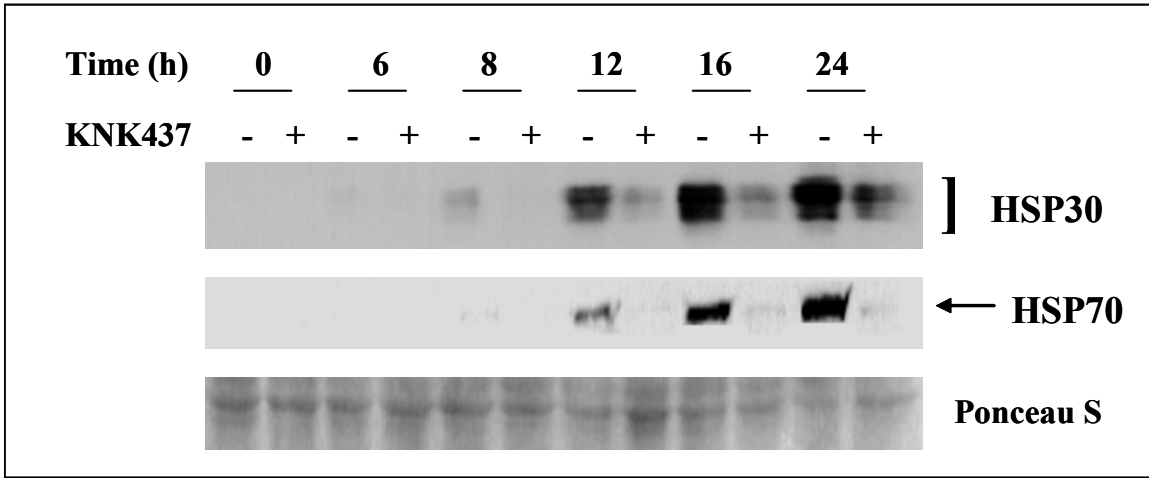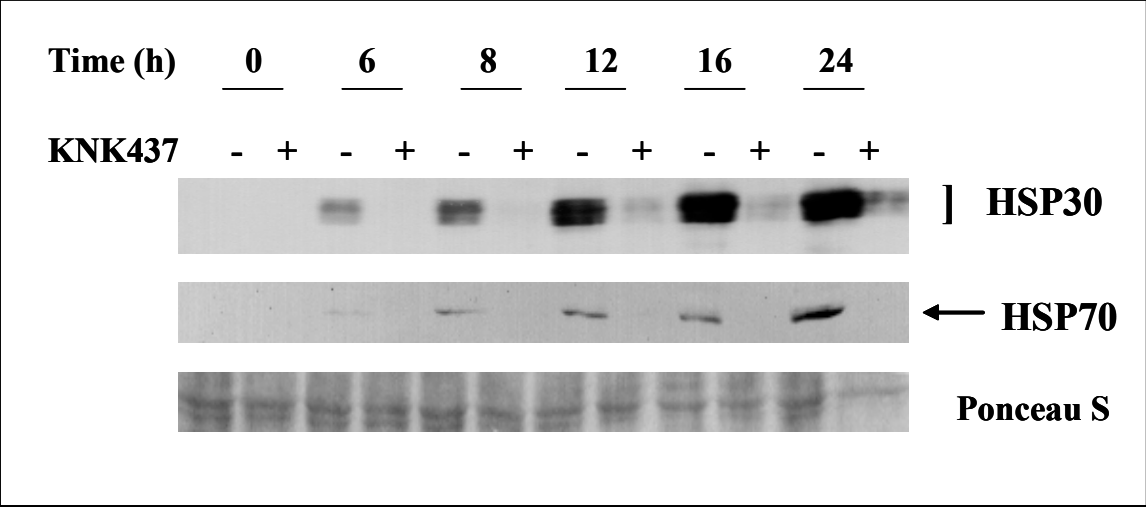
Figure 22. Inhibition of cadmium chloride-induced accumulation of HSP30 protein by KNK437. Image J software (1.38) was used for densitometric analysis of HSP30 protein accumulation after treatment with 200 µM cadmium chloride for 6-24 h, with or without a 2 h pre-treatment with 100 µM KNK437. The ability of KNK437 to inhibit HSP30 protein accumulation at each timepoint was graphed as % inhibition. Error bars represent standard error. At each timepoint, significant differences are indicated, (* = P<0.001).
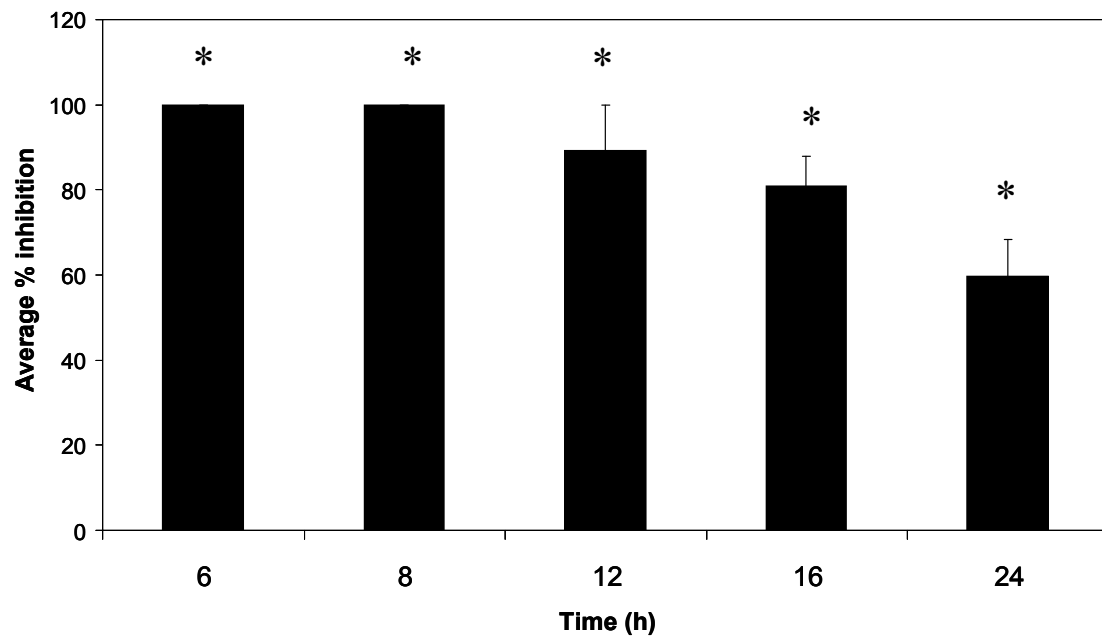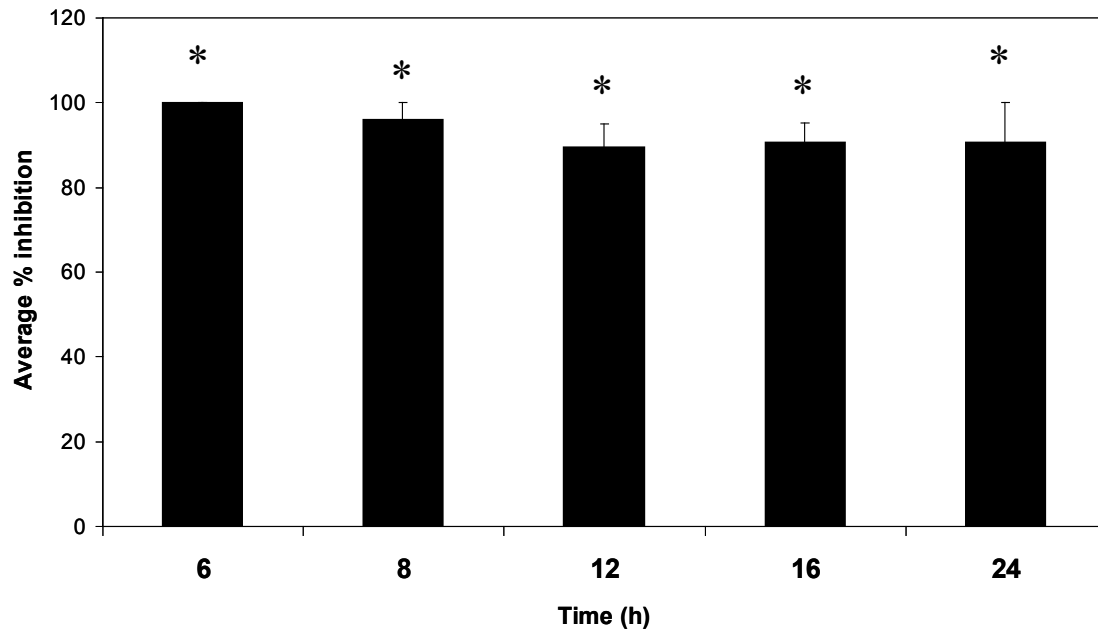
Figure 23. Effect of KNK437 on herbimycin A-induced accumulation of HSP30 and HSP70 protein in A6 cells.   A6 cells were treated with 1 μg/mL herbimycin A with (+) or without (-) a 2 h pre-treatment with 100 μM KNK437. After treatment, cells were harvested and protein was isolated.   Twenty or 40 μg of protein was then analyzed by western hybridization using an HSP30 or HSP70 antibody respectively.   A representative Ponceau S stain is shown in the bottom panel which was used to confirm equal loading and transfer.

Time (h)   0   6   8   12   16   24

KNK437   -   +   -   +   -   +   -   +   -   +   -   +

] HSP30

← HSP70

Ponceau S
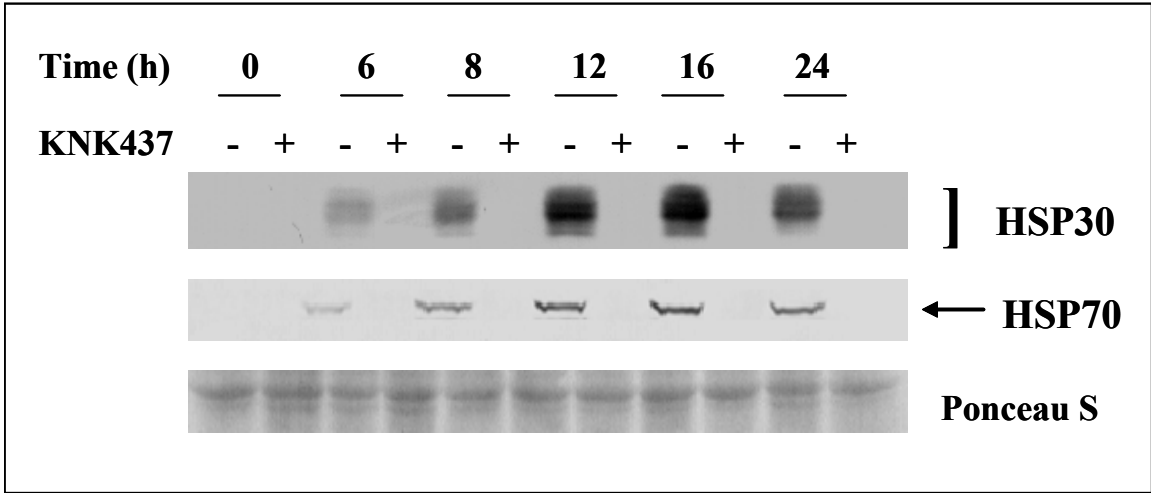
Figure 24. Inhibition of herbimycin A-induced accumulation of HSP30 protein by KNK437. Image J software (1.38) was used for densitometric analysis of HSP30 protein accumulation after treatment with 1 µg/ml herbimycin A for 6-24 h, with or without a 2 h pre-treatment with 100 µM KNK437. The ability of KNK437 to inhibit HSP30 protein accumulation at each timepoint is graphed as % inhibition. Error bars represent standard error. At each timepoint, significant differences are indicated, (* = P<0.001).
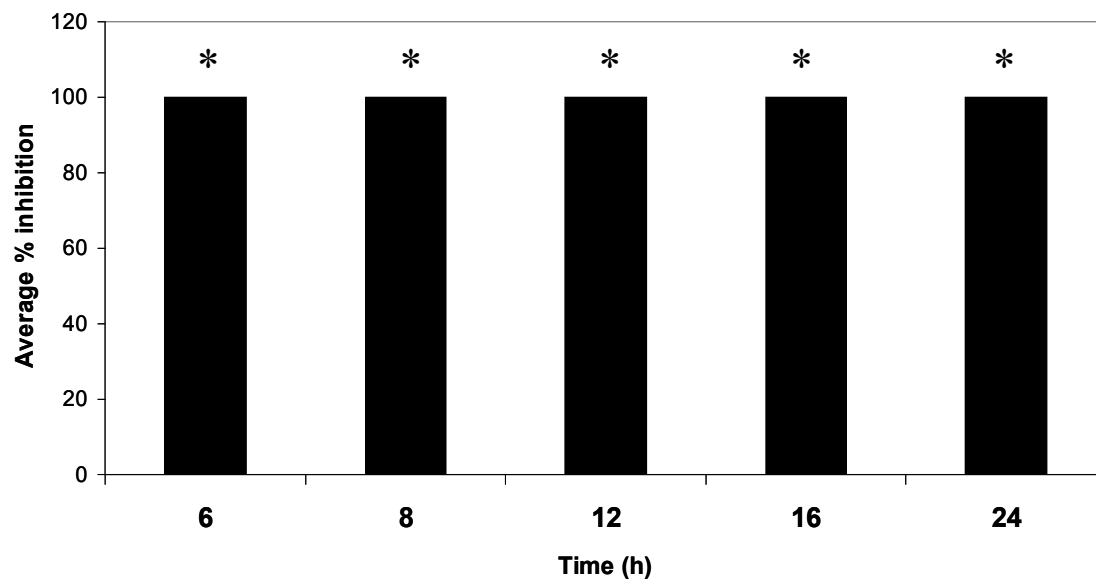
3.3 Effect of KNK437 on the intracellular accumulation of HSP30 protein in *Xenopus* A6 cells

Immunocytochemistry and laser scanning confocal microscopy (LSCM) were used to examine the effects of KNK437 on HSP30 accumulation and localization in A6 cells treated with heat shock, sodium arsenite, cadmium chloride or herbimycin A. Additionally, cell morphology, and the structure of the actin cytoskeleton were observed following exposure to the above-mentioned stressors. Initial experiments were performed with a 2 h pre-treatment of 100 μM KNK437 before treatment with heat shock or chemical stressors. For western blot analysis, a 2 h pre-treatment of 100 μM KNK437 was sufficient to reduce the accumulation of HSP30 significantly for all of the treatments mentioned above. However, this was not the case for immunocytochemical analysis which appeared to be more sensitive in detecting HSP30 accumulation (data not shown). Since accumulation of HSP30 protein was considerably reduced with a 6 h pre-treatment of 100 μM KNK437, this protocol was used in the following experiments.

A6 cells grown at 22°C displayed actin stress fibers that transversed the cell in axial bundles but did not accumulate detectable HSP30 protein (Figure 25 A, B). Treatment with 100 μM KNK437 did not result in the accumulation of HSP30 or disturb the pattern of actin stress fibers observed in control cells (Figure 25 C, D). A6 cells heat shocked at 33°C for 2 h resulted in the accumulation of HSP30 protein in nearly 100% of cells; located primarily in the cytoplasm with very little accumulation in the nucleus (Figure 26 A, C). With a 6 h pre-treatment of KNK437, there was no detectable heat shock-induced HSP30 protein accumulation (Figure 26 E). Also, there was no observable change in actin stress fibers in cells heat shocked at 33°C (Figure 26 B, D). However, when cells were pre-treated with KNK437 prior to heat shock at 33°C, there were occasional disruptions in the actin stress fibers (Figure 26 F). Heat shock at

85

35°C resulted in HSP30 accumulation in A6 cells in approximately 90% of the cells examined (Figure 27 A, C). While HSP30 protein was present in the cytoplasm, the majority of HSP30 staining was detected in the nucleus (Figure 27 A, C). When HSP30 and DAPI staining were overlapped, it was evident that HSP30 protein was not present in the nucleolus (B, D). When the cells were pre-treated for 6 h with 100 μM KNK437, there was no detectable HSP30 protein in the cells (E). In a small percentage of cells heat shocked at 35°C both with or without pre-treatment with KNK437, there was cell rounding possibly due to collapse of the actin cytoskeleton (data not shown).

Treatment with 20 μM sodium arsenite resulted in an abundance of HSP30 protein in the cytoplasm of all cells examined (Figure 28 A, C). There was a collapse of the actin cytoskeleton, with aggregation of actin at the periphery of the cell (Figure 28 D). A 6 h pre-treatment with 100 μM KNK437 resulted in a reduction of HSP30 in 40-75% of the cells (Figure 28 E). When the concentration of sodium arsenite was reduced to 10 μM for 14 h at 22°C, there was an accumulation of HSP30 in 90% of cells, located in the cytoplasm with an abundance near the periphery of the cell (Figure 29 A, C). Pre-treatment for 6 h with 100 μM KNK437 resulted in inhibition of HSP30 protein in 97-100% of the cells (Figure 29 E).

A 14 h treatment with 100 μM cadmium chloride at 22°C resulted in the accumulation of HSP30 in 80 - 100% cells. HSP30 was located in the cytoplasm and absent from the nuclei (Figure 30, A, C). In a small number of cells HSP30 was found in a punctate pattern (Figure 30 A, C). A 6 h pre-treatment with 100 μM KNK437 resulted in a decrease in HSP30 in 98 - 100% of the cells (Figure 30 E). Cadmium chloride on its own and when combined with KNK437 resulted in ruffling of the actin cytoskeleton at the periphery (Figure 30 D, F)

Finally, a 14 h treatment with 1 µg/mL herbimycin A at 22°C resulted in the accumulation of HSP30 in the cytoplasm of 60 - 80 % of cells (Figure 31 A, C). Cells pre-treated for 6 h with 100 µM KNK437 before exposure to 1 µg/mL herbimycin A had no detectable levels of HSP30 protein (Figure 31 E). Interestingly, a 2 h pre-treatment with KNK437 was also sufficient to fully inhibit HSP30 protein accumulation (data not shown). There was no observable change in the actin stress fibers when cells were treated with herbimycin A, both with and without the addition of KNK437 (Figure 31 B, D, F).

Figure 25. The effect of KNK437 on the intracellular accumulation of HSP30 in *Xenopus* A6 cells maintained at 22°C. A6 cells were grown on glass coverslips in L-15 media in the absence (A-B) or presence (C-D) of 100 μM KNK437. HSP30 was detected indirectly with an anti-*Xenopus* HSP30 primary antibody and Alexa-488 secondary antibody conjugate (green). Actin and nuclei were detected directly by staining with TRITC (red) and DAPI (blue), respectively. Cells were visualized using a Zeiss LSM 510 Meta laser confocal microscope. The left column indicates HSP30 fluorescence, while the right column shows a merged image of and HSP30, actin and DAPI fluorescence. Cells treated with KNK437 maintain the same shape and actin filament structure as the control cells. A 10 μm white scale bar is indicated.
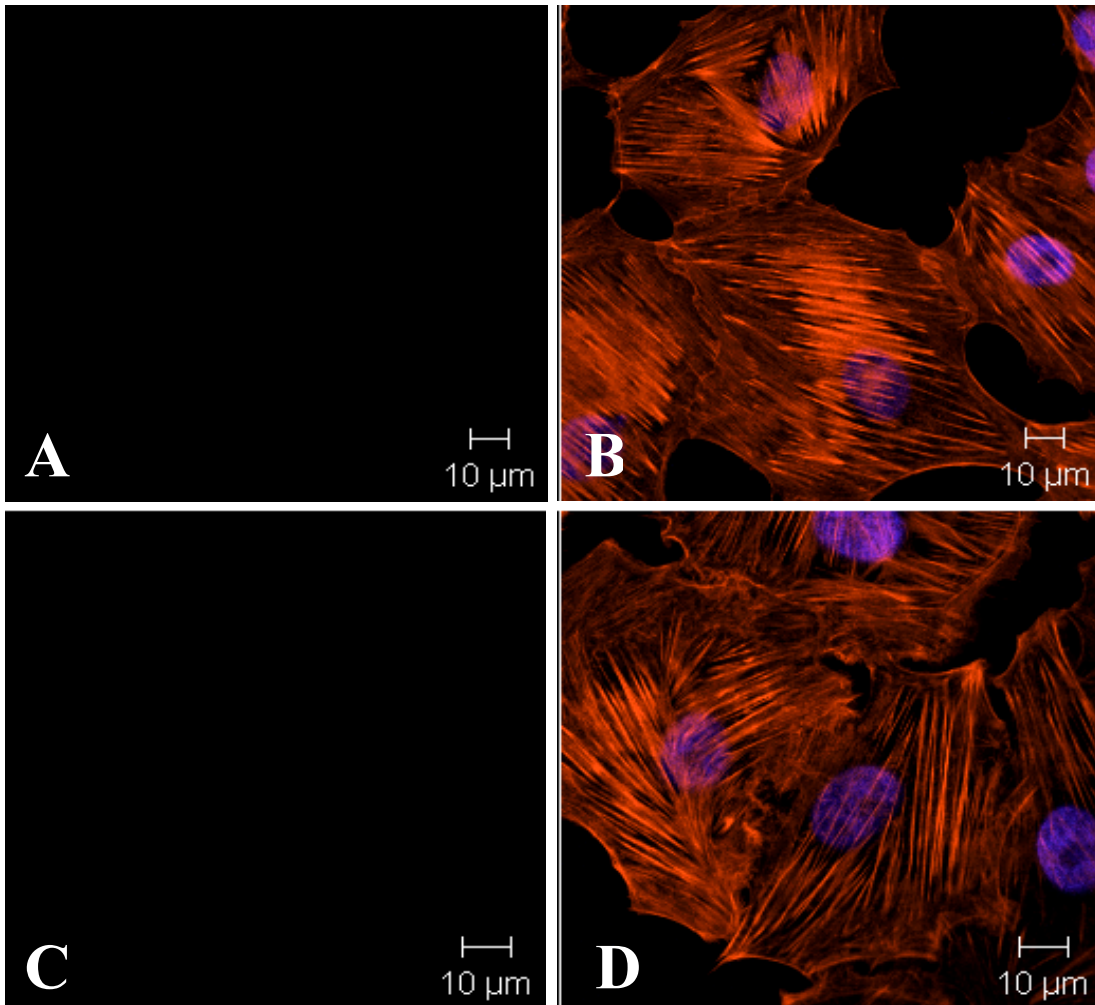
Figure 26. The effect of KNK437 on the intracellular accumulation of HSP30 in *Xenopus* A6 cells heat shocked at 33°C. A6 cells were grown on glass coverslips in L-15 media and were heat shocked at 33°C for 2 h followed by a 2 h recovery period at 22°C (A-F). Other A6 cells were pre-treated with 100 μM KNK437 before being heat shocked at 33°C for 2 h followed by a 2 h recovery period at 22°C (E-F). HSP30 was detected indirectly with an anti-*Xenopus* HSP30 primary antibody and Alexa-488 secondary antibody conjugate (green). Actin and nuclei were detected directly by staining with TRITC (red) and DAPI (blue), respectively. Cells were visualized using a Zeiss LSM 510 Meta laser confocal microscope. The left column indicates HSP30 fluorescence, while the right column shows a merged image of and HSP30, actin and DAPI fluorescence. A low magnification was used to demonstrate the relative proportion of cells expressing HSP30 (A-B). The same cells were then imaged using a higher magnification (C-D). Heat shock treatment of A6 cells at 33°C resulted in the accumulation of HSP30 in the cytoplasm (arrow), however it is absent from the nucleus in some cells (asterisk) and present in the nuclei in others (star). A 10 μm white scale bar is indicated.
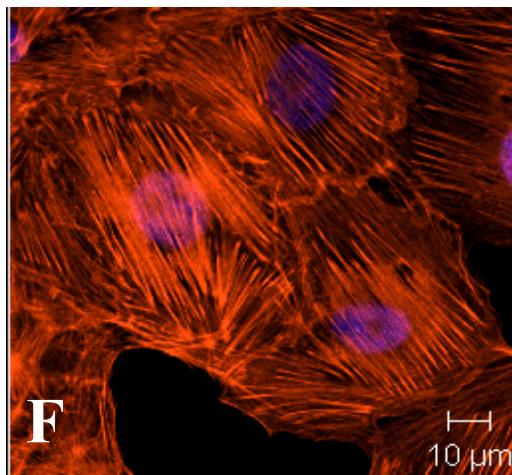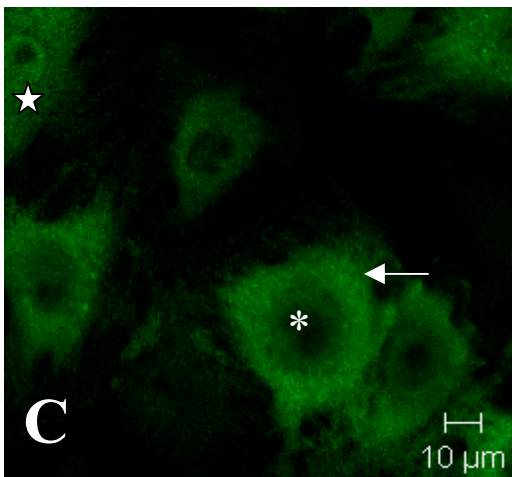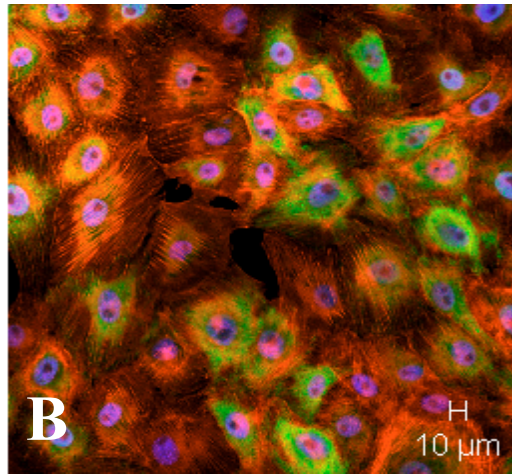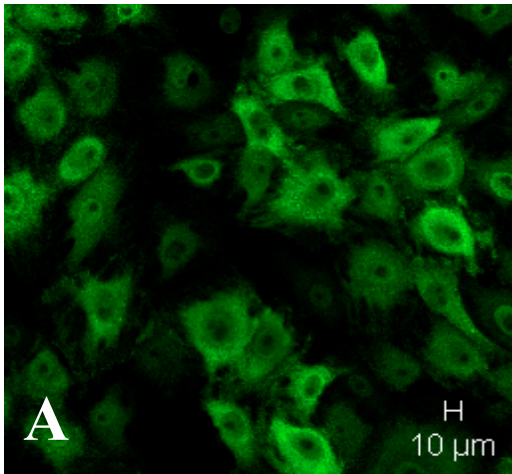
Figure 27. The effect of KNK437 on the intracellular accumulation of HSP30 in *Xenopus* A6 cells heat shocked at 35°C. A6 cells were grown on glass coverslips in L-15 media and were heat shocked at 35°C for 2 h followed by a 2 h recovery period at 22°C (A-F). Other A6 cells were pre-treated with 100 μM KNK437 before being heat shocked. HSP30 was detected indirectly with an anti-*Xenopus* HSP30 primary antibody and Alexa-488 secondary antibody conjugate (green). Actin and nuclei were detected directly by staining with TRITC (red) and DAPI (blue), respectively. Cells were visualized using a Zeiss LSM 510 Meta laser confocal microscope. The left column indicates HSP30 fluorescence, while the right column shows a merged image of and HSP30, actin and DAPI fluorescence. A low magnification was used to demonstrate the relative proportion of cells expressing HSP30 (A-B). The same cells were then imaged using a higher magnification (C-D). Heat shock treatment of A6 cells at 35°C resulted in the accumulation of HSP30 in the cytoplasm (arrow) and in the nucleus (asterisk) of over 50% of cells. A 10 μm white scale bar is indicated.
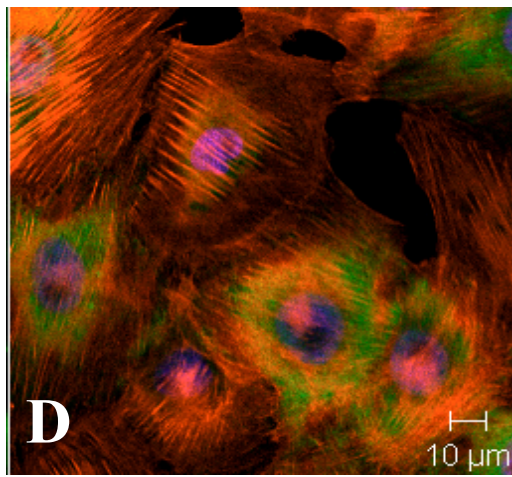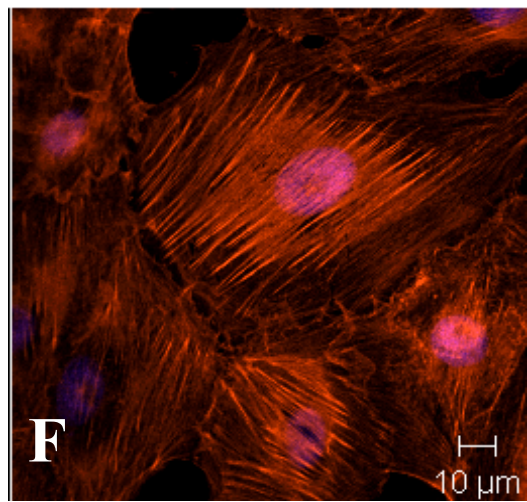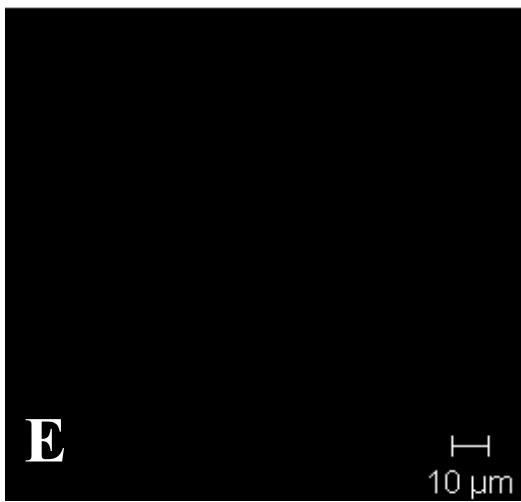
Figure 28. The effect of KNK437 on the intracellular accumulation of HSP30 in *Xenopus* A6 cells treated with 20 μM sodium arsenite for 14 h. A6 cells were grown on glass coverslips in L-15 media and were treated with 20 μM sodium arsenite for 14 h at 22°C (A-F). Other cells were pre-treated with 100 μM KNK437 before being treated with sodium arsenite (E, F). HSP30 was detected indirectly with an anti-*Xenopus* HSP30 primary antibody and Alexa-488 secondary antibody conjugate (green). Actin and nuclei were detected directly by staining with TRITC (red) and DAPI (blue), respectively. Cells were visualized using a Zeiss LSM 510 Meta laser confocal microscope. The left column indicates HSP30 fluorescence, while the right column shows a merged image of and HSP30, actin and DAPI fluorescence. A low magnification was used to demonstrate the relative proportion of cells expressing HSP30 (A-B). The same cells were then imaged using a higher magnification (C-D). Treatment of A6 cells with 20 μM sodium arsenite for 14 h at 22°C resulted in the collapse of the actin cytoskeleton (arrows).   HSP30 accumulation was present throughout the entire cytoplasm (circle arrow) and was absent from the nucleus (asterisk). A 10 μm white scale bar is indicated.

Figure 29. The effect of KNK437 on the intracellular accumulation of HSP30 in *Xenopus* A6 cells treated with 10 μM sodium arsenite. A6 cells were grown on glass coverslips in L-15 media and were treated with 10 μM sodium arsenite for 14 h at 22°C (A-F). Other cells were pre-treated with 100 μM KNK437 before being treated with sodium arsenite (E, F).   HSP30 was detected indirectly with an anti-*Xenopus* HSP30 primary antibody and Alexa-488 secondary antibody conjugate (green). Actin and nuclei were detected directly by staining with TRITC (red) and DAPI (blue), respectively. Cells were visualized using a Zeiss LSM 510 Meta laser confocal microscope. The left column indicates HSP30 fluorescence, while the right column shows a merged image of and HSP30, actin and DAPI fluorescence. A low magnification was used to demonstrate the relative proportion of cells expressing HSP30 (A-B). The same cells were then imaged using a higher magnification (C-D). Treatment of A6 cells with 10 μM sodium arsenite for 14 h 22°C resulted in the collapse of the actin cytoskeleton (arrow).   HSP30 accumulation was observed throughout the entire cytoplasm (circle arrow) and is absent from the nucleus (asterisk). A 10 μm white scale bar is indicated.
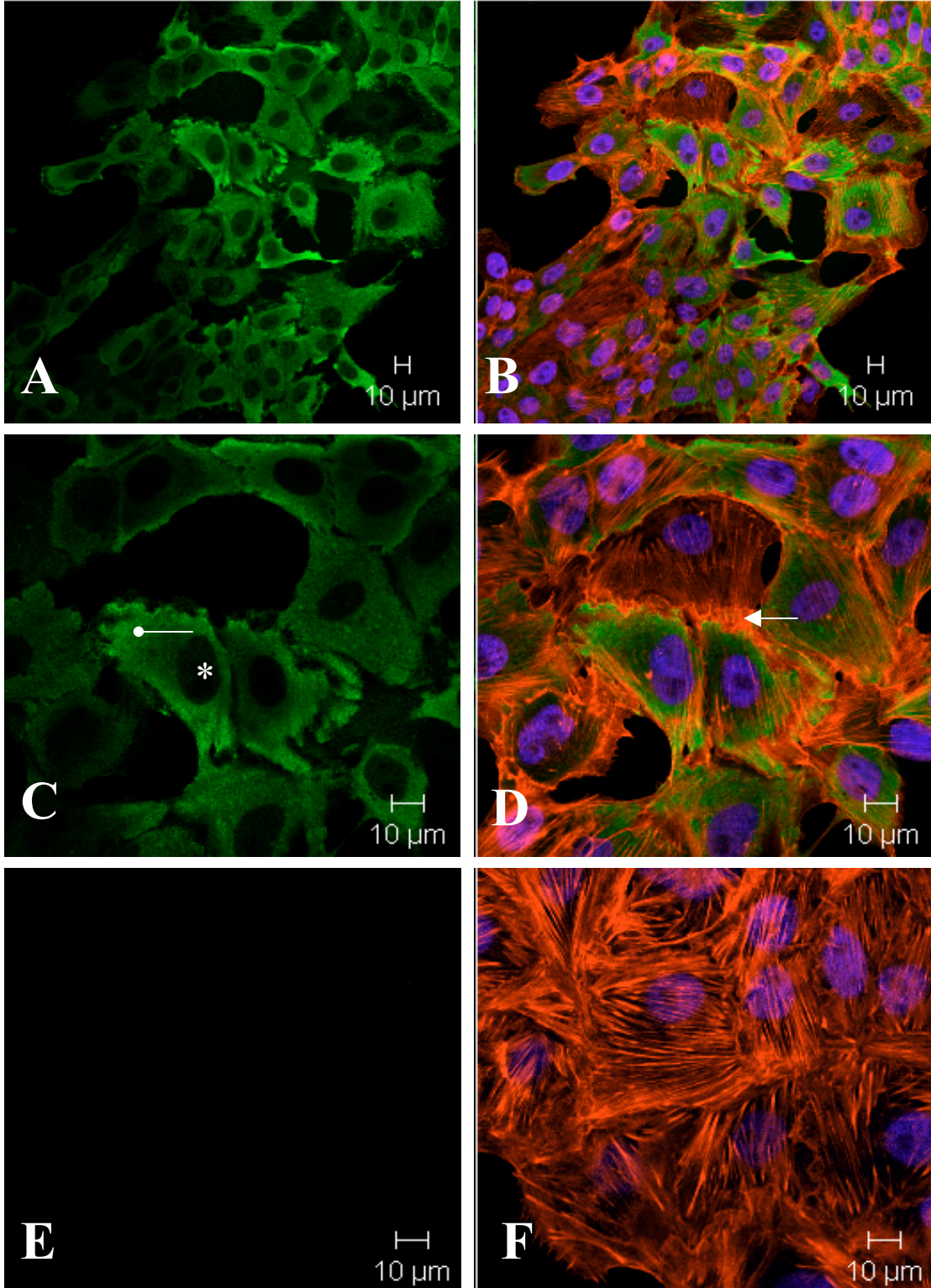
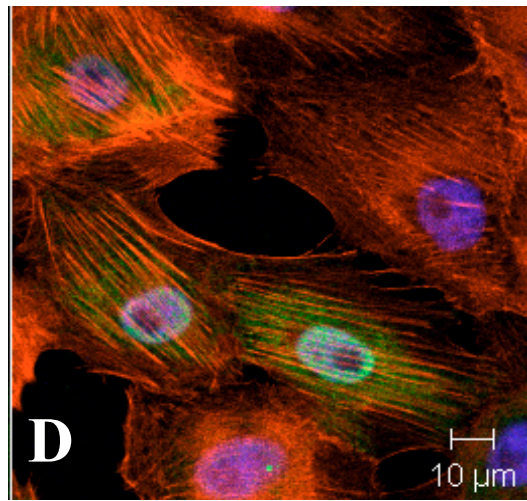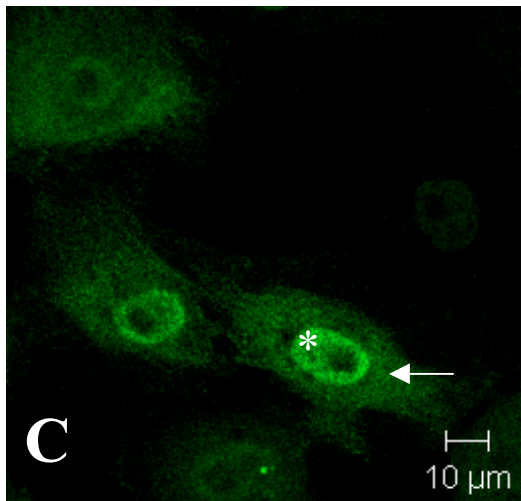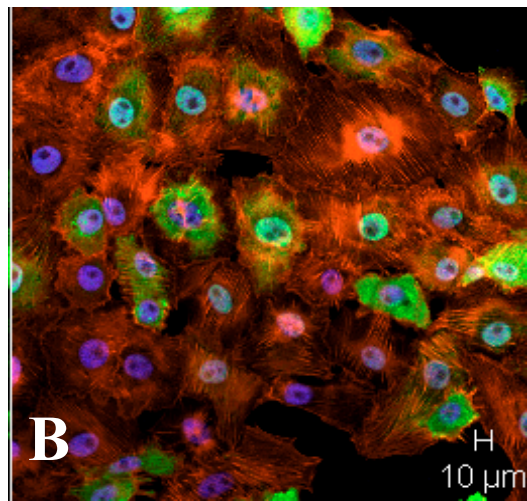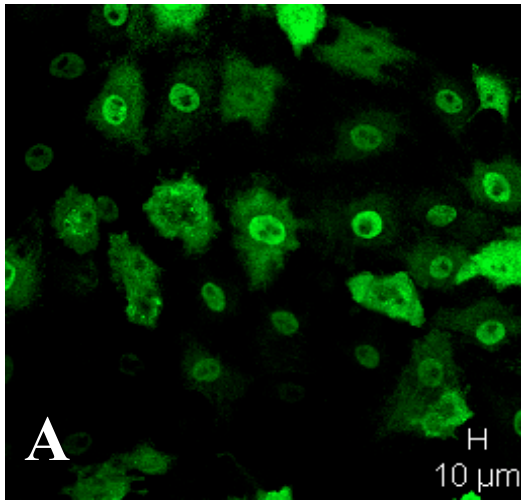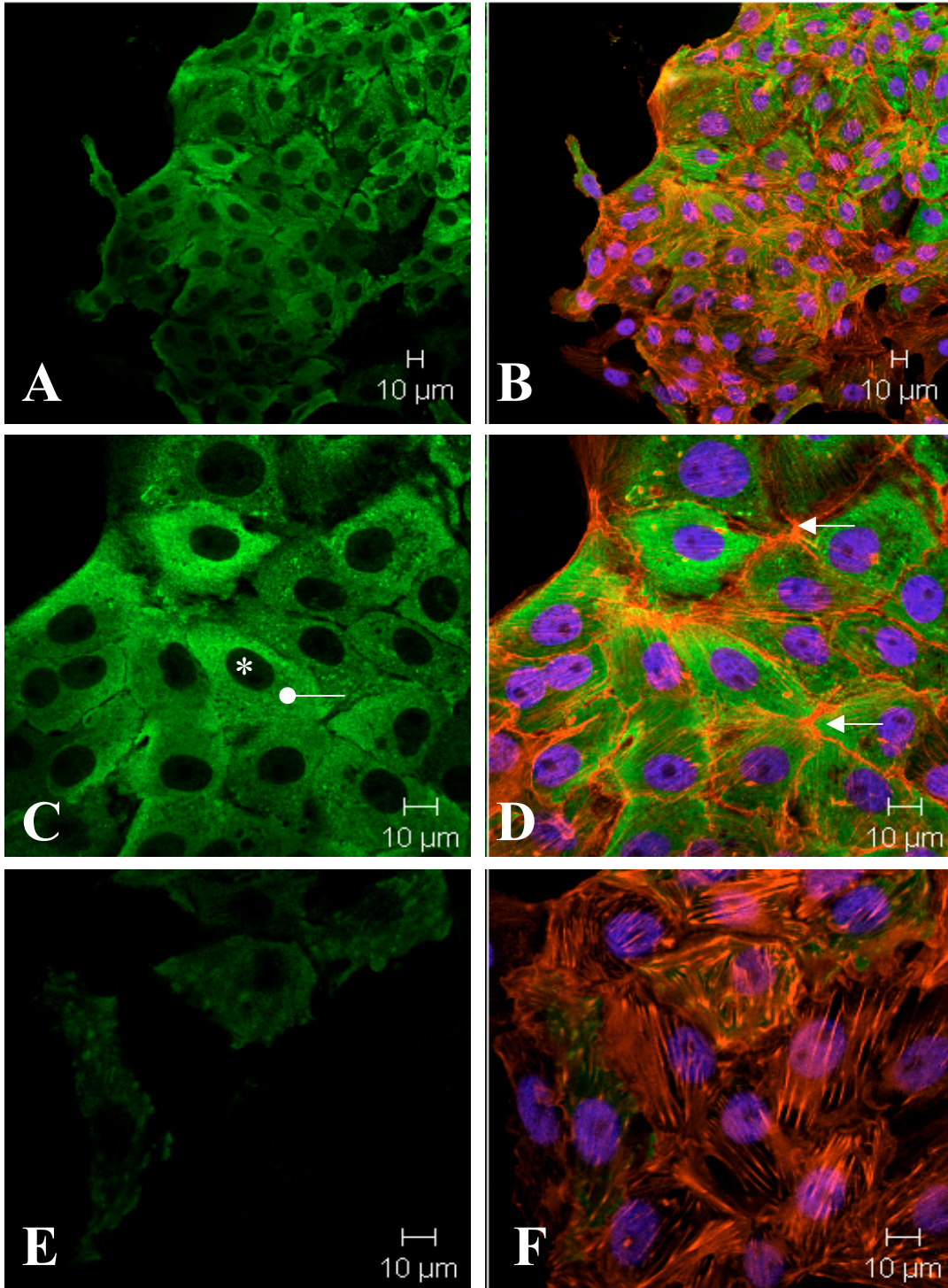**Figure 30. The effect of KNK437 on the intracellular accumulation of HSP30 in *Xenopus***
A6 cells treated with 100 μM cadmium chloride. A6 cells were grown on glass coverslips in

L-15 media and were treated with 100 μM cadmium chloride for 14 h at 22°C (A-F). Other A6

cells were pre-treated with 100 μM KNK437 before being treated with cadmium chloride (E-F).

HSP30 was detected indirectly with an anti-*Xenopus* HSP30 primary antibody and Alexa-488

secondary antibody conjugate (green). Actin and nuclei were detected directly by staining with

TRITC (red) and DAPI (blue), respectively. Cells were visualized using a Zeiss LSM 510 Meta

laser confocal microscope. The left column indicates HSP30 fluorescence, while the right

column shows a merged image of and HSP30, actin and DAPI fluorescence. A low

magnification was used to demonstrate the relative proportion of cells expressing HSP30 (A-B).

The same cells were then imaged using a higher magnification (C-D). Treatment of A6 with 100

μM cadmium chloride for 14 h at 22°C resulted in the accumulation of HSP30 in the cytoplasm,

particularly around the nucleus (arrow), however it was absent within the nucleus itself

(asterisk). HSP30 is occasionally present in a punctate pattern (diamond arrow). This treatment

both on its own and in conjunction with KNK437 resulted in the ruffling of the actin

cytoskeleton at the periphery of the cell (circle arrow). A 10 μm white scale bar is indicated.
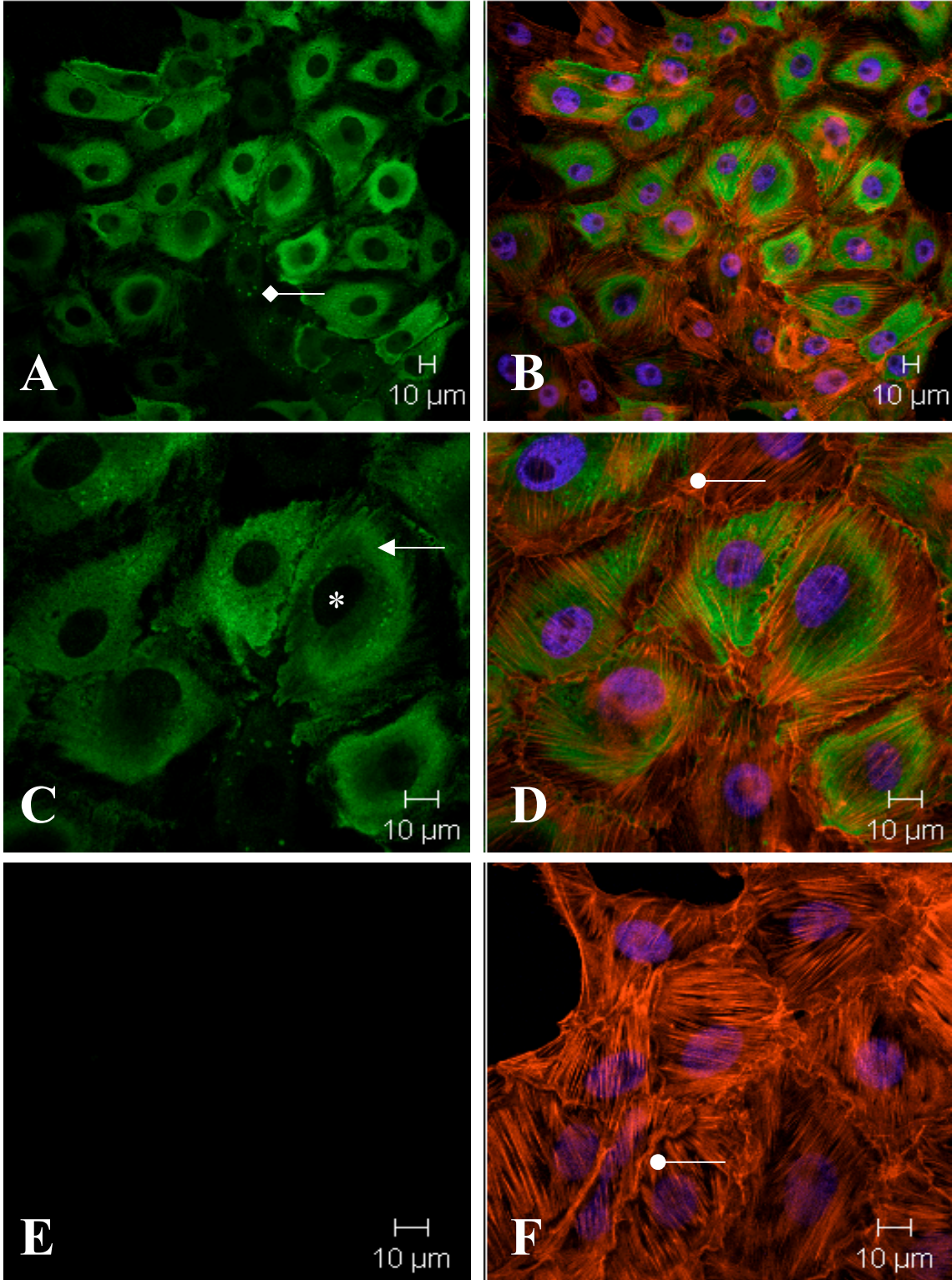
Figure 31. The effect of KNK437 on the intracellular accumulation of HSP30 in *Xenopus* A6 cells treated with 1 μg/mL herbimycin A. A6 cells were grown on glass coverslips in L-15 media and were treated with 1 μg/mL herbimycin A **for** 14 h at 22°C (A-F). Other cells were pre-treated with 100 μM KNK437 before being treated with herbimycin A. HSP30 was detected indirectly with an anti-*Xenopus* HSP30 primary antibody and Alexa-488 secondary antibody conjugate (green). Actin and nuclei were detected directly by staining with TRITC (red) and DAPI (blue), respectively. Cells were visualized using a Zeiss LSM 510 Meta laser confocal microscope. The left column indicates HSP30 fluorescence, while the right column shows a merged image of and HSP30, actin and DAPI fluorescence. A low magnification was used to demonstrate the relative proportion of cells expressing HSP30 (A-B). The same cells were then imaged using a higher magnification (C-D). Treatment of A6 cells with 1 μg/mL herbimycin A for 14 h at 22°C resulted in the accumulation of HSP30 evenly throughout the cytoplasm (arrow), and was absent from the nucleus (asterisk). A 10 μm white scale bar is indicated.
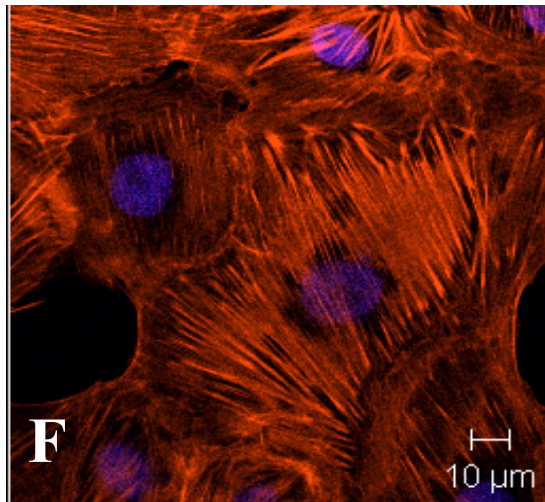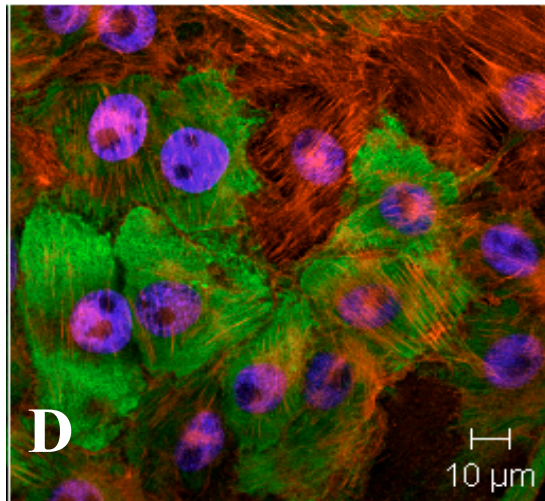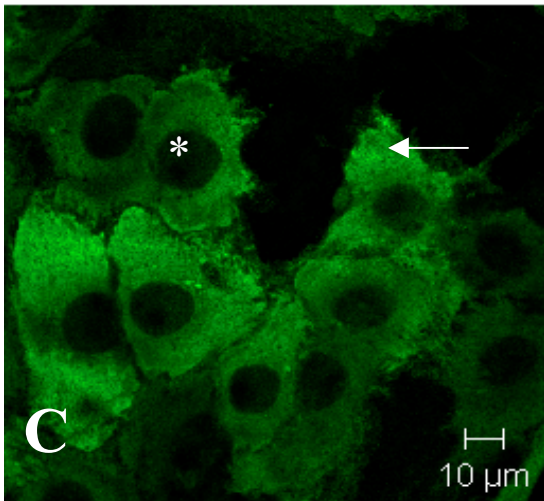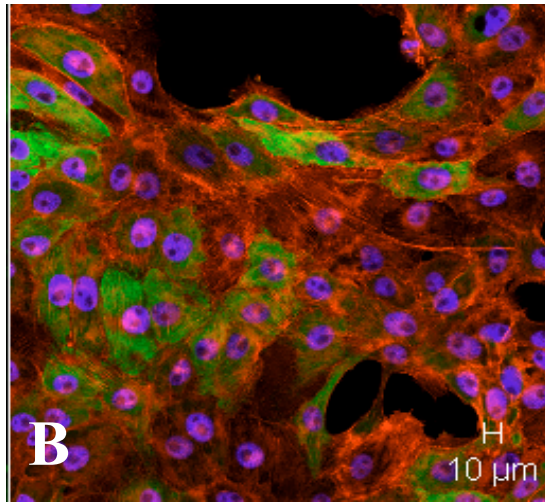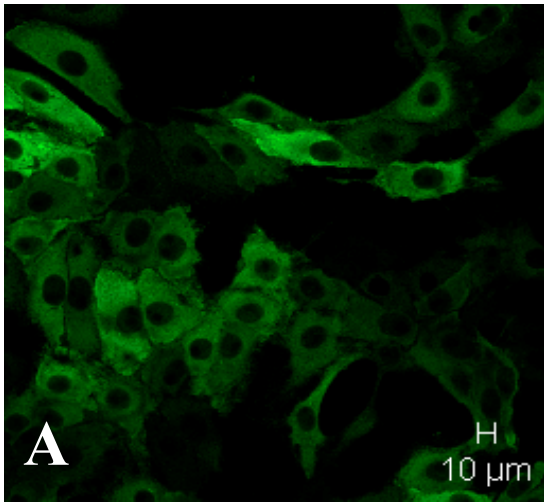
4 Discussion

This study has shown, for the first time, that the heat shock factor inhibitor, KNK437, inhibited the expression of chemical- induced *hsp* gene expression in a non-mammalian cell line; *Xenopus laevis* A6 cells. Initial studies demonstrated that treatment of A6 cells with sodium arsenite, cadmium chloride or herbimycin A resulted in the accumulation of *hsp30* mRNA as well as HSP30 and HSP70 protein. Previously, in our laboratory, it was reported that treatment of *Xenopus* A6 cells with sodium arsenite induced the accumulation of *hsp30*, *hsp70*, *hsp90* and *hsp110* mRNA as well as HSP30 and HSP110 protein (Darasch *et al*., 1988; Ali *et al*., 1996; Gellalchew & Heikkila, 2005; Gauley & Heikkila, 2006). Also, in *Xenopus* A6 cells, cadmium chloride induced the accumulation of *hsp70* and *hsp110* mRNA, while herbimycin A induced the expression of *hsp30* and *hsp70* genes (Briant *et al*., 1997; Gauley & Heikkila, 2006). These aforementioned chemical stressors have also been shown to induce *hsp* gene expression in mammalian cells (Hegde *et al*., 1995; Conde *et al*., 1997; Gaubin *et al*., 2000, Kim *et al*., 2001). The induction of *hsp* gene expression in *Xenopus* by these chemical stressors likely involves the activation of heat shock factor (HSF) and binding to the heat shock element (HSE) as determined for heat shock (Ovsenek & Heikkila, 1990). It has been demonstrated that treatment of *Xenopus* oocytes with sodium arsenite and cadmium chloride resulted in the specific binding of heat shock factor 1 (HSF1) to heat shock HSE as determined with gel shift assays (Gordon *et al*., 1997). Increased binding of HSF and HSE has also been shown in Schneider S2 cells when cells were treated with herbimycin A (Duncan, 2005). Although the exact pathways that lead to the induction of *hsps* when A6 cells are exposed to sodium arsenite, cadmium chloride or herbimycin A are unknown, the end result appears to be the induction of HSF1 which induces the expression of *hsp* genes.

KNK437 is a known inhibitor of HSF1 activity (Yokota *et al*., 2000). The present study has demonstrated that a 2 h pre-treatment with 100 µM KNK437 was sufficient to inhibit *hsp30* mRNA accumulation in A6 cells and completely prevent HSP30 and HSP70 protein accumulation in heat shocked A6 cells. Also, immunocytochemistry revealed that there was no detectable HSP30 accumulation in A6 cells that were pre-treated for 6 h with 100 µM KNK437 prior to heat shock. This confirms previous studies in our laboratory showing that KNK437 was capable of inhibiting heat-induced *hsp30*, *hsp47* and *hsp70* mRNA and HSP30 protein accumulation in A6 cells (Manwell & Heikkila, 2007). In mammalian cells, Yokota *et al*., (2000) found that KNK437 inhibited heat-induced HSP40, HSP70 and HSP105 accumulation. Also, in A-172 human squamous carcinoma cells and A549 human lung adenocarcinoma cells, KNK437 inhibited the heat shock induction of HSP27 and HSP72 (Ohnishi *et al.* 2004). Finally, KNK437 inhibited heat induced HSP27 accumulation *in vivo* in transplantable murine tumours in C3H/He mice (Koishi *et al*., 2001).

While the inhibition of heat-induced *hsp* gene expression by KNK437 has been reported in the literature, the inhibition of chemical stressor-induced *hsp* gene expression has not. In a single report, treatment of human COLO 320DM cells with KNK437 inhibited sodium arsenite-induced acquisition of thermotolerance (Yokota *et al*., 2000). However the effect of this agent on *hsp* gene expression was not demonstrated. In the present study, a 2 h pre-treatment of A6 cells with 100 µM KNK437 prior to a 20 µM exposure of sodium arsenite inhibited *hsp30* mRNA accumulation by 51% after 12 h and HSP30 protein accumulation by 59 % after 24 h. Also, KNK437 completely inhibited HSP70 accumulation after 24 h of sodium arsenite treatment. Immunocytochemistry revealed that A6 cells treated with 10 µM sodium arsenite resulted in HSP30 accumulation in the cytoplasm which was inhibited by KNK437 in

approximately 97-100% of the cells. Since sodium arsenite-induced expression of *hsp* genes involves the activation of *Xenopus* HSF1 (Gordon *et al*., 1997), it is likely that KNK437 is targeting this transcription factor.

In the present study, exposure of A6 cells to sodium arsenite caused a disruption in the actin cytoskeleton. Also in mammalian systems, sodium arsenite treatment of H9C2 myoblasts altered focal adhesion structure and function (Yancy *et al.*, 2005). Since focal adhesions consist of bundles of actin filaments and several adhesion-proteins, which attach the cytoskeleton to the extracellular matrix (Jockusch *at al.,* 1995), this may explain why *Xenopus* A6 cells treated at higher concentrations of sodium arsenite, displayed a rounded morphology. HSPs have also been implicated in the protection of focal adhesion kinases (Mao *et al*., 2003).

In the present study, a 2 h pre-treatment with KNK437 was sufficient to inhibit cadmium chloride-induced *hsp30* mRNA by 33% at 12 h and by 90% after a 24 h treatment. At the protein level, both HSP30 and HSP70 were markedly inhibited by KNK437. Immunocytochemistry revealed that treatment of A6 cells with cadmium chloride resulted in HSP30 accumulation in a punctate pattern, particularly in the perinuclear region. This accumulation pattern of HSP30 accumulation was inhibited in 98-100% of the cells by KNK437. As mentioned previously, the effect of KNK437 on cadmium-induced *hsp30* gene expression in A6 cells is likely the inhibition of HSF activity. Previous studies have shown that within cells, cadmium induced the accumulation of denatured or abnormal proteins by substituting for zinc or reacting with vicinal thiol groups in proteins (Waisberg *et al*., 2003). An accumulation of denatured protein is a signal for HSF activation.

Finally, this study revealed that a 2 h pre-treatment of A6 cells with 100 μM KNK437 inhibited *hsp30* mRNA, HSP30 and HSP70 accumulation induced by herbimycin A.

Herbimycin A, a benzoquinoid ansamycin antibiotic, has been shown to act as a tyrosine kinase inhibitor and an inducer of *hsp* gene expression (Hegde *et al*., 1995). While the mechanism of herbimycin A induction of *hsp* gene expression is not known, it is thought to bypass stress regulatory pathways in its activation of HSF. The present study has shown that KNK437 can inhibit *hsp* gene expression induced by pharmacological means.

Interesting differences were detected between heat- and chemically-inducible HSP30 localization in A6 cells. Treatment of A6 cells with a 33°C heat shock, sodium arsenite, cadmium chloride or herbimycin A resulted in HSP30 accumulation primarily in the cytoplasm. However, heat shock treatment of A6 cells at 35°C resulted in enriched HSP30 accumulation in the nucleus. At this time, it is not known how HSP30 enters the nucleus since it has no known nuclear localization sequence. Furthermore, it was previously shown in our laboratory that *Xenopus* HSP30C forms high molecular weight multimeric structures *in vivo* (Ohan *et al*., 1998). Since proteins without a nuclear localization signal can only diffuse through nuclear pores if they are smaller than 40 kDa (Breeuwer & Goldfarb, 1990), it is possible that HSP30 may be assisted by a protein that possesses one. Interestingly, HSP70 and HSC70, have been shown to play an essential role in the transport of proteins to the nucleus (Shi & Thomas, 1992). Since the HSP30 multimeric complex contains HSP70 (Ohan *et al*., 1998), it is possible that this molecular chaperone is involved in transporting HSP30 into the nucleus.

Although KNK437 inhibited *hsp30* mRNA and HSP30 accumulation induced by all of the chemical stressors used, the ability of KNK437 to inhibit *hsp* gene expression differed depending on the type of stressor employed. Herbimycin A, for example, induced a *hsp* response that was weaker when compared to sodium arsenite or cadmium chloride. This may result from the relative toxicity of sodium arsenite or cadmium chloride to A6 cells when compared to that of

herbimycin A. As herbimycin A is less toxic to the cells and it circumvents the stress regulatory

pathway (Hegde *et al*., 1995), it is possible that there is a reduced activation of HSF1 compared

to sodium arsenite or cadmium chloride. Also in comparison to heat shock, it was previously

shown in A6 cells that herbimycin A resulted in a weaker accumulation of *hsp30* and *hsp70*

mRNA (Briant *et al*., 1997).

In summary, the present study has shown that KNK437 is an inhibitor of *hsp* gene

expression induced not only by heat shock but also by sodium arsenite, cadmium chloride or

herbimycin A. In future studies, it would be beneficial to perform a gel shift assay to verify that

KNK437 affects the binding of HSF1 to HSE in *Xenopus* A6 cells. Previously, it was

demonstrated that KNK437 inhibited the heat shock-induced acquisition of thermotolerance in

A6 cells (Manwell and Heikkila, 2007). Thus it would be interesting to determine whether

KNK437 is able to inhibit the acquisition of thermotolerance or stress resistance in A6 cells

induced by the various chemical stressors used in the present study.

To date, studies employing KNK437 to inhibit *hsp* gene expression have only been

carried out in cultured cells. It would be interesting to examine its effect in a developmental

system. Previous studies in our laboratory have shown that *hsp30* mRNA was present

constitutively in the cement gland of early and midtailbud embryos and that HSP30 protein was

not present until the early tadpole stage. In heat shocked embryos, *hsp30* mRNA and HSP30

protein accumulation was enriched in the cement gland, somitic region, lens placode and

proctodeum at the early to midtailbud stages. It is likely that HSF1 was involved in the induction

of heat induced *hsp30* gene expression (Heikkila *et al*., 2003). However, it is not known whether

HSF1 is involved in the constitutive accumulation of *hsp30* mRNA in the cement gland of

Xenopus embryos. Thus, additional experiments could examine the effect of inhibiting HSF1 by

KNK437 on the viability, morphology, and pattern of *hsp* gene expression in control and heat shocked *Xenopus laevis* embryos during early development.

The discovery of KNK437 as an HSF inhibitor has proved to be a valuable tool in the optimization of cell death in tumour cells by combining radiation with thermal therapy (Yokota *et al*., 2000; Sakurai *et al*., 2005). While these experiments have been beneficial to the field of cancer research, KNK437 has been used very little as a tool for examining the role of HSPs in certain cellular or developmental processes. Also, since KNK437 is a universal inhibitor of HSF1, it would be useful for research examining *hsp* gene expression in non-model systems.

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