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Identification and functional characterization of unique single domain antibodies against pro-apoptotic protein Bax.

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Identification and Functional Characterization of Unique Single Domain Antibodies Against Pro-apoptotic Protein Bax

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Abstract

Neuronal cell death as a result of increased oxidative stress is involved in many neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, stroke and ischemia. Bax is a well established pro-apoptotic protein of the Bcl-2 family. It is critically involved in the initial phase of apoptosis under oxidative stress.

In this study we aimed to develop specific blockers against the pro-apoptotic Bax protein that would in turn block cell death. After screening a llama phage-display library for specific single domain antibodies (V_HH) against purified recombinant Bax protein, we isolated six genes that code for V_HH clones capable of blocking Bax function *in vitro* (when expressed as antibodies) and *in vivo* (when expressed as intrabodies).

We have generated mammalian cell lines stably transfected with anti-bax intra-body genes that are virtually resistant to apoptosis induced by oxidative stress. These genes and the protein coded by them are unique and specific biochemical tools with potential to inhibit proteins in their native structure. This is particularly advantageous for future biochemical mechanism studies to study the role of Bax in various cell death pathways. Specifically, these antibodies can be used to study the interaction of Bax protein to various other pro-and anti-apoptotic Bcl-2 family proteins. In addition, intrabodies have potential to be used either directly (as in gene therapy) or as models for the development of therapeutic agents, for disease involving cell death induced by oxidative stress.

Dedication:

I would like to dedicate this thesis to my future husband Matthew and parents who have always supported and encouraged me to keep pushing forward and never to let go of my goals and dreams.

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List of Abbreviations

AD	Alzheimer's disease
AIF	apoptosis inducing factor
ANT	adenine nucleotide translocase
APAF-1	apoptosis activating factor 1
ATP	adenosine triphosphate
BH(1-4)	Bcl-2 homology domains 1-4
BSA	bovine serum albumin
CARD	caspase recruitment domain
CHAPS	3-[3-(Cholamidopropyl)dimethylammonio]-1-proanesulfonate
CNS	central nervous system
Cyp D	cyclophilin D
DISC	death inducing signaling complex
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
EDTA	ethylenediamine-tetraacetic acid
EGTA	Ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid
FADD	Fas associated death domain
FBS	fetal bovine serum
Fv	variable region fragment (of an antibody)
Fc	constant region fragment (of an antibody)
HEPES	N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
His	histadine

HRP	horseradish peroxidase
IAP	inhibitors of apoptosis
IgG	immunoglobin
IMS	inner membrane space (referring to mitochondria)
iRNA	inhibitor RNA (ribonucleic acid)
MDA	malonaldehyde
MIM	mitochondrial inner membrane
MOM	mitochondrial outer membrane
MOMP	mitochondrial outer membrane permealization
MS	Multiple Sclerosis
OD	ocular density
PD	Parkinson's disease
PHPA	parahydroxy-phenylacetate
PP2A	protein phosphatase 2A
PTP	permeability transition pore
ROS	reactive oxygen species
rTPA	recombinant tissue plasminogen activator
scFv	single chain variable fragment
SDS-PAGE	sodium diodecyl sulphate- polyacrilamide gel electrophoresis
Ser	serine
SHSY-5Y	human neuroblastoma cell line
Smac	secondary mitochondrial derived activator of caspases
SOD	superoxide dismutase

Chapter 1: Introduction

1.1 Apoptosis

Cell division, differentiation and death are important physiological processes which help control the number of cells in an organism (Raff, M, 1992). Until the last 25 years the majority of research has focused on the former two processes. However since this time, work on apoptosis has allowed for the various discoveries in understanding the importance of balance in cell number control for maintaining a healthy system (Kerr, J *et al.*, 1972). Overall, apoptosis is understood to be vital for development and homeostasis in healthy organisms. Specifically, in development apoptosis is vital for proper organ and limb formation (Saunders, J, 1966), regulating cell numbers thus preventing uncontrolled growth (common to cancer) (Raff, M, 1992) and removal of damaged and thus potentially harmful cellular debris (Cohen, J *et al.*, 1992).

Of particular importance was the discovery that cell death in multicellular organisms was subject to genetic control (Ellis, H *et al.*, 1986). Improper regulation of these genes was since been shown to result in inhibition of apoptosis resulting in various diseases such as cancer (Strasser, A *et al.*, 1990) and autoimmune diseases (Watanabe-Fukunaga, R *et al.*, 1992). However, inappropriate activation of apoptotic pathways can have similarly detrimental effects as seen in various neurodegenerative diseases such as Alzheimer's Disease (AD), Parkinson's Disease (PD) and Stroke (Price, D, 1999). Research in understanding, elucidating and interfering with various apoptotic pathways has since grown exponentially, in an effort to combat these diseases.

1.2 Apoptosis vs. Necrosis

Apoptosis is both physically and biochemically distinct from cellular death occurring by accidental damage also known as necrotic death. Necrotic death is a passive form of cell death which occurs from direct injury to a cell (Fig.1.1A). This results in the swelling and lysis of the cell, followed by inflammation due to the release of cytoplasmic material into the surrounding tissue (Steller, H, 1995; Pollack, M *et al.*, 2001). Specifically, swelling occurs because the injury to the cell destroys the cells ability to control its fluid and ion balance, water and positively charged ions which are normally pumped out are allowed to enter the cell resulting in its rupture. In an effort to limit infection and clear cellular debris, macrophages and other white blood cells congregate at the rupture site resulting in inflammation which can have negative implications on surrounding tissues (Duke, R *et al.*, 1996).

In contrast apoptosis is an active process divided into three unique phases: induction, effector and degradation/elimination phase (with distinct morphological features) (Fig.1.1B). The induction phase may vary depending on the cell type involving a death inducing signal which may arise from within the cell or an external source. Some examples of apoptosis inducers include reactive radicals (reactive oxygen species and nitrogen intermediates), tumor necrosis factor-ά, activation of Ca^{2+} pathway and upregulation or downregulation of pro- and anti-apoptotic proteins respectively (Pollack, M *et al.*, 2001). Despite the numerous activation pathways, the tight regulation of each pathway in healthy cells is a feature shared by all.

The second phase in the apoptotic pathway is the effector phase, where the cell becomes committed to die as key apoptotic pathways become activated including the

extrinsic pathways via death domain activation on the cell surface (Baker, S *et al.*, 1998) and intrinsic pathways involving the mitochondria (Kluck, R *et al.*, 1997).

Theses events are subsequently followed by a degradation phase involving activation of caspase cascades (cystein proteases) resulting in enzymatic cleavage of cellular components. Plasma membrane flipping detected by the redistribution of phosphatidyl serine between the inner and outer leaflet of the plasma membrane is well studied feature of apoptosis (Fadok, V *et al.*, 1992). This is followed by changes at the nuclear level, specifically the breakdown of the nuclear envelope and chromatin condensation while activated endonucleases fragment DNA (Kerr, J *et al.*, 1972). In the last stages following these events apoptosis is characterized by unique morphological changes including plasma membrane blebbing, as the cell is fragmented into small apoptotic bodies, followed by phagocytosis by neighboring cells which prevents inflammation and damage to surrounding tissue (Pollack, M *et al.*, 2001).

1.3 Apoptosis Pathways

Activation of apoptosis involves tight regulation of numerous pathways stimulated by both external and internal signals, resulting in extrinsic and intrinsic pathways.

1.3.1 Extrinsic Apoptosis Pathway: Receptor Mediated Apoptosis

Tumor necrosis factor superfamily (TNFR) receptors are a key component of this apoptotic pathway. Two important ligands which can bind to these receptors are TNF-α and Fas ligand (FasL) (commonly present in immune cells). Binding of these ligands to the cell membrane receptor results in activation of a specific group of proteases

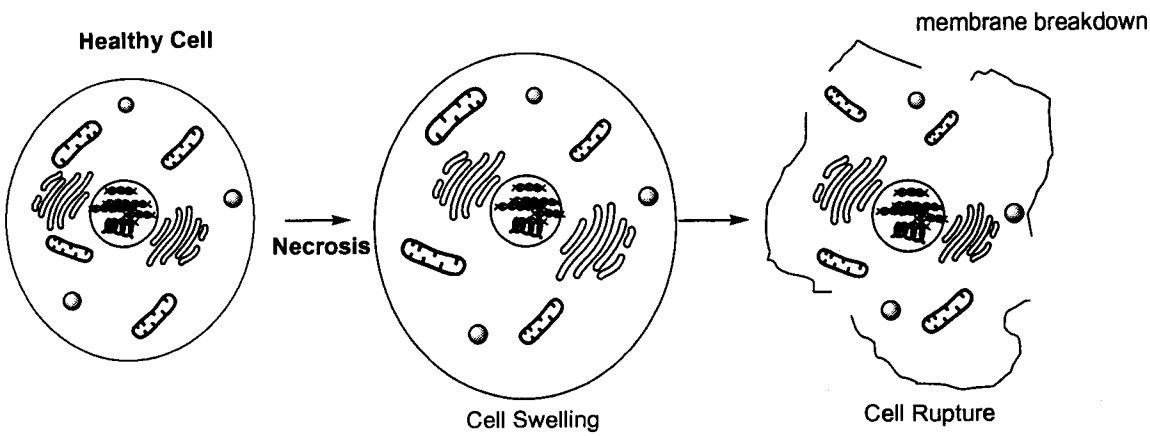

Figure 1.1A Cell death through necrosis

Necrosis is a passive process occurring due to accidental cell injury resulting in cell rupture and inflammation.

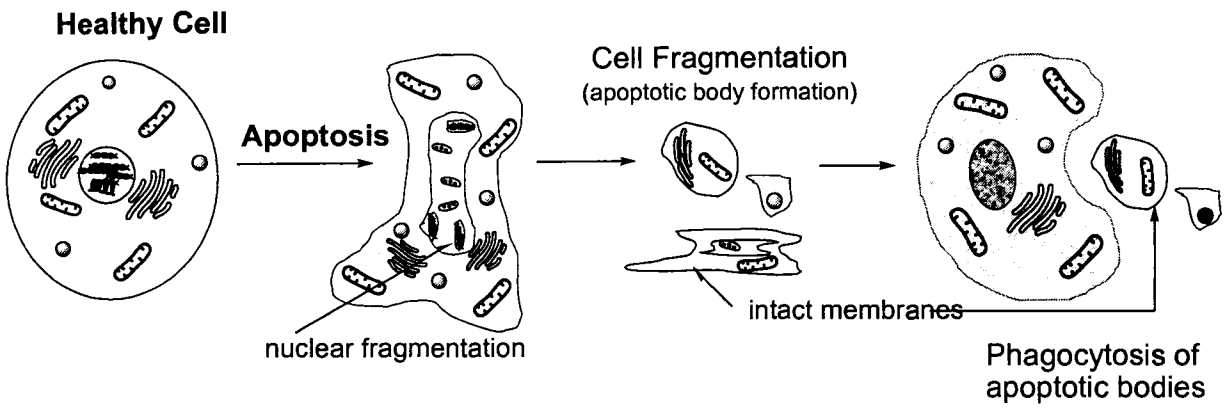

Figure 1.1B Cell death by apoptosis

Apoptosis is an active process in which the cell is signaled to dye by an external or internal signal. Upon activation of apoptosis nuclear condensation and fragmentation is noted followed by fragmentation of the entire cell into apoptotic bodies. Finally the apoptotic bodies are removed by phagocytosis from neighboring cells. In this process cell membrane is kept intact preventing cytosolic components from leaking into the outside and resulting in inflammation.

responsible for the execution of apoptosis (Baker, S *et al.*, 1998). These proteins are more specifically known as caspases, a set of cysteine dependent, Asp-specific proteases which can function to activate other caspases (initiator caspases) or can be directly involved in cellular degradation (executioner caspases) (Lee, D, 2000). Receptor mediated induction of apoptosis (shown in Fig 1.2) begins with the binding of FasL to the Fas receptor on the plasma membrane. This causes a conformational change in the Fas receptor leading to the, recruitment of procaspase 8 to the cell membrane, by the Fas Associated Death Domain (FADD) bound to the Fas receptor. Procaspase 8 recruitment undergoes self activation resulting in the recruitment of this protein and formation of a death inducing signaling complex (DISC) at the plasma membrane (Salvesen, G *et al.*, 1999). Activated caspase 8 can then directly lead to executioner caspase 3 activation, which results in cleavage of key proteins on the plasma and nuclear membrane leading to overall structural breakdown of the entire cell (Nicholson, D, 1999). In addition caspase 8 has also been shown to mediate the cleavage and thus activation of pro-apoptotic protein Bid, which exerts its apoptotic effect by facilitating mitochondrial membrane permealization in association with Bax (Desagher, S, 1999).

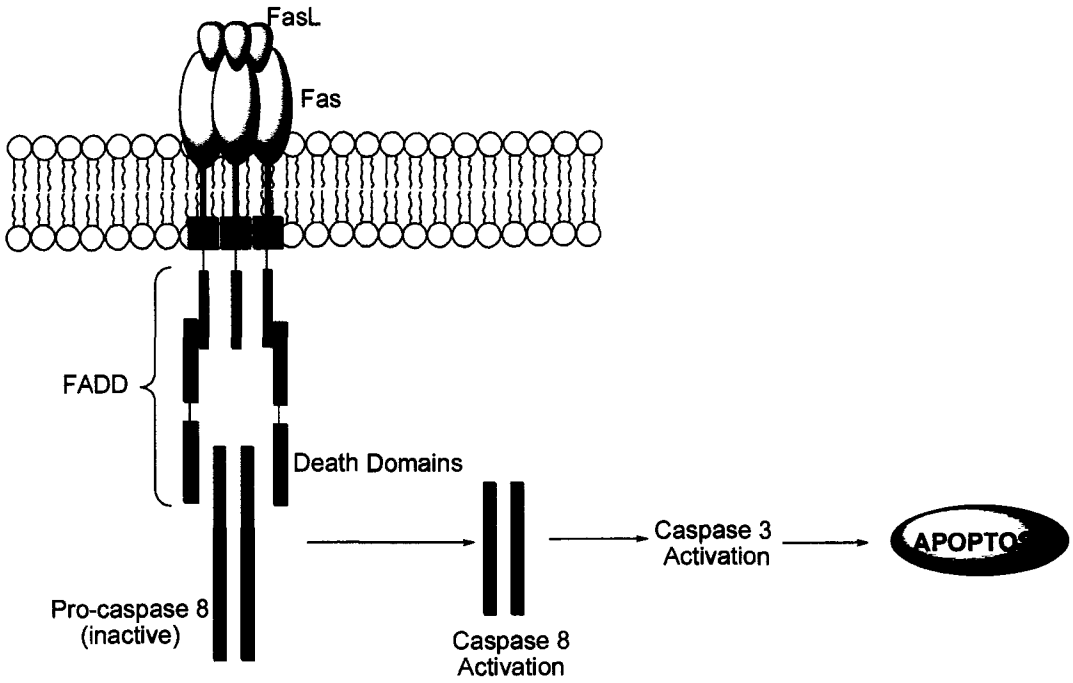

Figure 1.2 Receptor mediated induction of apoptosis

Overview of receptor mediated apoptosis resulting from FasL binding to Fas receptor and subsequent activation of initiator caspase 8 and executioner caspase 3 resulting in apoptosis of the cell.

1.3.2 Intrinsic Apoptosis Pathway: Role of Mitochondria

Mitochondria are involved in many processes which are essential to cell survival, from energy production to redox control, in addition to maintaining a balance in various metabolic and biosynthetic pathways (Bouchier-Hayes, L *et al.*, 2005). As the study of apoptosis has progressed, a great deal of evidence has been presented placing the mitochondria at the heart of apoptosis (Kerr, J *et al.*, 1972; Cohen, J *et al.*, 1992; Steller, H, 1995; Zamzami, N *et al.*, 1995; Raff, M, 1996). The central event which leads to apoptosis involves the mitochondrial outer membrane permealization (MOMP) and subsequent leakage of pro-apoptotic proteins from the inner membrane space (IMS) such as cytochrome *c* and apoptosis inducing factor (AIF). This process may occur as a consequence of a number of pro-apoptotic stimuli including activation of pro-apoptotic proteins of the Bcl-2 family such as Bid, Bak and Bax, inactivation of anti-apoptotic proteins of the Bcl-2 family such as Bcl-2 and Bcl-x_L and increased reactive oxygen species levels (Nomura, K *et al.*, 2000; Adhihetty, P *et al.*, 2003)

The induction of apoptosis through this pathway requires only a few mitochondria to be affected or for the membrane opening to occur transiently, resulting in release of pro-apoptotic proteins while still maintaining ATP synthesis. As previously discussed, apoptosis is an active process which requires ATP, therefore if MOMP occurs for a prolonged period of time the mitochondria will uncouple and will be left unable to synthesize this molecule. To further complicate this situation, ATPase reverses direction and begins to actively hydrolyse cellular ATP, critically decreasing ATP levels. This leads to disruption of metabolic pathways and activation of degradative enzymes (proteases, nucleases, phospholipases) resulting in cell swelling and rupture (necrotic

death) and neighboring tissue damage. Therefore, the mitochondria play an important role in assessing cellular damage and inducing appropriate cell death (apoptosis). In cases of extensive damage, which cannot be repaired, apoptosis is induced in order to prevent damage to surrounding tissue (Halestrap, A, 2002).

1.3.2.1 Molecular Mechanism of MOMP: Regulation by Bcl-2 Family of proteins

Currently there are two hypothesis presented which attempt to explain the principal behind MOMP regulation. In the first model, MOMP is understood as a process which is specific to only the mitochondrial outer membrane (MOM) and is regulated by the Bcl-2 pro- and anti-apoptotic proteins, which can promote or prevent pore formation, respectively (Fig.1.3A). This evidence has been presented following studies which have shown that this family of proteins is able to affect the permeability of the (MOM) in cell free systems, by using vesicles formed from MOM. It has been proposed that pro-apoptotic proteins such as Bax and Bak may form a pore in the MOM either by homo or heterodimerization or activation by other BH3 proteins such as Bid, discussed above (Kuwana, T, *et al.*, 2002).

Alternatively, the second model which attempts to explain the mechanism of mitochondrial destabilization and its role in apoptosis involves the formation of a permeability transition pore (PTP) (Fig. 1.3B). There are several components to the PTP, which span from the inner to the outer membrane of the mitochondria. The voltage dependent anion channel (VDAC) protein is found to transverse the MOM while two other components cyclophilin D (cyp D) a soluble matrix protein and the adenine

nucleotide translocase (ANT) protein are found on the mitochondrial inner membrane (MIM) (Halestrap, A, 2002).

The opening of the PTP (which leads to release of cytochrome *c* and consequently apoptosis) is also under debate. Some evidence supports the idea that increased Ca^{2+} levels can overwhelm the mitochondria and force the PTP into high conductance. This process is believed to be irreversible once the PTP is activated and is completely dependent on the saturation of all calcium binding sites of the PTP (Ichas, F *et al.*, 1998).

Other studies have shown that pro-apoptotic proteins such as Bax and Bak undergo translocation to VDAC from the cytosol upon activation (homo/heterodimerization or Bid activation) resulting in cytochrome *c* release (Murphy, K *et al.*, 2000). In addition, both Bcl-2 (anti-apoptotic) and Bax have also been shown to interact with the MIM component, ANT to regulate cytochrome *c* release and apoptosis , as well as the MOM component, VDAC (Brenner, C, 2000).

Despite the debate over the role and mechanism of the PTP in apoptosis, a great deal of evidence has emerged in support of the theory that the PTP facilitates the release of proapoptotic proteins and that the association of Bcl-2 family of proteins appear to play an important role in the PTP regulation.

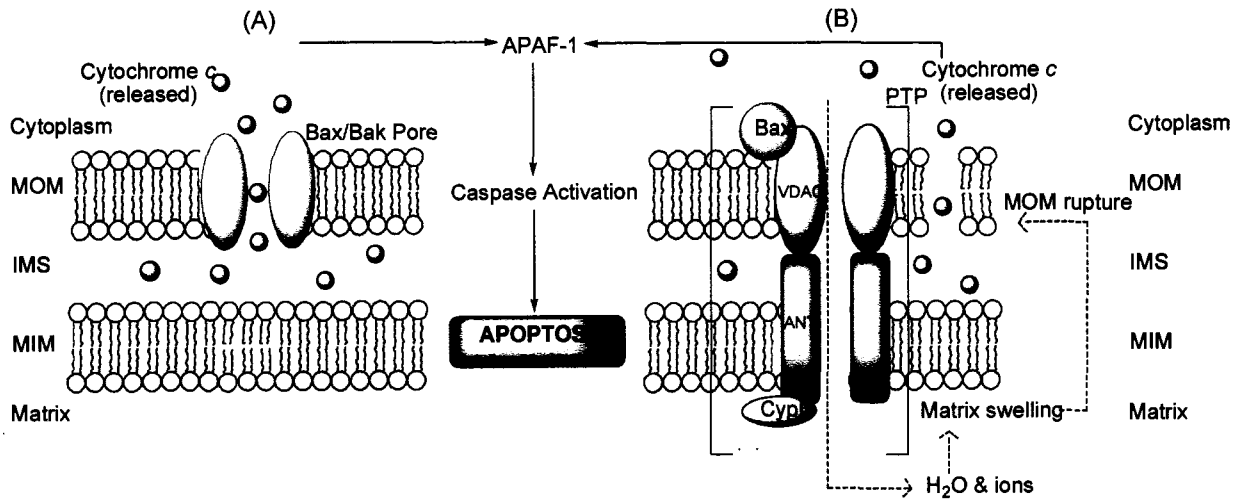

Figure 1.3 Molecular mechanism models of MOMP

The hypothesis presented suggest that pro-apoptotic proteins such as Bax and Bad play an important role in the leakage of MIM protein release. (A) In the first model Bax or Bak are thought to forms small pores only on the MOM. (B) The second model involves a permeability transition pore which found to more commonly associate with Bcl-2 family proteins to control its permeability and subsequently apoptosis.

1.3.2.2 Consequences of MOMP

Despite the various causes of MOMP, in all cases this event most commonly results in release of the IMS protein cytochrome *c* (normally functioning as vital component of the electron transport chain) and ultimate cell death (Fig.1.4). Once in the cytoplasm, cytochrome *c* binds to apoptotic activating factor-1 (APAF-1), an active process involving ATP. Subsequently, this association forces APAF-1 to undergo a conformational change exposing its caspase recruitment domain (CARD) resulting in the accumulation of procaspase 9 which can undergo self activation. This initiator caspase is then able to activate (via proteolysis) executioner caspase 3 which in turn activates Caspase activated DNase leading to DNA fragmentation and ultimate cell death (Li, P *et al.*, 1997). It should be noted however that cells may also express various caspase inhibitors, which may prevent apoptosis and act as death checkpoints for the cell. For example, one group of proteins termed inhibitors of apoptotic proteins (IAPs) contain both upstream and downstream inhibitory targets such as caspase 9 and caspase 3, respectively (Deveraux, Q, 1998).

In order to proceed with the apoptotic pathway, such inhibitors must be inactivated. This task is also accomplished by proteins released from the inner mitochondrial space (IMS) such as Smac (second mitochondrial derived activator of caspases, DIABLO) which work to inactivate endogenous caspase inhibitors and AIF (apoptosis inducing factor) which migrates to the nucleus to induce chromatic changes (Green, D *et al.*, 1998). These finding provide further insight into the tight regulation which is involved in the active process of apoptosis as opposed to its passive necrosis counterpart.

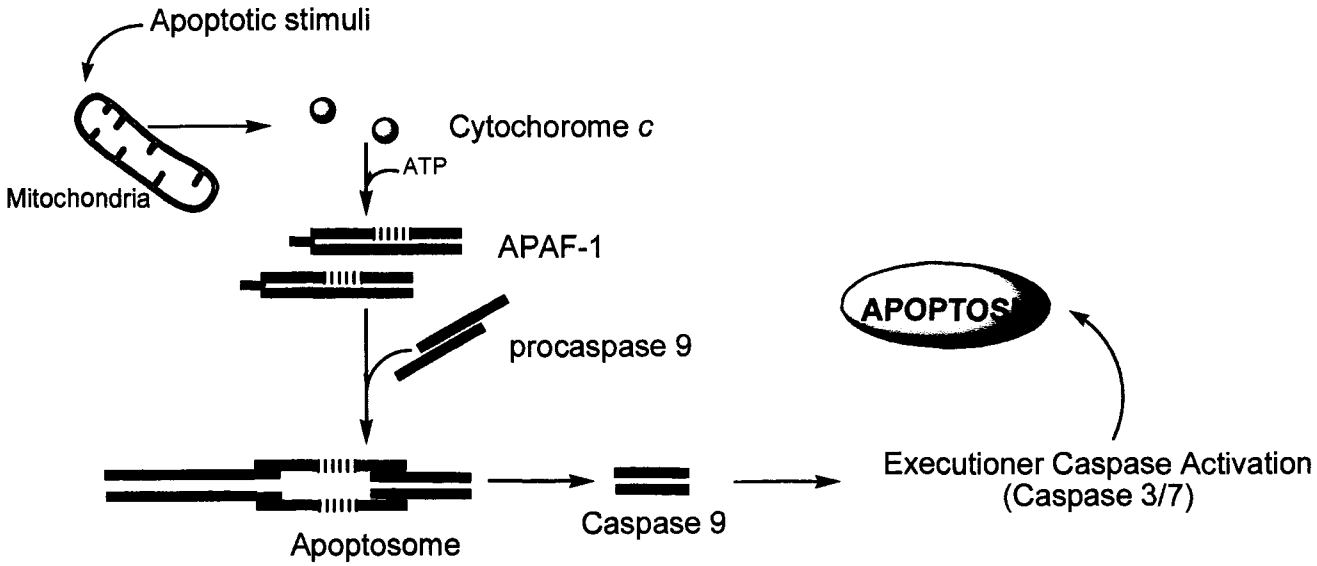

Figure 1.4 Effects of cytochrome *c* release from the mitochondrial IMS

Once cytochrome *c* is released from the mitochondrial inner membrane space, it forms a complex with the apoptosis activating factor which is responsible for the recruitment and activation of effector caspase 9. Subsequent activation of executioner caspases follows resulting in apoptosis of the cell.

1.4 Bcl-2 Family Proteins

The growing family of Bcl-2 proteins can be divided into two groups: anti-apoptotic such a Bcl-2, Bcl-xL, Bcl-w and pro-apoptotic such as Bax, Bak, Bcl-xS. These proteins share homology within 3-4 conserved regions termed Bcl-2 homology (BH1-4) domains and act as intracellular checkpoints in the cell death pathway (Korsmeyer, S *et al.*, 1998). The presence and accessibility of these domains strongly influences their apoptotic character. With a few exceptions mammalian anti-apoptotic proteins contain BH domains 1-4, while pro-apoptotic proteins generally contain BH domains 1-3 (Fig.1.5).

These proteins play an important role in controlling mitochondrial permealization. Intracellularly, a balance exists between the pro- and anti-apoptotic Bcl-2 proteins and the ratio of pro-apoptotic to anti-apoptotic is important for determining the susceptibility of a cell to apoptosis. In the event of over-expression of Bax (or similar pro-apoptotic members) apoptosis is accelerated, as noted in tissues where cell death occurs as part of the normal maturation process (e.g. epidermis). In contrast, over-expression of Bcl-2 (or similar anti-apoptotic members) leads to heterodimerization (with Bax) and repression of death induction (Oltvai, Z *et al.*, 1993).

There are four Bax isoforms which result from alternative gene splicing of *bax*, including Bax α (21kDa), β (24 kDa), γ(5kDa), and δ(16kda) of which only Bax α and Bax β contain the BH3 domain necessary for induction of apoptosis. Of these four isoforms, Bax α has the necessary C-terminus transmembrane domain involved in permealization of the mitochondrial membrane leading to apoptosis, and varies from the

similar Bax β in the C-terminus (Oltvai, Z *et al.*, 1993; Apte, S *et al.*, 1995; Suzuki, M *et al.*, 2000).

Yeast two hybrid work using various deletion mutants of Bax have proven that Bax uses its BH3 domain for homodimerization and heterodimerization with other members of Bcl-2 (pro- and anti-apoptotic), as mutation in only this region rendered the protein unable to dimerize. More specifically, Bax is understood to bind in a parallel (tail-to-tail) manner using this domain to itself and other Bcl-2 members. However, the apoptosis antagonists Bcl-2 proteins dimerize to each other using a head-to-tail conformation involving the BH4 domains of one protein and the BH1 and BH2 domains of the second. In heterodimerizing with the pro-apoptotic partners, BH1 and BH2 domains of the anti-apoptotic protein interact with the BH3 domain of the pro-apoptotic partner (Zha, H *et al.*, 1995).

In order to understand the whether the death promoter (Bax) resides upstream or downstream from the death antagonist (Bcl-2), in a genetic apoptosis pathway, knockout mouse models were utilized. From such work three models were proposed to account for the activity of the Bcl-2 family proteins. Firstly, if Bcl-2 acts as a primary suppressor of apoptosis, its activity could be inhibited by upstream action of Bax. Conversely, if Bax acts as a downstream regulator of death its homodimerization and subsequently activity could be quenched by upstream regulation of Bcl-2. Lastly, both proteins have also been shown to act independently in the control of apoptosis despite the *in vivo* competition between these proteins (Knudson, C *et al.*, 1997).

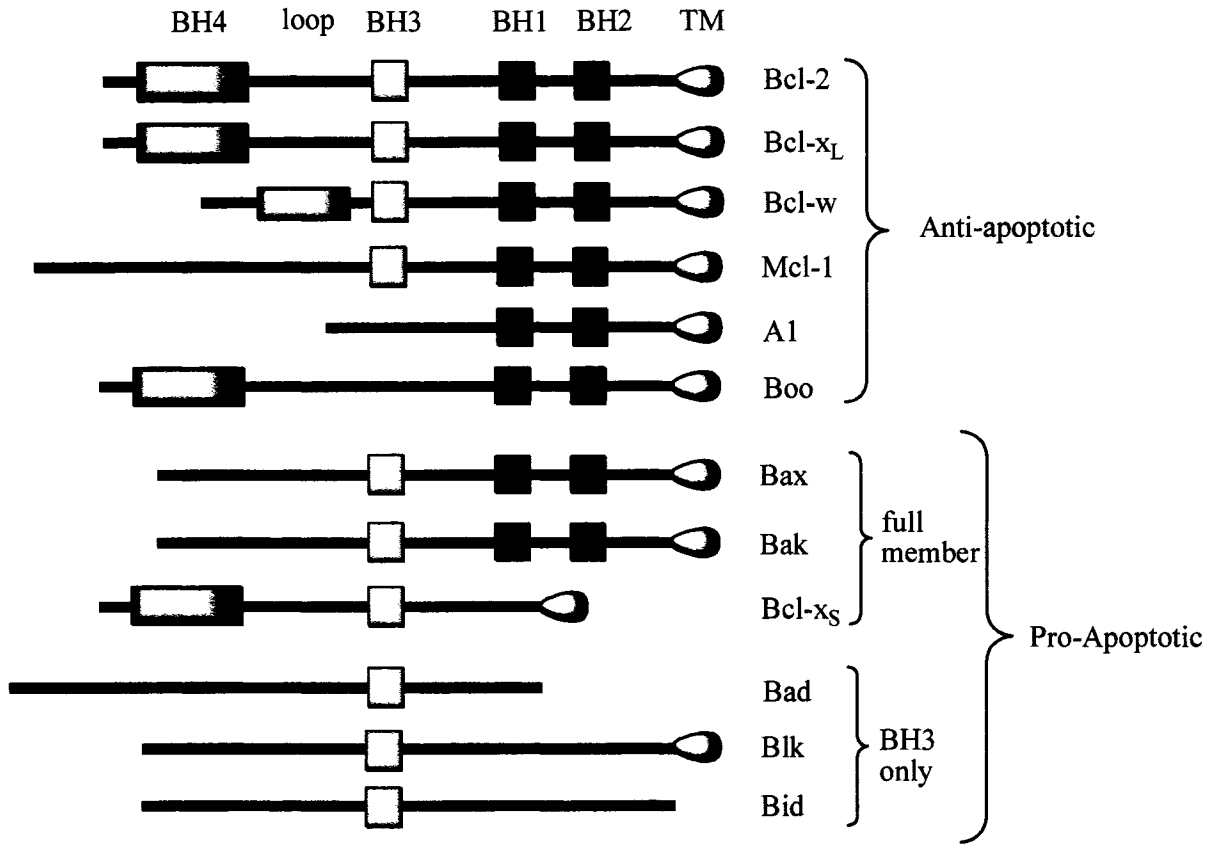

Figure 1.5 Selected Bcl-2 family proteins

With a few selected exceptions the anti-apoptotic group contains a unique BH4 domain absent in almost all pro-apoptotic members.

There is some debate on the ability of other members of the multigene family to substitute for missing Bcl-2 or Bax proteins using knock out studies. Studies using *Bcl-2-/-* knockout mice have shown a significant developmental defects as the majority of newborn mice with this mutation die within a few weeks of age (Veis, D *et al.*, 1993), while those with mutations *Bcl-x-/-* result in embryonic death at approximately day 13 (Motoyama N, *et al.*, 1995). These results suggest that these anti-apoptotic proteins, are not interchangeable. Conversely, Bax-/- knockout mice studies by Lindsten *et al.* (2003) have demonstrated the complementary role of Bax and Bak. Here, knock out in only one gene resulted in no obvious neurological phenotypic changes when compared to the doubly deficient mice, suggesting a possible a redundancy in the Bax and Bak protein role in apoptosis (Lindsten, T *et al.*, 2003)

Further characterization has recently been published by Xin *et al.* 2006 showing the effects of nicotine induced phosphorylation of Bax at Ser (S) 184 by protein kinase AKT. This post-translational modification works to inactivate Bax specifically in lung cancer cells. Protein phosphatase 2A (PP2A) was found to be an active regulator in these cell types, working to both dephosphorylate Bax and also to break up the Bax-Bcl-2 heterodimers allowing Bax to homodimerize and permealize the mitochondria inducing apoptosis (Xin, M *et al.*, 2006).

1.5 Role of Reactive Oxygen Species (ROS) in Apoptosis

As previously discussed, apoptosis in dividing tissue is vital for the health of an organism, as unnecessary, damaged or dangerous cells are replaced by newly formed healthy cells. However, inappropriate induction of this cellular process can become

equally detrimental, leading to loss of healthy cells. When this occurs in post-mitotic tissue such as the brain which is composed of specialized non-dividing cells (neurons), the overall function of this organ can become seriously compromised as seen in multiple neurodegenerative disease such as Alzheimer's disease (AD), Parkinson's disease (PD) and stroke. Oxidative stress due to the production of reactive oxygen species from the mitochondria is a persistent threat to mammalian cells.

Reactive oxygen species are oxygen containing molecules which possess unpaired electrons (free radicals), thus increasing their ability to react (bond) with other molecules. Biologically relevant free radicals include superoxide ion ($O_2^{-}\bullet$), perhydroxyl radical ($HO_2^{-}\bullet$), hydroxyl radical (•OH) which is the most reactive and least selective of the radicals and the nitric oxide ion (NO•). Cellular toxicity due to reactive oxygen species arises from both direct reactions of these molecules with biological targets (lipids, proteins, and DNA) and secondary reactions leading to formation of other radicals (Cuzzocrea, S *et al.*, 2001).

Superoxide ion is among the most prevalent radical in biological systems, and is known to be formed by the enzyme NAD(P)H oxidase. As a means for coping with this threat, cells produce numerous anti-oxidant complexes (glutathione, uric acid and vitamins C & E) and enzymes (supersuperoxide dismutase (SOD), catalase and glutathione peroxidase). Specifically, the clearance of superoxide radical is accomplished by the superoxide dismutase enzyme (SOD), converting $O_2^{-}\bullet$ into hydrogen peroxide (H_2O_2). Several enzymes such as catalase or glutathione peroxidase can then safely break down hydrogen peroxide into water, however in the presence of reduced

transition metals (ferrous ions), H_2O_2 can be converted into the reactive hydroxyl radical (•OH), contributing to the oxidative stress of an organism (Chance, B *et al.*, 1979).

In addition to these anti-oxidant mechanisms, cells have also adapted to moderate levels of oxidative stress by involving ROS in common physiological functions: control of ventilation, erythropoietin production and similar hypoxia-inducible functions, signal transduction and oxidative stress responses (Droge, W, 2002).

Despite the various coping mechanisms, in pathological cases leading to the aforementioned neurodegenerative diseases, an abnormal increase in reactive oxygen species overwhelms these protective measures resulting in often irreversible damage to tissues, as healthy cells become mistakenly apoptotic; a phenomenon commonly observed following reperfusion after ischemic stroke.

1.6 Neurodegenerative Disease and ROS

Neurodegenerative diseases affect the central nervous system (CNS) and include but are not limited to epilepsy, multiple sclerosis (MS), Alzheimer's disease (AD), Parkinson's disease (PD) and stroke. Though these diseases affect various tissues and also vary in course length and pathology, there are some important similarities. In all cases, one of the primary reasons behind the detrimental effects of such diseases is the post-mitotic nature of the cells (neurons) in the target tissue (CNS). Once injury to the CNS particularly in the brain has occurred, neurons which die cannot be replaced, leaving the patient with some type of physical or mental functional limitation, depending on the specific area of the brain which becomes affected (Price, D, 1999).

Oxidative stress caused by elevated levels of ROS in the brain is one of the major factors, particularly for AD, PD and stroke. In comparison to other organs, the brain is the most vulnerable organ to oxidative stress. This phenomenon is supported by the fact that brain cells make up approximately 2% of the total body mass but consume 20% of the total oxygen intake as they rely almost solely on oxidative phosphorylation for ATP generation and thus generate larger quantities of free radicals compared to other organs. In addition, certain brain regions contain high iron contractions which as mentioned above are able to catalyze free radical production. The brain is also rich in unsaturated lipids, which are an important target for lipid peroxidation and finally, the low antioxidant systems in the brain (compared to organs such as liver and kidney) offer minimal defense to elevated levels of ROS (Dringen, R, 2000). As a result, minimizing and/or eliminating oxidative stress is immensely important in the development of therapeutics against neurodegenerative diseases.

1.7 ROS role in Ischemia/Reperfusion Injury due to Stroke

Ischemic stroke in humans is the third most common cause of death in most industrialized countries, after heart disease and cancer. Caused by an interruption of blood flow to the brain, the effects of stroke can range significantly depending on the particular region of the brain which is affected. Functions such as memory, personality, cognition, language and motor skills can be either temporarily or permanently damaged, and in approximately 25% of cases patients are left in a vegetative state or die (Moore, R *et al.*, 1993). In cases of Stroke, neurons directly devoid of blood flow, found in the core region of injury are killed within minutes of this effect, while neurons in the surrounding

region termed the penumbra retain their potential for survival for hours or even days. Specifically, this latter region can be saved by reperfusion when blood flow is returned, however, this is certainly not without consequences, discussed below (Schaller, B *et al.*, 2004).

Over the years three general treatment methods have been established, and used in combination for treatment of stoke: eliminating the blood clot to restore blood flow, minimizing the recurrence of clots through blood thinner drugs and most recently, neuroprotection though preventing the formation or scavenging free radicals formed as a result of reperfusion.

The primary aim in cases of ischemic stoke is to eliminate the blood clot and restore essential nutrients carried by the blood to affected areas. Currently, physicians have several ways of accomplishing this including thrombolytic recombinant tissue plasminogen activator (rTPA), which is effective in minimizing brain damage if administered within 3h of stroke onset. Unfortunately, many patients (especially the elderly) who suffer a stroke may not recognize the signs in this short period of time and thus do not receive proper care. If left untreated for a greater period than 3h, despite the possibility of spontaneous reperfusion, permanent and fatal brain damage will most likely occur. Recently two other drugs with similar functions have also been introduced to help restore proper blood supply (reperfusion) to injured areas: pro-urokinase and intravenous ancrod containing fibrinogen lowering properties (Lee, J *et al.*, 1999). The second category for treatment of stroke is the use of blood thinners which can minimize the recurrence of clot formation to avoid stroke recurrence.

Therapeutic approaches based on reperfusion, although important for restoring much needed blood flow, consequently result in damage to surrounding and often healthy cells. After blood supply becomes available to neurons at the injury site, these cells often take up too much oxygen at one time leading to a drastic increase in free radical (ROS) production and oxidative stress which may spread to surrounding healthy cells. Subsequently, various pro-apoptotic proteins such as Bax become activated (in cells near the injury site but not directly affected by the stroke) leading to apoptosis of post-mitotic cells. Such damage continues to occur up to 72 h after onset of stroke, and therefore, neuroprotection by preventing the production or eliminating free radicals has recently become an important target in therapeutic research (Rotham, S *et al.*, 1986).

1.8 Intrabody Application in Therapeutics for Neurodegenerative Diseases

Intrabodies are antibody molecules which are expressed intracellularly, through gene transfection, in order to target specific cellular components. Such complexes are therapeutically relevant as they can lead to phenotypic knockout of a specific target within the cell (Kontermann RE, 2004). Since only the antigen binding site is required for activity of intrabodies single chain Fv (scFv) which represent the smallest recombinant antigen binding fragment are among the most commonly applied. However, multiple other formats including Fab fragments, scFv-C_K fusion proteins, single chain diabodies (V_H-C_H fragments) and most recently single domain antibodies (V_HH) from camellid species have proven equally if not more effective (Hudson, P, 1998).

Direct binding of intrabodies to target proteins has been reported to either inhibit or activate these target proteins depending on their particular native function

(Kontermann RE, 2004). For example, recent work on Parkinson's Disease reported by Emadi *et al.* 2004, involved the use of an anti-monomeric-α-synuclein scFv (intrabody) which binds to and prevents formation of high molecular weight oligomers and aggregates of this protein which are important characteristic features of PD and other dementia related diseases (Emadi, S *et al.*, 2004). Similar work was also reported by several other independent groups showing the decrease in these aggregation by specific intrabodies, for use in both understanding and treating this condition (Maguire-Zeiss, K *et al.*, 2004; Zhou, C *et al.*, 2004; Emadi, S *et al.*, 2004).

Intrabodies have also been applied for the activation of pro-apoptotic proteins such as caspase 3 as demonstrated by Tse *et al.* (2000), using a unique indirect approach. In this case, scFv-fusion protein consisting of an anti-β-galactosidase scFv fused to caspase 3 was able to lead to forced dimerization and thus autoactivation of caspase 3, inducing apoptosis, when co-expressed with β-galactosidase (Tse, E *et al.*, 2000).

For the last number of years, intrabodies have become a major focus in research of therapeutics against numerous diseases in addition to those affecting neurodegeneration, including (but not limited to) cancer, targeting areas such as cell cycle regulation (Strubea, R *et al.*, 2002) and anti-apoptotic proteins such as Bcl-2 (Piche, A *et al.*, 1998). Intrabodies for treatment of HIV-1 infections have also been introduced with targets such as viral coat proteins gp120 (Chen, S *et al.*, 1994) and gp41 (Zhou, P *et al.*, 1998) and transcription proteins cyclin T1 (Bai, J *et al.*, 2003).

Although phenotypic knockout can be carried out using other methods such as RNA interference (iRNA) acting at the level of mRNA and subsequent inhibition of protein synthesis, there are several advantages to using intrabodies for understanding

cellular functions and developing therapeutic agents. Non-specific effects (binding alternate mRNA sites) are among the major safety concern in the use of iRNA, which have imposed a blockade on numerous studies using this technology (Jackson, A *et al.*, 2004). Similarly, in 2004, Snove *et al.* reported that 75% of the 359 published siRNA sequences (at that time) posed a risk for eliciting non-specific responses, while Persengiev *et al.* further reported that by using a conventional 21-bp iRNA to treat cells, over 1000 genes involved in various and unrelated cellular functions were stimulated or repressed (Persengiev, S *et al.*, 2004; Snove, O *et al.*, 2004). This risk is reduced when using intrabodies, since binding does not occur simply through nucleotide base pairing, which can occur more readily in comparison to the interaction between an intrabody and their highly ordered protein targets.

Furthermore, when target complexes have multiple binding/interacting sites, repression of the gene will lead to the alteration of multiple functions, which may not necessarily be desired. However, by designing or screening only those intrabodies with the potential to bind to one specific site on multidomain targets, this problem can be eliminated, resulting in higher specificity of the intrabody technology (Heng, B *et al.*, 2005).

In addition, to blocking various binding interactions, intrabodies can also be used to attach components such as localizing signals(to target complexes in order to relocate these other subcellular locations) or ubiquitin-proteosome pathway substrates (to targets for their selective degradation) (Cattaneo, A *et al.*, 1999; Zhou, P *et al.*, 2000).

Research into intrabody technology is a growing field, with important application potential in both academic and therapeutic discovery settings. Specifically, this

technology can be applied for knock out studies at the protein level in order to understand specific protein functions, without altering DNA. Furthermore, intrabodies can also be advantageous in treating diseases (such as cancer and HIV as discussed above) which result from genetic mutations leading to in specific protein mutations.

1.9 Single Domain Antibodies (V_HH) as Potential Blockers for Pro-apoptotic Bax

Single domain antibodies are designed to contain only the active variable heavy region (V_HH). These antibodies (or intrabodies when expressed intracellularly) are derived from camellia or llama species which naturally lack the light chains expressed in conventional antibodies (Fig.1.6). The absence of the light chains allows researchers to isolate a smaller antigen binding active complexes termed single domain antibodies (sdAbs), which are at least half the size of the scFvs isolated from conventional IgGs .

This smaller size is advantageous in structural studies, therapeutic application requiring tissue penetration, and recombinant antibody technology. In addition, when expressed as intrabodies the significantly smaller sdAb may prevent non-specific interactions and disturbances within the cell (Tanha, J *et al.*, 2002). sdAbs also offer greater solubility than even heavy chain variable domains of classic IgGs, which has been understood to occur due to the presence of hydrophilic residues instead of hydrophobic residues which are normally present between the light and heavy variable regions of IgGs (Desmyter, A *et al.*, 1996). The overall comparable affinity of sdAbs to conventional scFvs but higher solubility, stability and expression levels offers greater advantage for use in research at both mechanistic and therapeutic levels of research.

Antibody phage display libraries have been established from total RNA of naïve llama lymphocytes, containing phages which display a specific sdAb on their surface and the gene for this sdAb. Through a panning technique a sdAb and its gene can be identified for almost any target antigen of interest (Tanha, J *et al.*, 2002). Obtaining the gene for the sdAb selected becomes immensely important as this can easily be transfected inside most cells to study the effects of sdAb inside cells as intrabodies.

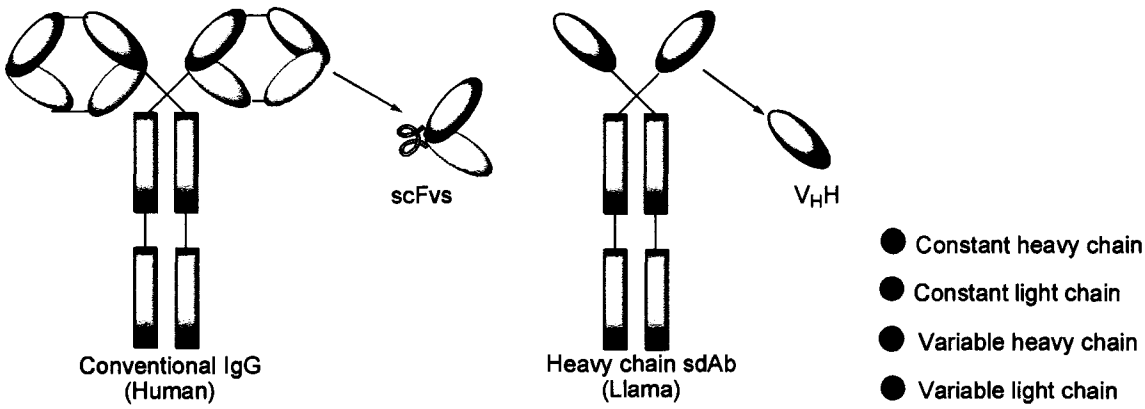

Figure 1.6 Comparing single domain antibodies to conventional human antibodies

Unlike the conventional human antibodies, single domain antibodies (sdAbs) lack both sets of light chains; therefore the antigen combining sites are formed only from the variable heavy chain, (V_HH).

1.10 Objectives

Pro-apoptotic proteins such as Bax which can become activated during oxidative stress-induced apoptosis play a central role in death of post-mitotic cells in neurodegenerative diseases. In order to prevent inappropriately induced apoptosis, we plan to use sdAb to inhibit the activity of Bax.

The objectives of this study are as follows:

1) To isolate and purify recombinant Bax protein from a culture of transformed E.coli.
2) In collaboration with Dr.J.Tanha (NRC-IBS) to use a llama phage display library for a panning assay to isolate Bax specific V_HH clones by using immobilized recombinant.
3) To establish and standardize an *in vitro* assay for Bax activity on isolated mitochondria.
4) To functionally characterize the anti-Bax V_HHs *in vitro*.
5) Finally, to express the anti-Bax V_HH genes through transfection inside human neuroblastoma cells (SHSY-5Y) to study their efficacy in blocking oxidative stress induced apoptosis.

Chapter 2: Materials & Instrumentation

2.1. Chemical and supplies

2.1.1. Cell Lines

Human Neuroblastoma (SH-SY5Y) cells and transformed kidney cells (Hek293) were purchased from the American Type Culture Collection (ATCC), Manassas, VA, USA.

2.1.2. Media

DMEM F12 HAM was purchased from Sigma Chemical Company, Mississauga, ON, Canada. L-Glutamine, Gentamycin, Fetal bovine serum (FBS) and Geneticin were purchased from Gibco BRL, VWR, Mississauga, ON, Canada.

2.1.3. Other chemicals

Most of the chemicals including BSA, CHAPS, EDTA, EGTA, hydrogen peroxide, HRP, HEPES, MDA, $MgCl_2$, Na_2EDTA, TRIS-HCl, Triton X-100, parahydroxyphenylaceticacid (PHPA), succinate, thiobarbituric acid (TBA), trichloroacetic acid, trypsin, horse radish peroxidase-conjugated anti-mouse antibodies, monoclonal anti-green fluorescent protein, Bacto tryptone, Bacto yeast extract, lyzosyme, DNase, $NiSO_4$, imidazole and chlormphenicol were purchased from Sigma Chemical Company, Mississauga, ON, Canada.

DMSO, NaOH, NaCl, $NaHCO_3$ and sucrose were purchased from BDH Inc., Toronto, Canada. Glycine was purchased from EM Sciences, NJ, USA. Hoechst,

Annexin-V (red and green) were obtained from Molecular Probes, Eugene, OR, USA. Protein assay reagent, acrylamide, prestained gel standards and ammonium persulfate were purchased from Bio Rad, Ontario, Canada.

JC-1 kit, Dual Sensor: MitoCasp kits, Apo 3/7 HTS caspase assay kits were purchased from Cell Technology, Mountain View, CA, USA. Monoclonal anti-cytochrome *c* IgGs and anti-Bax IgGs (for Western Blot) were purchased from Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA. ChemiGlow West kit were obtained from Alpha Innotech Corporation, San Leonardo, CA, USA. Fugene 6 Transfection Reagent was purchased from Hoffmann-La Roche Ltd., Mississauga, ON, Canada.

2.2 Instrumentation

Fluorescent and phase contrast pictures were taken using a fluorescent microscope (Leica DM IRB, Germany). Fluorescence measurements were conducted in multiwell plate reader with the help of Spectra Max Gemini XS (Molecular Devices, Sunnyvale, California). Absorbance was measured by a UV-Visible Spectrophotometer (Agilent Technologies). Absorbances in 96 well micro-titer plates were measured using a Bio-tek ELx 808ru Ultra Microplate Reader.

Cell culture was conducted under sterile conditions in the class-II type A/B3 Biosafety cabinet (Nuaire), and all cultures were maintained in a CO_2 incubator containing a HEPA filter (Thermo Forma). Centrifugation was done using low speed centrifuge (Jouan) and DESAGA (Sarstedt-Gruppe).

A pH Meter (VWR, Model 8100) with buffer solutions from VWR, an Adventurer ^{TM} balance (OHAUS), Vortex Jr. Mixer from Scientific Industries Inc, 1296-

002 DELFIA ^{R} plate shaker from Wallac, a heat block (Gibco BRL, VWR, Canada) Rocking platform model 200 from VWR, a Corning stirrer from Fisher Scientific (Toronto, Ontario), a Dounce homogenizer from Kontes Glass Company (NJ, USA), freezer vials (VWR) and Eppendorf pipettes (Fisher Scientific).

Dounce homogenizer was purchased from Kontes Glass Complany, NJ, USA. Cell culture supplies including culture dishes, flasks, pipettes, tubes, freezer vials were obtained from Sarstedt Inc, Montreal, Quebec, Canada.

Fluorescence microscope (Zeiss Axioskope 2 Mot plus, Gottingen, Germany) was used to monitor cellular staining (described in Methods) and fluorescence pictures were taken using a camera (QImaging, Gottingen, Germany). The images were processed using Improvision Open*Lab* v3.1.2, Jasc Paint Shop Pro v8.00 and Adobe Photoshop v8.0.

Chapter 3: Methods

3.1 Isolation of Bax

3.1.1 Expression of Bax Protein

A culture of E.coli transformed with *bax gene* containing a His_6 tag, (for purification purposes discussed below) was obtained from Bruno Antonsson. The culture was used to inoculate 100ml of a 1L LB media stock (10g Bacto tryptone, 5g Bacto yeast extract and 10g NaCl) supplemented with 20μg/ml Chlormphenicol. The flask was incubated overnight on a shaker at 37°C and then transferred to the remaining 900ml of LB stock and incubated on a shaker at 37°C. The culture was monitored using UV-Vis spectroscopy until it reached an absorbance of approximately 0.5 OD_{280} at which point the expression of Bax was induced using Arabinose (1g/L). The final culture was incubated overnight on a shaker at room temperature and the samples were then centrifuged at 20 000xg at 4°C for 15 min. The supernatant was discarded and the pellet was stored at -20°C for a maximum of 5 days before purification.

3.1.2 Purification of Bax Protein

The Bax expressing E.coli pellet described above was suspended in lysis/loading buffer (0.02 M Phosphate Buffer (NaH_2PO_4), 100μg/ml Lysozyme, 50μg/ml DNase, 2mM PMSF, 1% TritonX100, 0.05M Imidazole) and incubate over ice for 45min, followed by sonification at 4°C. Samples were then centrifuged at 20 000xg for 10min at 4°C and the supernatant containing protein fractions was collected and stored on ice. Bax was purified through affinity chromatography using Hi-Trap Nickel Chelating Column. The column was prepared by running 2.5ml of 0.1M $NiSO_4$ solution, followed by 20ml

ddH_2O and lastly 30ml of loading buffer at an elution rate of 5ml/min. The protein solution was then loaded into the column at an elution rate of 2ml/min, and Bax was bound to the column through the interaction of the His tag interaction with the stationary Ni^{2+} phase. Following this approximately 30ml of loading buffer containing 0.05M imidazole (which can also interact with Ni^{2+} stationary phase) was also loaded and run through the column at the same elution rate to eliminate non- specific binding interaction which may have occurred between bacterial proteins and the stationary phase. This elution was collected in 5ml fractions whose protein estimation was determined using UV-Vis spectroscopy (described below), once the total protein concentration decreased and remained steady at low levels in the final fractions, it was assumed that the majority of non-specifically interacting proteins were removed. Bax was then allowed to elute out by loading elution buffer (0.02M Phosphate buffer and 0.5M Imidazole). The increased concentration of imidazole allows for the displacement and release of Bax which is collected and analyzed through SDS-PAGE and Western Blot (described below).

3.2 Protein Estimation

The concentration of proteins present in the total cell lysate sample was estimated using the protocol from BioRad Laboratories. The protein estimation was carried out by taking 2.5-5μL aliquots of each total cell lysate sample, 797.5-795 μL of water (respectively) and 200 μL of BioRad protein assay reagent to a total volume of 1 mL in plastic cuvettes. The mixtures were vortexed and allowed to stand for 10 minutes at room temperature. The absorbance was then taken using a UV-Visible Spectrophotometer and

analyzed at 595 nm. The standard curve was prepared by using various amounts of a standard protein solution (BSA) and recording the absorbance in identical condition.

3.3 Isolation of sdAb(V_HHs) against recombinant Bax protein by panning a llama phage display library

The following work was carried out by Dr.J.Tanha and associates at the National Research Council of Ottawa (Institute for Biological Sciences).

A llama V_HH phage display library described previously was used in panning experiments (Tanha, J *et al.*, 2002). Panning against recombinant Bax protein was performed as described by Tanha *et al.* 2002, with the following changes. In the second and the third rounds, the phage elution additionally involved $MgCl_2$/HCl treatment. First, the bound phages in the microtiter wells were eluted with 200 µl and neutralized with 100 µl 1 M Tris-HCl pH 7.4. Then, the emptied wells were subsequently incubated with 100 µl of 4 M $MgCl_2$ at room temperature for 15 min. The eluted phage was removed and the wells were incubated with 100 µl of 100 mM HCl for five min at room temperature. The $MgCl_2$/HCl-eluted phages were pooled, neutralized with 1.5 ml of 1 M Tris-HCl pH 7.4 and combined with the triethylamine-eluted phages. One ml of the combined phages was used to infect *E. coli* for overnight phage amplification and the remaining 1 ml was stored at -80°C for future reference. V_HH clones were identified from the titer plates by plaque-polymerase chain reaction and sequencing as described (Tanha, J *et al.*, 2003). Following panning, phage clones from titer plates were amplified in microtiter wells and screened for binding to Bax protein by standard ELISAs using a horse radish

peroxidase/anti-M13 monoclonal antibody conjugate (GE Healthcare, Baie d'Urfe, QC, Canada) as the detection reagent.

3.3.1 *E. coli* protein expression and purification of V_HHs

V_HH genes were cloned from the phage vector into the expression vectors by standard cloning techniques. *E. coli* expression of V_HHs and subsequent purification by immobilized metal affinity chromatography were performed as described (Tanha, J *et al.*, 2003). Protein concentrations were determined by A_{280} measurements using molar absorption coefficients calculated for each protein (Tanha, J *et al.*, 2002).

3.3.2 Preparation of sdAb (V_HH) fusion constructs

V_HH genes were inserted in the Hind III/BamH I sites of pEGFP-N1 (V_HH-green fluorescent protein (GFP) fusion), pDsRed1-N1 (V_HH-red fluorescent protein (RFP) fusion) or Hind III/Not I site of pEGFP-N1 (V_HH) (BD Biosciences, Mississauga, ON, Canada). The V_HH recombinant vectors were propagated in *E. coli* and were purified using QIAprep® Spin Miniprep kit according to the manufacturer's instructions (QIAGEN, Mississauga, ON, Canada) by Dr.Tanha and associates in NRC-IBS Ottawa.

3.4 Cell Culture

3.4.1 Propagation of Cell Lines

Human neuroblastoma (SHSY-5Y) cells and transformed human kidney cells (Hek 293) were grown in complete medium consisting of DMEM Ham's F12 media with the addition of 2 mM L-glutamine and 10% (v/v) fetal bovine serum and 20 μg/ml gentamycin. Cells transfected for formation of stable cell lines were also supplemented with 200 μg/ml Geneticin (G418). The cells were incubated at 37°C with 5% CO_2 and 95% humidity.

3.4.2 Cell line sub-culturing

Cells lines were grown in 25cm^2 sterile flask and sub-cultured by removing the culture medium through aspiration followed by the addition of 1ml of 0.15% Trypsin and incubation for 1-2 min at 37°C until the cells were able to detach with minimal force from the flask wall. After this, approximately 4-6 ml of fresh media was added to the cell solution and the cells were aliquoted in various culture dishes as needed.

3.4.3 Transfection of SHSY-5Y

Transfection of mammalian SHSY-5Y cells with the purified anti-bax and control V_HH plasmids (described above) was carried out using Fugene 6 Transfection Reagent (Hoffmann-La Roche Ltd., Mississauga, ON, Canada) following the manufacturer's protocol. Briefly, under sterile condition in a laminar flow hood, 7.5μl of Fugene was added to 100μl of incomplete DMEM-F12 Ham media in a plastic eppendorff tube and

left to stand for 10-15min. Approximately 4μg of DNA was then added to this mixture followed by a second incubation in the hood for 30-40 min, after which the entire mixture was added to the cells (in completed medium). SHSY-5Y cells used for transfections were sub-cultured into 25cm^2 flasks 24h prior to transfection for optimum uptake of the DNA. Forty eight hours after transfection, cells were transferred to complete DMEM media (as described above) containing 300 μg/ml Geneticin for selection of positive transfected cells for 1-2 weeks. Stable cell lines were subsequently maintained in complete DMEM media as described above with 200 μg/ml Geneticin.

3.5 Preparation of post-nuclear cytoplasmic fraction

SHSY-5Y cells were grown to 70% confluence in 10-ml Petri dishes. The post-nuclear cytoplasmic fraction was obtained from these cells using a previously published method (Li, N *et al.*, 2003; Naderi, J *et al.*, 2006). Specifically, cells were harvested by mechanical dislodging using a rubber policeman. Cells were then centrifuged at 500xg for 5 minutes. The supernatant was removed and the pellet was then washed twice with 1X PBS pH 7.4 and centrifuged again at 500xg for 5 minutes. The supernatant was removed and the pellet was resuspended and incubated on ice in hypotonic buffer (10mM EDTA, 10mM Hepes, 50mM Sucrose), followed by homogenization to rupture cell membranes. The homogenate was then centrifuged at 800xg at 4°C for 5min to separate the cytoplasmic (supernatant) and nuclear (pellet) fractions.

3.6 Isolating Mitochondria

Post nuclear supernatant (described above) was collected and centrifuged at 13000xg at 4°C for 10 min, and the pellet (crude mitochondrial fraction) was resuspended in isolation buffer (250mM Sucrose, 1mM $MgCl_2$, 10mM HEPES, 20mM Succinate) kept on ice and used within 1-2h for experimentation.

3.7 Measurement of reactive ROS production from isolated mitochondria

Mitochondrial ROS generation is measured by H_2O_2 generation rate, determined fluorimetrically by measurement of the oxidation of para-hydroxy phenyl acetate (PHPA) coupled to the reduction of H_2O_2 by horseradish peroxidase (HRP), based on a previously published protocol (Li, N *et al.*, 2003; Naderi, J *et al.*, 2006). Crude mitochondrial extract was suspended in isolation buffer (described above) with the addition of PHPA (4mg/ml) and aliquoted evenly in a 96 well plate (~20μg/well) and incubated forming the following four fractions: (a) mitochondria alone (negative control), (b) mitochondria with Bax only (positive control), (c) mitochondria and one anti-Bax V_HH (total of six factions) and (d) mitochondria with Bax and one anti-Bax V_HH (total of six factions). Approximately 2μg Bax and 4μg of anti-Bax V_HH was used. A total of 0.4 units of HRP was also added to each fraction and after 30 minutes of incubation, the fluorescence of oxidized PHPA (excitation 320 nm, emission 400 nm) was measured in a 96 well micro-titer plate using the Spectra Max Gemini XS. Mitochondrial hydrogen peroxide production was determined by interpolation from the standard curve generated by reagent hydrogen peroxide. Total mitochondrial protein was estimated using BioRad with bovine serum albumin as a standard and the results were expressed per microgram of protein.

Microsoft Excel version 6.0 software was used for data representation and statistical analysis.

3.8 Cytochrome *c* release assay

Isolated mitochondria was divided into three equal fractions (60μg protein each) and incubate in three conditions: alone, with Bax (1μg) and with Bax (1μg) and V_HH 5-2 (2μg), for 20 min at room temperature, on a shaker. The samples were then centrifuged at 500xg for 10 min and the pellet and supernatant were resolved separately on SDS-PAGE, followed by Western Blot (described below) using monoclonal anti-cytochrome *c* as a primary antibody in a 1:500 dilution.

3.9 Western Blot Assay

All western blots were performed using the following protocol, with variations in the primary antibody as needed (monoclonal anti-Bax, monoclonal anti-cytochrome *c*, monoclonal anti-VDAC and monoclonal anti-green fluorescent protein). Protein samples from both *in vitro* and *in vivo* experiments were initially resolved using 10% or 12% SDS-PAGE technique, using 30-50μg of sample per well. Gels were transferred onto a nitrocellulose membrane which was subsequently blocked using a non-fat milk solution (5% milk in TBST) for 45 min on a shaker at room temperature. The membrane was then incubated with primary antibody of choice at a dilution of 1:500 in 2% non-fat milk solution, overnight at 4°C (12-18h). After this incubation period the membrane was washed with TBST three times and subsequently incubated with the secondary antibody (anti-mouse IgG (whole molecule) peroxidase conjugate) at a dilution of 1:2000 in 2%

milk solution and incubated at room temperature on a shaker. The blots were developed using a ChemiGlow West kit and recorded using an Alpha Innotech Corporation Imaging System.

3.10 Induction of Oxidative Stress

3.10.1 Hydrogen Peroxide Method

Working solutions of H_2O_2 was made by diluting a 10 M stock of H_2O_2 solution with distilled water to a concentration of 100 mM. SHSY-5Y cells were grown to approximately 70% confluence. Oxidative stress was induced by incubating the cells in complete media containing either 100 μM or 200 μM H_2O_2 for 1 h at 37°C. The media was then replaced with fresh, complete media (without H_2O_2) and the cells were incubated for different time periods to monitor apoptotic features and oxidative stress parameters.

3.10.2 Hypoxia/Hypoglycemia

Cells grown between 50-70% confluence were placed in a oxygen free chamber in control salt solution (110mM NaCl, 5.4mM KCl, 0.8mM $MgCl_2$, 1.8mM $CaCl_2$, 15mM $NaHCO_3$ 15mM HEPES, 50mM Glycine pH 8) for 12-24h at 37°C as needed. After this treatment, cells were removed from the chamber and the salt solution was replaced with fresh medium and cells were analyzed in 24h.

3.10.3 Serum Deprivation

Cells were grown to 50-70% confluence in complete medium as described above. Subsequently, the medium was replaced with DMEM-F12 Ham supplemented with only 20 μg/ml gentamycin lacking both L-glutamine and FBS and observed for up to 72h.

3.11 Monitoring Apoptosis

3.11.1 Cellular Staining Techniques

3.11.1.1 Monitoring nuclear morphology

Nuclear morphology was monitored as an indicator for apoptosis in cells by staining cells with Hoechst 33342 to a final concentration of 10 μM. After incubating for 10 min at 37°C, the cells were then examined under a fluorescence microscope where brightly staining nuclear condensation was understood to indicate apoptotic cells. Fluorescence pictures were taken processed using Improvision Open*Lab* v3.1.2, Jasc Paint Shop Pro v8.00 and Adobe Photoshop v8.0.

3.11.1.2 Monitoring mitochondrial membrane potential

Mitochondrial membrane potential was detected using JC-1 mitochondrial specific dye. The cells were treated with 10 μM JC-1 and incubated for 40 min at 37°C, in dark. The cells were observed under the fluorescent microscope and fluorescence pictures were taken and processed as described above.

3.11.1.3 Monitoring plasma membrane flipping

Annexin V was used to monitor plasma membrane flipping in cells according to manufacturers instructions. Briefly, Annexin V dye was added to Annexin-Binding Buffer (10mM HEPES, 140mM NaCl and 2.5mM $CaCl_2$ at pH 7.4) in a 1:2000 dilution and added to cells (following the aspiration of original culture media). After incubating for 15 min at 37°C in dark, the cells were examined under the fluorescence microscope and fluorescence pictures were taken and processed as described above.

3.11.2 Monitoring Protease and ROS activity *in vivo*

3.11.2.1 Lipid peroxidation determination

Lipid peroxidation in cells was determined using the thiobarbituric acid-reactive substances (TBARS) reaction with malonaldehyde and related compounds as previously described (Cereser, C *et al.*, 2001). Total cell lysates were extracted as described above and 10-25µl of each fraction was incubated with 1ml of thiobarbituric acid on a 100°C heat block for 15 minutes, developing a yellow colour. After cooling, absorbance at 535 nm was measured using a spectrophotometer. Lipid peroxidation levels were determined running internal standards of malonaldehyde (MDA). Results were calculated per microgram of protein. Protein concentration was measured using the Bio Rad protein assay reagent with bovine serum albumin as a standard (mentioned earlier). Microsoft Excel 6.0 software was used for data representation and statistical analysis.

3.11.2.2 Caspase 3/7 activation

The activation of Caspase 3/7 was measured in cells using Apo 3/7 HTS ™ High Throughput Screen Assay kit (Cell Technology Inc, Mountain View, CA). Pre-cultures cells were trypsinized, collected and centrifuged at 500xg for 7 min at room temperature. Cell pellet was resuspended in 500µl PBS/ $2.5x10^6$ cells and aliquoted into a black 96 well plate as needed. To each fraction Caspase detection reagent (prepared as per manufacturers instructions) was added in a 1:1 dilution and plates were incubated at 37°C on a shaker for 45min. Measurements of Caspase 3/7 activation were taken using a fluorescence plate reader at 488nm excitation and 520nm emission, and results were calculated as fluoresce per µg of protein, where high values indicated caspase activation. Protein concentration was measured using the Bio Rad protein assay reagent with bovine serum albumin as a standard (mentioned earlier). Microsoft Excel 6.0 software was used for data representation and statistical analysis.

3.11.2.3 MitoCasp Assay for detecting general caspase activation and mitochondrial membrane potential

General caspase activation and mitochondrial membrane potential were detected in cells using a Dual Sensor: MitoCasp™ Assay Kit following manufacturers protocol (Farkass, D *et al.*, 1989). Briefly, for both assays cells were harvested and suspended in PBS as described above. Cell suspensions from each of each group (50-100µl) was transferred in a eppindorff tube and mixed with Caspase Reagent or Mitochondrial Membrane Potential Dye (prepared as per manufacturers instructions). The final mixture was incubated at 37°C for 45min on a shaker. Cells were then centrifuged at 500xg for

10 min at room temperature and the supernatant was removed. The pellet was washed with 1X wash buffer provided in the assay kit, centrifuged at 500xg for 10 min at room temperature, followed by removal of supernatant and repeating this step a second time. Cells were then resuspended in the wash buffer and transferred to a black 96 well plate for analysis by a fluorescence plate reader. Caspase activity was measured first, taking measurements at 488nm excitation and 520nm emission, followed by MMP activity measured at 549nm excitation and 574 emission. Results were calculated as fluoresce per μg of protein, where high values indicated caspase activation. Protein concentration was measured using the Bio Rad protein assay reagent with bovine serum albumin as a standard (mentioned earlier). Microsoft Excel 6.0 software was used for data representation and statistical analysis.

3.11.2.4 Immunoprecipitation

Immunoprecipitation was carried to detect the possible interaction of anti-Bax intrabodies with VDAC as described by (Adachi M *et al.*, 2004), with the following changes. Mitochondrial fractions were extracted as described above for cells expressing V_HH 5-2 and non-transfected SHSY-5Y. A solution of 1μl anti-VDAC monoclonal antibody with 10μl protein G-Sepharose beads was made in 300μl RIPA buffer (20mM Tris-OH, 150mM NaCl, 10mM KCl and 1% triton X-100) and incubated for 1h at 4°C. Approximately 60μg of mitochondrial protein from each cell type was added to the pre-incubated anti-VDAC solution described here, and both samples were incubated for ~18h at 4°C on a shaker. Immunoprecipitates were collected by centrifugation (500xg) for 2 min at 4°C followed by washing the pellet three times using the RIPA buffer. After the

final wash the pellet was suspended in 30µl of RIPA buffer and mixed with 10µl of SDS-sample buffer and the samples were analyzed using SDS-PAGE and Western Blot as described previously.

Chapter 4: Results

4.1 Purification of Recombinant Bax for anti-Bax-sdAb (V_HH) identification

In order to carry out a panning assay to identify sdAb (V_HH) against the pro-apoptotic protein Bax, this antigen was isolated and purified from a culture of E.coli cells transformed with *bax* containing a N-terminal polyhistidine-tag, as described in Chapter 3, through affinity chromatography, using a Ni^{2+} chelating column. The presence of the N-terminal polyhistidine-tagged protein allowed for the use of a metal affinity resin as a method for purifying the recombinant Bax. The resin uses a tetradentate metal chelator to bind the Ni^{2+} ions by occupying four of the six coordination sites of the octahedral binding structure of the metal ion. The remaining sites are left available for interactions with His tails of recombinant Bax. Using low and high concentrations of imidazole which also has a potential to interacting with Ni^{2+} all non-specific proteins were eluted followed by release of purified Bax, respectively.

Bax expression in E.coli following induction with Arabinose was monitored by SDS-PAGE, loading 20μg of protein per well and stained with Coomasie Blue (Fig. 4.1). The supernatant and pellet fractions of induced cells (containing cytosolic and membrane bound proteins (S+ and P+) respectively) were compared to the supernatant and pellet fractions of non-induced cells (S- & P-, respectively). Highest Bax expression (detected by the ~21kDa band) was seen in the induced supernatant fraction (S+) as expected since Bax remains in the cytosol in its monomeric form.

Following purification, the pure Bax fraction was analyzed by SDS-PAGE to ensure additional (non-Bax) proteins (bands) were eliminated (Fig.4.2A). Bax is identified as the 40kDa band which was further confirmed using Western Blot indicating

that it was isolated in dimer form due to the presence of non-ionic lipids such as Triton X used during the purification process, which force strong homodimer formation of Bax (Hsu, Y-T *et al.*, 1997) (Fig.4.2B). For this procedure the protein fraction was first resolved using SDS-PAGE, loading 20μg protein as above and transferred onto a nitrocellulose membrane. The membrane was probed with monoclonal anti-Bax IgG as the primary antibody (with recognition for the N-terminus of Bax, common to isoforms α, δ, and β) in a 1:500 dilution in 2% non-fat milk solution. The blot was further probed with a secondary monoclonal anti-mouse HRP conjugate (1:2000 dilution) and developed using ChemiGlow West kit, showing a positive band at 40kDa for Bax. The experiment was subsequently repeated for each individual purification with similar results.

4.2 Panning a Llama phage display library for anti-Bax V_HHs

Six anti-Bax recombinant V_HH showing high affinity binding to recombinant Bax (with intact His tag) and no binding to BSA as a control were obtained from Dr. Jamshid Tanha of the Institute for Biological Sciences, National Research Council of Canada. The anti-Bax V_HHs 1 to 5-2 were isolated by the processes of panning described in Methods. They were tested for their affinity and specificity for Bax using ELISA and Biacore studies (data not shown).

4.3 *In vitro* analysis of six unique anti-Bax V_HHs efficacy

The six unique anti-Bax V_HHs identified in the panning assay were initially tested to understand if binding of Bax would translate in the inhibition of its function. In order

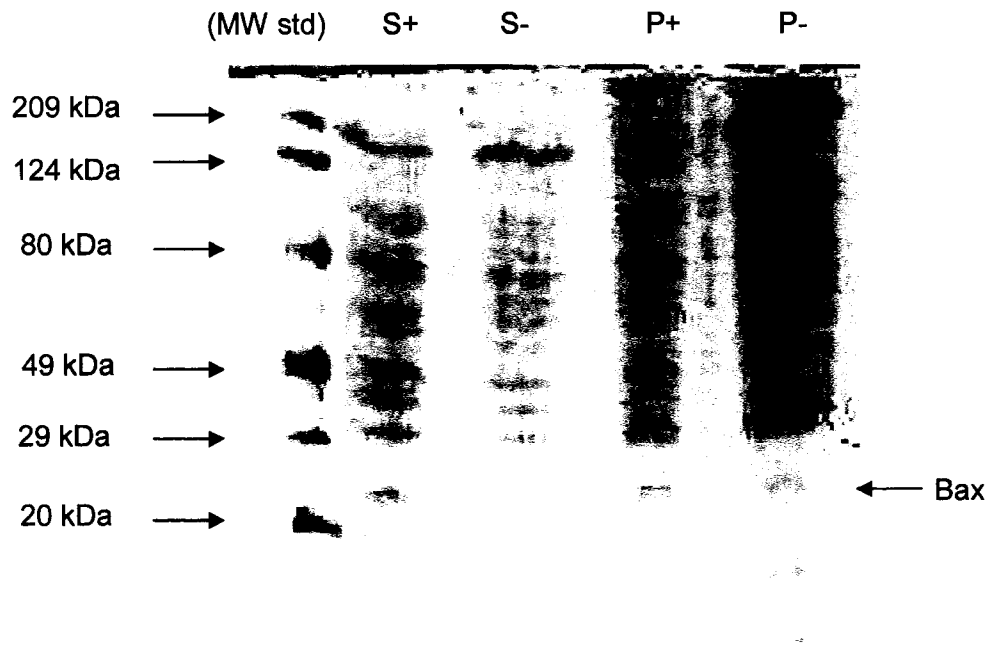

Figure 4.1 Detection of Bax expression in induced vs. non-induced fractions

(A) Recombinant Bax expression was induced from a transformed E.coli culture. Following induction using Arabinose cells were collected and lysed through sonification to separate cytosolic proteins in the supernatant (S) and membrane bound proteins in the pellet (P). Superantant and pellet fractions from induced (+) and non-induced (-) cells were compared to detect Bax expression using SDS-PAGE and staining with Coomasie Blue. Higher expression of Bax was detected in the induced supernatant fraction (S+) compared to the non-induced supernatant (S-) by the noted ~21kDa band. Both the induced and non-induced pellet fractions (P+ and P- respectively) show less Bax expression as expected since this protein is cytosolic in its monomer form.

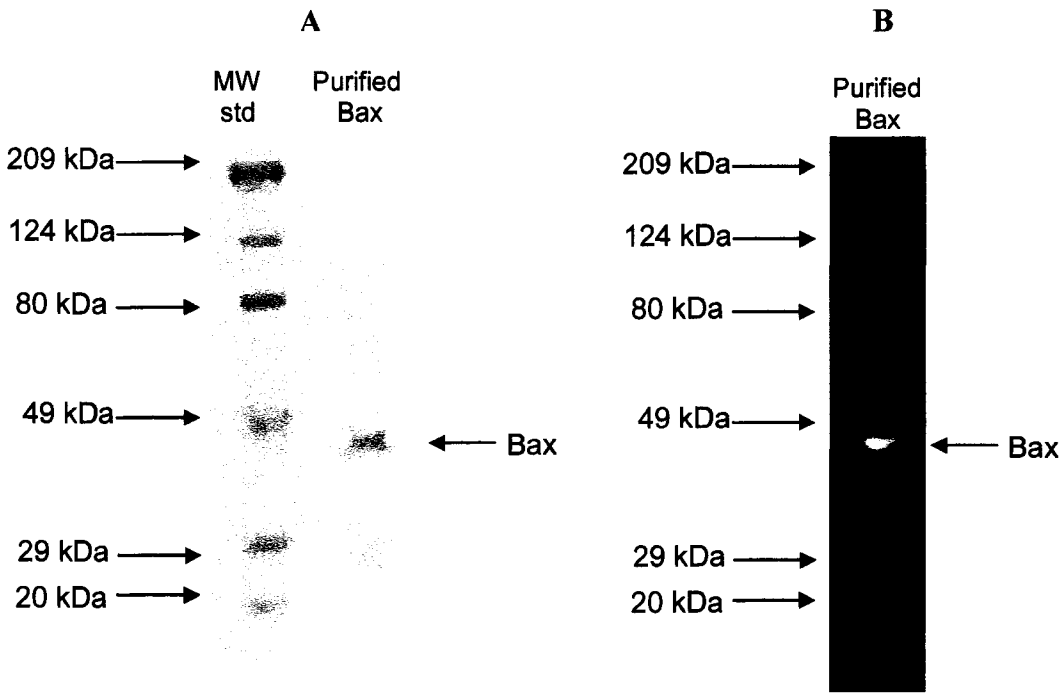

Figure 4.2 Detection of Purified Bax through SDS-PAGE and Western Blot

(A) After isolation and purification from a transformed E.coli culture, the presence of purified Bax was confirmed using SDS-PAGE and staining with Coomasie Blue. Bax is isolated as a dimer at 40kDa following purification. (B) Purified Bax fractions were further anaylyzed through Western Blot using a monoclonal anti-Bax primary antibody which was detected after incubation with secondary anti-body ((anti-mouse IgG (whole molecule) peroxidase conjugate) and developed using a ChemiGlow West kit.

to investigate if the anti-Bax V_HHs could prevent the permealization of the outer mitochondrial membrane, we monitored two parameters associated with Bax induced mitochondrial destabilization: elevation of ROS production and release of proteins from the mitochondria inner membrane space (IMS).

4.3.1 Anti-Bax V_HHs inhibit Bax-induced dysfunction of isolated mitochondria

Since there is no direct measure of Bax activity on mitochondria, we chose to measure the ROS production by the mitochondria. Previous work in our laboratory has shown that in presence of isolated mitochondria, Bax is able to permealize the MOM and subsequently lead to increase in ROS production (Naderi, J *et al.*, 2006). ROS is detected fluorimetrically by measurement of the oxidation of PHPA coupled to the reduction of H_2O_2 by horseradish peroxidase (HRP). Therefore, if by binding to Bax the six anti-Bax V_HHs were able to individually block its destructive effect on the mitochondria we would expect lowered fluorescence in those sample containing isolated mitochondria, Bax and anti-Bax V_HH compared to controls of mitochondria and Bax alone.

The data in figure 4.3 represents a compilation of five independent experiments. In each individual experiment, the fluorescence detection for the negative control (mitochondrial alone) was subtracted from each subsequent fraction. In addition, the positive control fraction (mitochondria with Bax only) was taken as 100% fluorescence and all test samples (mitochondria, Bax and anti-Bax V_HH clones) were calculated relative to this control. The results showed a decrease in ROS production in all fractions containing anti-Bax V_HHs compared to the positive control (mitochondria and Bax alone). Anti-Bax 5-2 produced the most significant results, decreasing the fluorescence

and thus the ROS production to approximately 10%, indicating strong protection of the mitochondria from Bax activity. Anti-Bax V_HHs incubated with mitochondria (in absence of Bax) did not affect the ROS level and even showed lower ROS generation that fractions containing mitochondria alone. This is possible since the anti-Bax V_HH could be binding and inhibiting endogenous Bax isolated from mitochondria of SHSY-5Y cells used. In addition, in order to show that Bax binding and inhibition is not occurring due to non-specific interactions, a non-specific single domain antibody (raised against Caspase 3) was also used as a control. This complex showed no consistent ability to prevent Bax induced mitochondrial destabilization. These results indicate that the anti-Bax V_HH clones are binding and more importantly inhibiting Bax, by preventing specific Bax induced mitochondrial damage.

4.3.2 Anti-Bax V_HHs prevent cytochrome *c* release from inner mitochondrial space

Another measurable consequence of Bax-induced mitochondrial damage is the release of proteins from the mitochondrial inner membrane space (IMS). Since anti-Bax 5-2 was able to strongly inhibit Bax induced ROS production form the mitochondria, we further tested its ability to prevent the release of cytochrome *c* which normally resided in the IMS. As in the previous experiment, isolated mitochondria were divided into three equal fractions (60μg protein each) and incubated in three conditions: (a) mitochondria with reaction buffer only (b) with Bax (1μg) alone, (c) with Bax (1μg) and V_HH 5-2 (2μg), for 20 min at room temperature. The fractions were then separated into supernatant fraction (containing proteins released from Bax-permealized mitochondria)

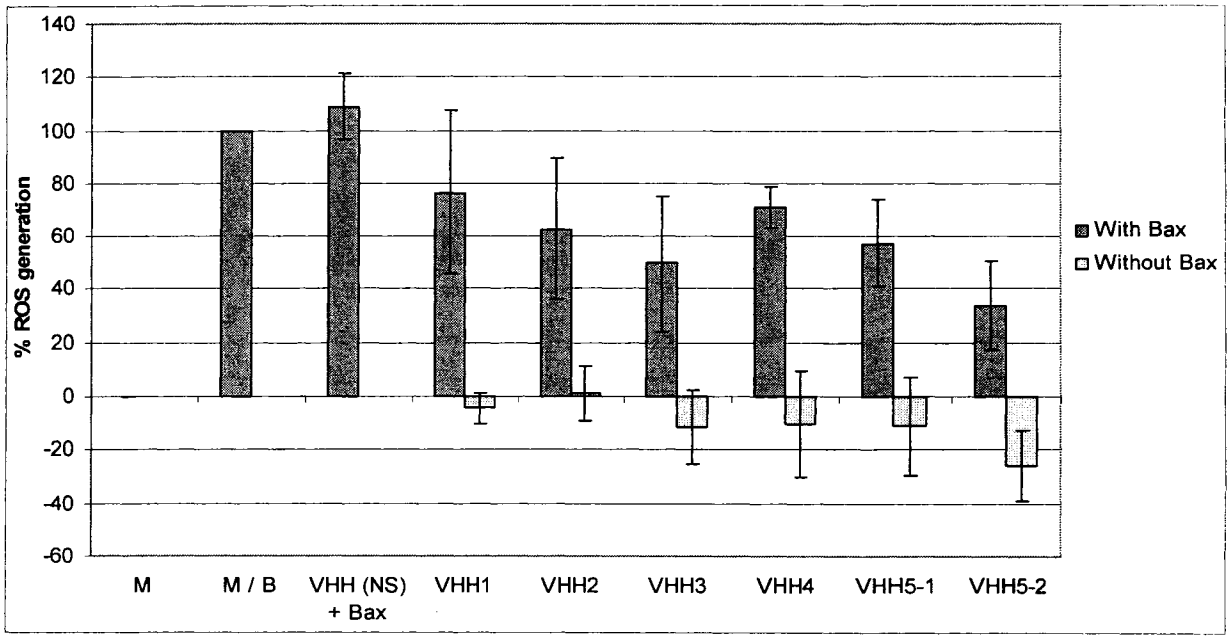

Figure 4.3 Measurement of ROS generation in isolated mitochondria (Mitoch)

Isolated mitochondria were incubated with Bax either in the presence or absence of different V_HHs in a reaction buffer. ROS generation was measured as described in Methods. ROS generated by Bax alone was taken as 100%. Different V_HHs inhibited ROS production caused by Bax to different degrees with the best effect seen with Bax5-2 V_HH. The standard error shown as error bars was calculated using Microsoft Excel program and the data obtained from five separate experiments.

and pellet fractions (containing proteins retained in mitochondria). After resolving and analyzing these samples using (10%) SDS-PAGE and Western Blot, the presence of anti-Bax V_HH 5-2 reduced the amount of cytochrome *c* released, by Bax destabilization of the MOM, compared to the positive control (mitochondria and Bax alone). These results were quantified by measuring the integrated density of each band, which showed greatest cytochrome *c* release in fractions containing mitochondria and Bax, alone (Fig.4.4A). As expected, anti-Bax V_HH 5-2 also increased the amount of cytochrome *c* retained in the pellet fraction, shown in the Western Blot and the integrated band density graph. Equal protein loading was confirmed by Ponseau S staining of the nitrocellulose membrane prior to blocking and incubation with the primary antibody (anti-cytochrome *c* IgG). All lanes showed similar intensity staining indicating equal protein loading and therefore eliminating the possibility that cytochrome *c* intensity changes in the developed Western Blots (Fig.4.4 A & B) are caused by greater starting concentrations of this protein (in a particular fraction). This further confirms the ability of the anti-Bax V_HH to not only bind Bax but also to prevent its interaction and destabilization of the mitochondrial outer membrane, which can ultimately lead to apoptosis as described in Chapter 1.

4.4 *In vivo* analysis of six unique anti-Bax V_HHs for preventing apoptosis

The *in vitro* functional characterization of the anti-Bax V_HHs are important positive preliminary results which suggest that these single domain antibodies can both bind Bax and inhibit its effect on isolated mitochondria. In the next phase of this project, we set out to investigate the effect of all six anti-Bax V_HHs when expressed inside human neuroblastoma cells, SHSY-5Y. Specifically, we planned to investigate whether these

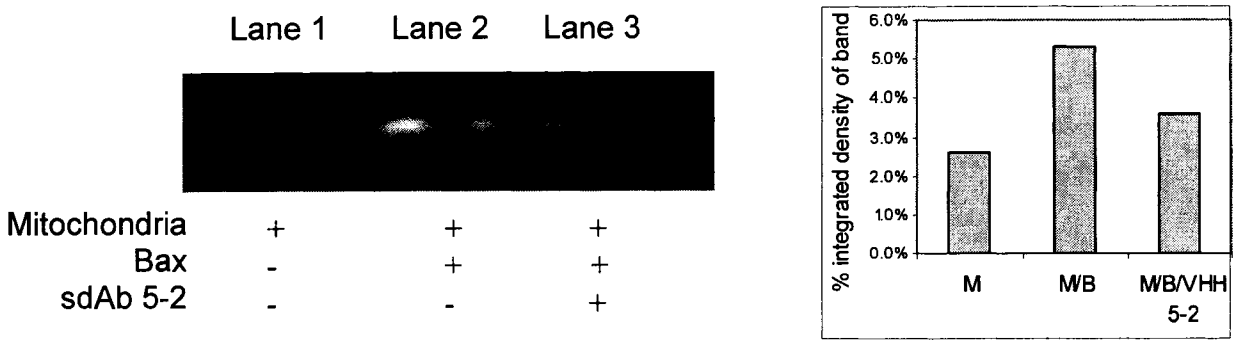

Figure 4.4A Cytochrome *c* released in supernatant (protein released from mitochondria)

The fractions resolved by SDS-PAGE and further analyzed by Western blot represent cytochrome *c* released from the mitochondria. Lane 1 represents the negative control (mitochondria) showing low cytochrome *c* detection. Lane 2 represents the positive control fraction (mitochondria and Bax) showing the highest detection of cytochrome *c*, which is lowered when anti-Bax V_HH 5-2 is present with Bax and mitochondria Lane 3. These results were further confirmed by generating integrated density values of each band, with greatest cytochrome *c* release in the fraction containing mitochondria and Bax alone.

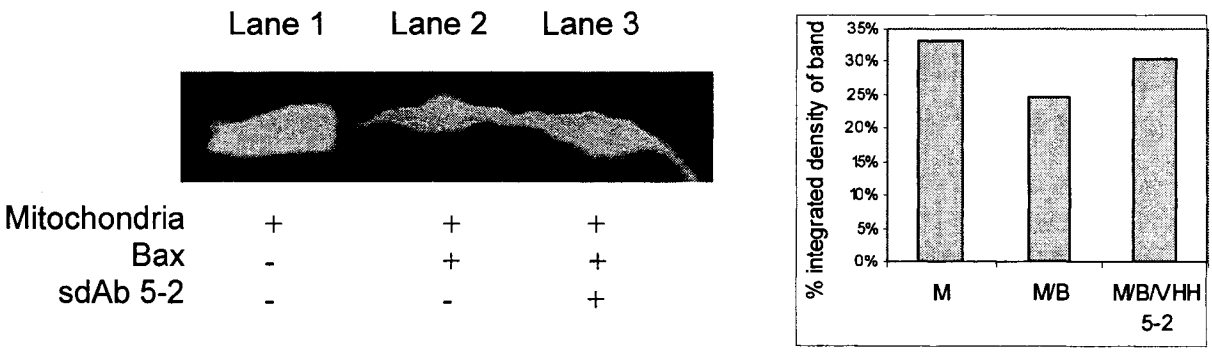

Figure 4.4B Cytochrome *c* detected in pellet (protein retained in mitochondria)

The pellet fractions from the same experiment as described in Fig.4.4A, were subjected to the same analysis. Lane 1 represents negative control (mitochondria alone) which retains a large amount of cytochrome *c*. Lane 2 represents the positive control (mitochondria and Bax), showing a decrease in the retention of cytochrome *c*, indicating mitochondrial destabilization. Lane 3 contains mitochondria in presence of Bax and anti-Bax V_HH 5-2, which also has similar retention detected as in mitochondria alone (lane 1), indicating that this V_HH can inhibit Bax activity and limit mitochondrial permealization. The integrated density values for the bands shown also confirm that there was least amount of cytochrome *c* retention in fractions containing mitochondria and Bax alone.

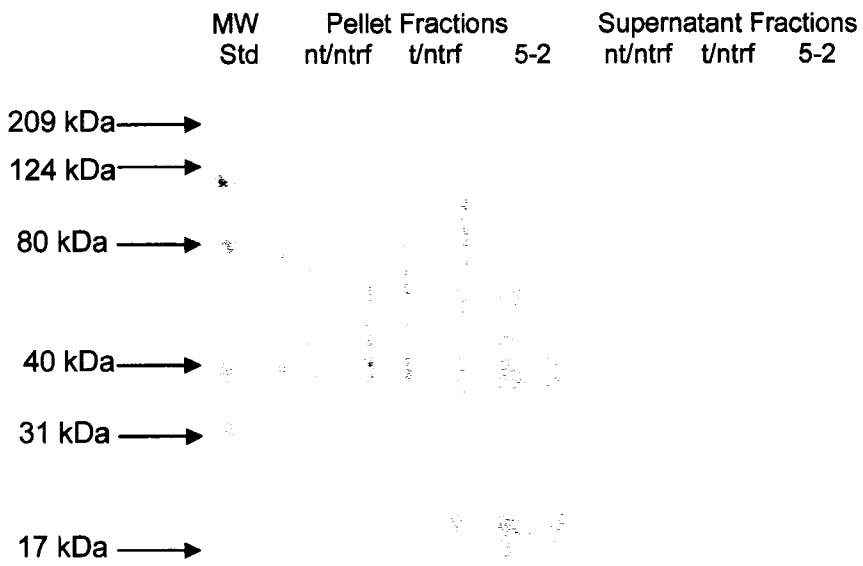

Figure 4.4C Ponseau S staining of Pellet and Supernatant Mitochondrial Fractions

To ensure that equal amount of protein were loaded in each lane for the detection of cytochrome *c* in the pellet (retained in IMS) or supernatant (released from IMS), the nitrocellulose membrane (onto which the gel was transferred) was stained with Ponseau S. Since the staining intensity in all fractions is relatively equal we can conclude that change in cytochrome *c* noted by the developed Western Blots (Fig. 4.4 A and B) were not attributed to overloading in a particular lane, but were due to the changes in cytochrome *c* localization within the cell.

anti-Bax V_HHs have the potential to prevent oxidative stress induced-apoptosis in this cell line. The anti-Bax V_HHs were expressed inside this cell line through both transient and stable transfection using constructs containing the V_HH gene obtained from Dr. Jamshid Tanha, NRC-IBS.

4.4.1 Transient Transfection of anti-Bax V_HH plasmids in SHSY-5Y cells

Transient transfection was carried out as described in Methods, and positively transfected cells were confirmed through detection of the RFP or GFP fluorescent marker protein, which were co-expressed with the V_HH genes. Oxidative stress was induced within 24 h of the transfection by administering H_2O_2 to a final concentration of 100μM. The transfected cells were exposed for 1 h and qualitatively analyzed though cellular staining using Hoechst and JC-1 dye after 6h (data not shown) and 24h, to monitor nuclear morphology and mitochondrial membrane potential, respectively (Fig. 4.5). Cells expressing an anti-Bax V_HH in fusion with GFP showed resistance to apoptosis under these oxidative stress conditions which have been shown in this and other studies in our laboratory to induce approximately 50% cell death (24 h after treatment) in SHSY-5Y.

4.4.2 Stable Transfection of anti-Bax V_HH plasmids in SHSY-5Y cells

Through transient transfection we were able to qualitatively show that the anti-Bax V_HHs were able to reduce apoptosis due to H_2O_2 induced oxidative stress. However, as transiently transfected cells make up only 5-10% of the cell culture in each plate, it becomes difficult to quantify these results. Therefore, the next phase in this project involved forming stable cell lines which express each of the six anti-Bax V_HH clones (individually) and monitoring their apoptotic resistance though cellular staining, protease

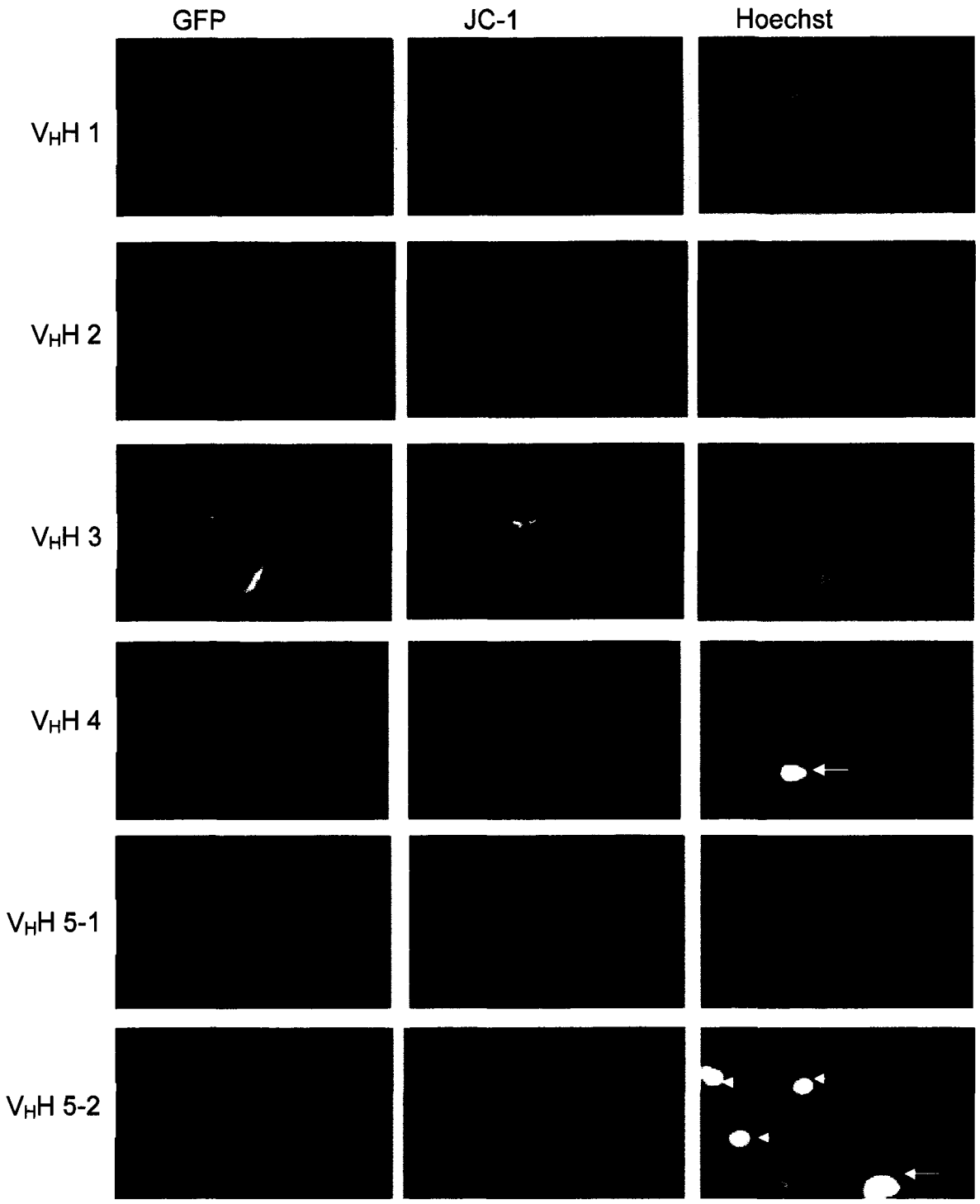

Figure 4.5 Transiently transfected cells show resistance to oxidative stress induced apoptosis

The construct for each anti-Bax V_HH in fusion with GFP or RFP (data not shown) was transiently transfected in SHSY-5Y cells. Oxidative stress was introduced by the addition of 100μM H_2O_2 for 1 h and cells were monitored after 24 h. Positively transfected cells, detected by presence of GFP (column 1) generally show stable mitochondrial membrane potential confirmed by red punctuate JC-1 (column 2). Healthy nuclei are also seen in these cells through Hoechst staining (column 3). A brightly stained and apoptotic nuclei is detected for one of the cells transfected with V_HH 4 (white arrows). Multiple apoptotic nuclei are detected among cells transfected with V_HH 5-2, however these nuclei belong to non-transfected cells which are floating and not expressing the V_HH 5-2 intrabody (yellow arrows) since they are not GFP positive.

activation detection, ROS production (measuring ROS and monitoring lipid peroxidation). To ensure the specificity of our anti-Bax V_HHs, we also created three control stable cell lines expressing GFP or RFP fluorescent proteins (alone) and a non-specific V_HH (PTH_{50}) fused with RFP.Stable cell lines were formed by taking advantage of the antibiotic resistance gene (Neo^r) therefore the addition of G418 allowed for the selection and propagation of positively transfected cells. By maintaining this antibiotic in the media at all times, the survival of each cell depended on the presence of the resistance gene (in fusion with the V_HH gene) therefore this prevents the cell from removing the plasmid after 48-72h as occurs with transient transfection.

4.4.2.1 Confirming the expression of anti-Bax V_HHs in stable cell lines

To ensure that cells in the newly formed stable cells lines are indeed expressing the specific anti-Bax V_HH, we initially used the GFP or RFP fusion proteins as a maker for positive transfection. Since the V_HH gene and GFP are expressed as fusion proteins cells which express the GFP or RFP must also express the V_HH clones. Stable cell lines were therefore confirmed by detecting these fluorescent proteins through fluorescence microscopy (Fig. 4.6). The fluorescent protein markers were also useful to ensure that all non-transfected cells (no GFP/RFP expression) were eliminated by the G418 present in the culture medium. A culture of non-transfected SHSY-5Y were also subjected to identical variations of G418 (staring at 400μg/ml and gradually reduced to 200μg/ml) in parallel to the stable cell lines to ensure that non-transfected SHSY-5Y were not tolerating the antibiotic and becoming resistance during this time.

Though the detection of GFP or RFP fusion markers is a relatively facile way to verify that no non-transfected cells are present among our stable cell line, we also had to consider the possibility that the V_HH gene can be excised, resulting in the sole expression of the marker protein (GFP or RFP) and not the anti-Bax V_HH. We monitored the presence of the intrabodies inside the SHSY-5Y cells through Western Blot, however one challenge for this experiment was the lack of primary antibody for the unique anti-Bax V_HH. Since the anti-Bax V_HH clones were expressed in fusion with GFP, we were able to probe our Western blot with anti-GFP in order to detect the change in the molecular mass of marker protein, which would account for the presence of the intrabody.

The anti-bax V_HH are approximately 10-12kDa therefore we expected to find the GFP band from each of the six cell lines at a higher position than cells expressing GFP alone. Figure 4.7 confirmed these results as the band for each of the six anti-Bax V_HH appears approximately 10-12kDa) above the control GFP only expressing cells, which accounts for the molecular weight of the V_HH clones (12kDa.

4.4.2.2 Cell viability following hypoxia/hypoglycemia induced stress

Hypoxia/hypoglycemia was induced in cells expressing anti-Bax intrabodies for 12h (as described in Methods), after which point cells were rescued (placed in glucose media and exposed to oxygen) and monitored for signs of apoptosis through nuclear morphology staining using Hoechst reagent to count cells to quantitatively determine cell viability (Fig.4.8). This experiment is a close model for studying damage caused by reperfusion, which occurs following a stroke. During the reperfusion phase of stroke, there is a noted increase in reactive oxygen species which may spread to surrounding

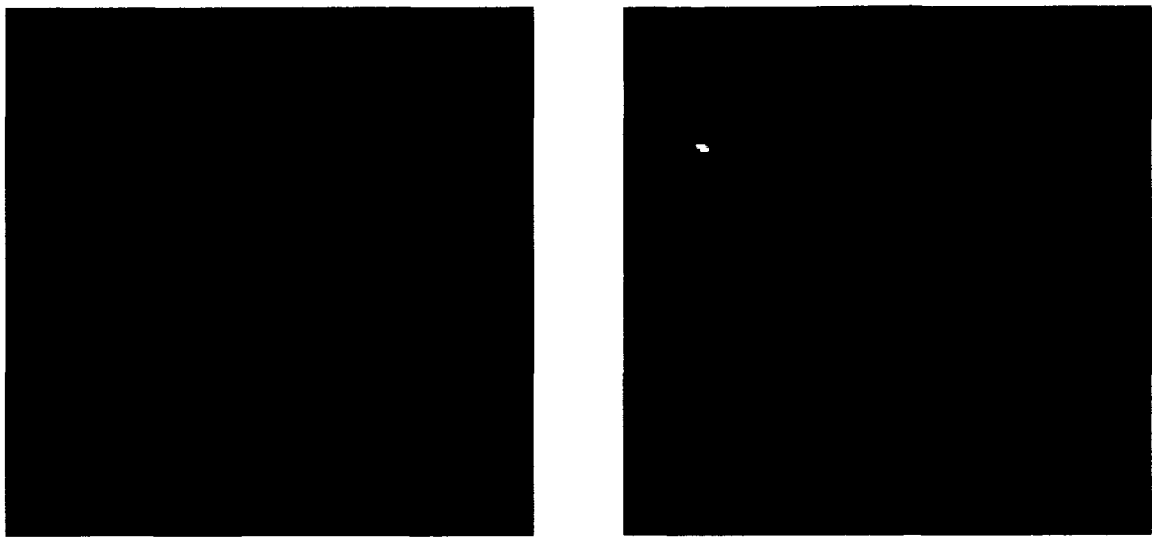

Figure 4.6 RFP expression

Following transfection of the anti-Bax V_HH clones in fusion with RFP (shown here), cells were placed in complete medium containing G418 at a decreasing conentration (400μg/ml to 200μg/ml) for approximately two weeks, and after all non-transfected cells were eliminated (since they do not contain the G418 resistance gene) cells were maintained in G418 media at 200μg/ml. The expression of RFP was used as a marker for the expression of anti-Bax V_HH (3 and 5-2 shown here).

Figure 4.7 Confirming Expression of V_HH by change in molecular weight of GFP

Western blot using anti-GFP primary antibody was used to detect the expression anti-Bax V_HHs co-expressed with GFP. Lanes 1-6 represent GFP detected from cell lysates of stably transfected cells (expressing V_HH 1 - 5-2 respectively) and lane 7 represents GFP detected in GFP only expressing cells (no V_HH). Co-expression of all six anti-Bax V_HHs (with GFP) was confirmed as the GFP band appears approximately 10-12kDa above the band for GFP only (lane 7), which accounts for the weight of the anti-Bax V_HH clones (12kDa).

healthy neurons causing over expression and activation of Bax leading to inappropriately induced apoptosis of these cells (Margaill, I *et al.*, 2005). Therefore we monitored the ability of the anti-Bax intrabodies to inhibit Bax activity under similar conditions in order to prevent apoptosis. Following the induction of hypoxia/hypoglycemia, Hoechst staining was used to count apoptotic vs. healthy nuclei, generating a cell viability graph. The variance in the cell viability rates of cells transfected with the anti-Bax intrabodies and the non-transfected cells was found to be statistically significant ($p<0.05$). Therefore by inhibiting the pro-apoptotic function of Bax the anti-Bax intrabodies were able to limit oxidative stress-induced apoptosis.

4.4.2.3 Caspase 3/7 activation is inhibited following hypoxia/hypoglycemia in cells expressing V_HH 5-2

Cells transfected with anti-bax intrabody 5-2 were also tested for their ability to prevent Caspase 3/7 activation (as previously described) following hypoxia/hypoglycemia treatment for 12h (Fig. 4.9). As expected, these cells exhibited low Caspase 3/7 activity (represented as fluorescence/μg protein) comparable to the non-treated/non-transfected (nt/ntrf) control cells. Contrastingly, cells exposed to the same stress condition which were non-transfected (t/ntrf) exhibited higher Caspase 3/7 activity, suggesting that the anti-Bax intrabodies offer protection against apoptosis caused by oxidative damage.

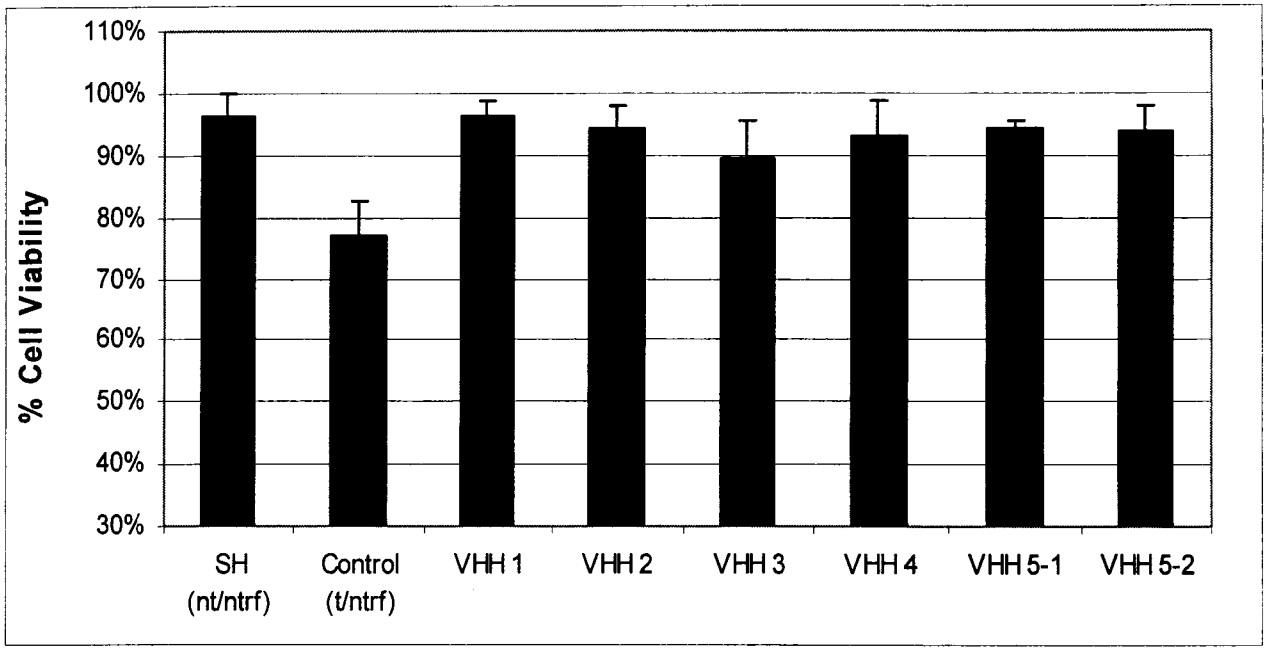

Figure 4.8 Hypoxia/Hypoglycemia induced oxidative stress inhibited by anti-Bax intrabodies

Following oxidative stress induced by hypoxia/hypoglycemia for 12h, cells were stained with Hoechst after 24h, and cell viability was determined by counting apoptotic and healthy nuclei. Cells expressing the anti-Bax intrabodies were able to overcome the damage caused by exposure to hypoxia/hypoglycemia, showing cell viabilities which were statistically different that the treated non-transfected cells ($p<0.05$).

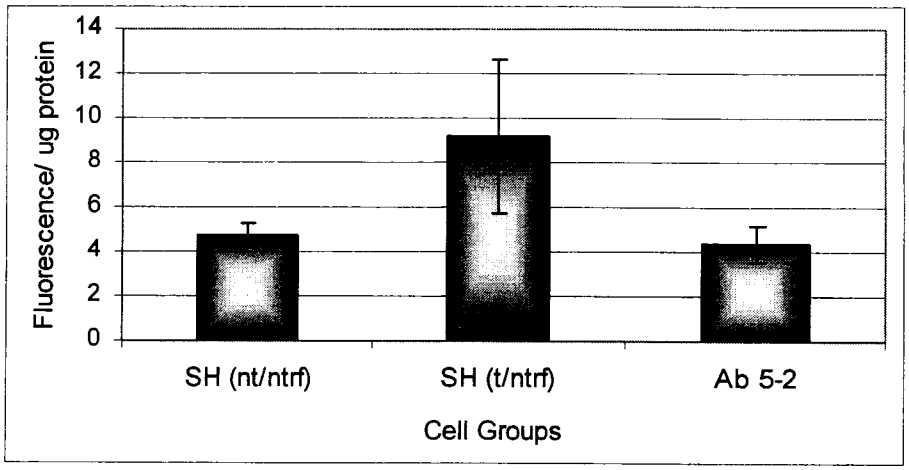

Figure 4.9 Caspase 3/7 remains iinactive in cells expressing V_HH 5-2

Following hypoxia/hypoglycemia treatment for 12 h cells expressing anti-Bax intrabody 5-2 exhibit low caspase 3/7 activation almost identical to SHSY-5Y cells which were non-treated/non-transfected (nt/ntrf). Cells which do not express any intrabodies but underwent the same treatment (t/ntrf) show twice the level of Caspase 3/7 activation.

4.4.2.4 Monitoring nuclear morphology following 100μM H_2O_2 treatment

Stable cell lines containing each of the six anti-Bax V_HH as well as four control cells lines (non-tranfected (ntrf), GFP only, RFP only and PTH_{50} (non-specific V_HH)) were exposed to 100μM H_2O_2 for 1h and monitored after 24h for signs of apoptosis through Hoechst staining to monitor nuclear condensation indicative of apoptotic nuclei (Fig. 4.10). The control cell lines (described above) which were exposed to the above treatment (t), showed poor resistance to apoptosis confirmed by numerous brightly stained nuclei. In addition, many of the cells in these samples were already detached and floating at this time point and not detected. Contrastingly, cells expressing any of the anti-Bax V_HH showed strong resistance to this stress factor as very few apoptotic nuclei can be detected.

Quantitative results for percent cell viability were attained by counting number of brightly stained, condensing nuclei compared to total nuclei using 5-10 fields from 3-5 individual experiments (Fig. 4.11). In each experiment, the control and experimental cell lines were treated in parallel under the same conditions to ensure accuracy. Here control cell lines show a decrease in cell viability compared to non-treated SHSY-5Y (nt/ntrf) by approximately 50%. However, cells expressing the anti-Bax V_HHs (in fusion with GFP or RFP) show viabilities between 90-95%, after exposure to this stress level, which is comparable to non-transfected SHSY-5Y which were not treated (nt/ntrf), whose viability in culture is approximately 96%. Using statistical analysis the cell viability difference in cells expressing the anti-Bax intrabodies to all treated control cells (ntrf, GFP, RFP and PTH) were found to statistically significant ($p<0.05$). This indicated that the expression

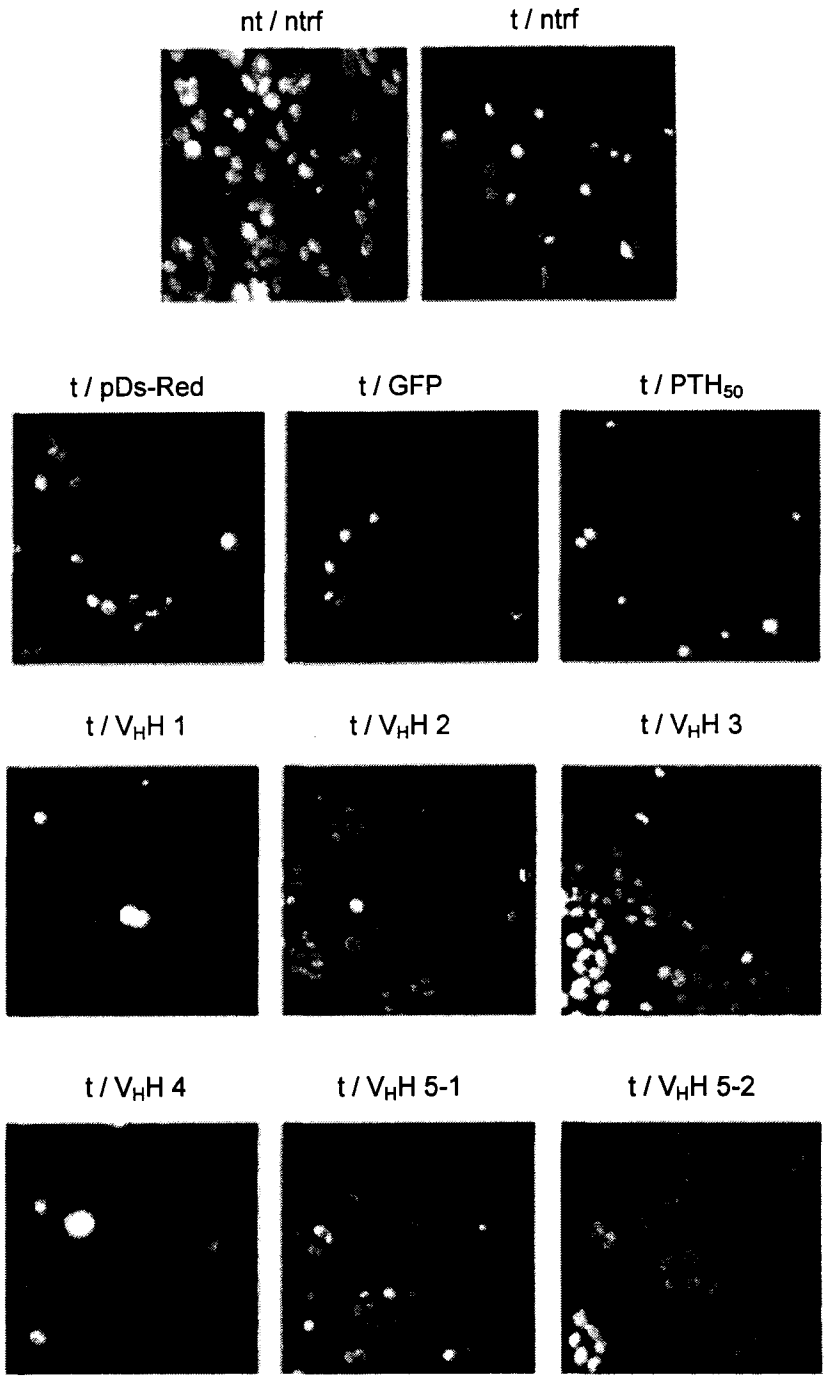

Figure 4.10 Nuclear morphology following 100μM H_2O_2 treatment

Cells transfected with anti-Bax V_HH and treated with 100μM H_2O_2 for 1h (t/V_HH (1- 5-2) were stained with Hoechst reagent after 24h, which showed a small percentage of apoptotic nuclei (bright staining) similar to the non-treated/non-transfected cells (nt/ntrf). In contrast a substantial increase in apoptotic nuclei is seen in all the positive control cell lines exposed to this treatment (t) including cells which were non-transfected (t/ntrf), and those transfected with GFP only (GFP), RFP (pDs-Red) and a non-specific V_HH (PTH_{50}).

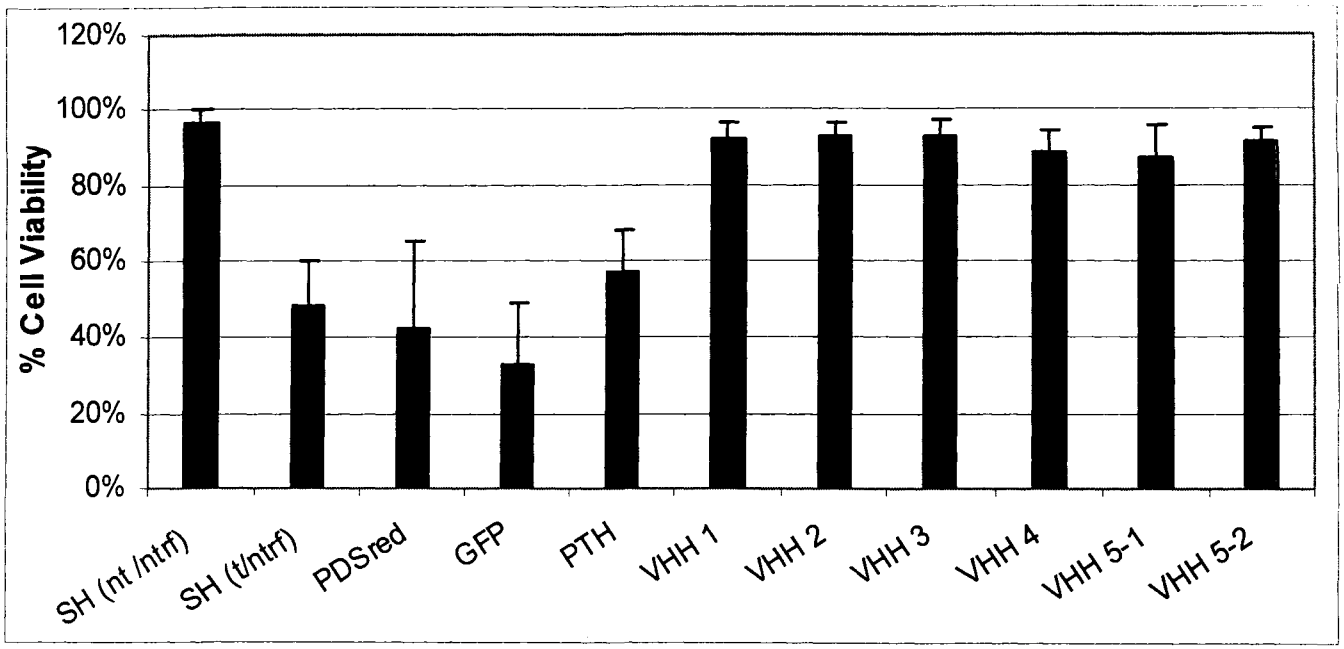

Figure 4.11 Quantifying cell viability following 100μM H_2O_2 treatment

Cell lines transfected with anti-Bax V_HH were treated with oxidative stress (100 μM H_2O_2 for 1 h). Non-treated / non-transfected (nt/ntrf) cells were used as a negative control while all other control and experimental cell lines were exposed to the oxidative stress described. After 24 h cultures were stained with Hoechst reagent (as described in Fig. 4.8). Healthy and apoptotic nuclei from three separate experiments were counted using 6-10 fields/cell line/experiment, and the number of healthy cells was plotted as a percentage of all the cells counted as Cell Viability. SHSY-5Y cells transfected with anti-Bax V_HHs (fused with GFP or RFP) show strong resistance to apoptosis, with cell viability values which are statistically higher than all the treated control cell lines ($p<0.05$).

of the anti-Bax intrabodies is not only non-toxic to these cells but is able to significantly reduce oxidative stress-induced apoptosis.

4.4.2.5 Monitoring plasma membrane flipping following 100μM H_2O_2 treatment

Control and experimental cells lines exposed to oxidative stress using 100μM H_2O_2 for 1h were also monitored for a plasma membrane flipping through Annexin V binding 24 hours after treatment. Annexin V measures an early feature of apoptosis: the phosphatidyl serine flipping across the plasma membrane. Annexin V green conjugate was used for cells expressing the anti-Bax intrabodies with RFP marker proteins as well as for the RFP only control cell line. Annexin V red conjugate was used for experimental and control cell lines expressing the GFP marker protein. The four treated control cell lines (non-transfected, GFP, RFP, PTH_{50}) all show substantial levels of Annexin V binding (Fig. 4.12) which appears as a fluorescent outline of cells in the early phase of apoptosis.

In the experimental cell lines expressing anti-Bax intrabodies, there is limited Annexin V detected similar to non-treated/ non-transfected cells (Fig.4.13). Here the complimentary healthy nuclei are also shown through Hoechst staining, to confirm the presence of numerous cells in the fields shown. Since protection against oxidative stress is limited in control cell lines, we can assume that the intrabodies alone are responsible for this action.

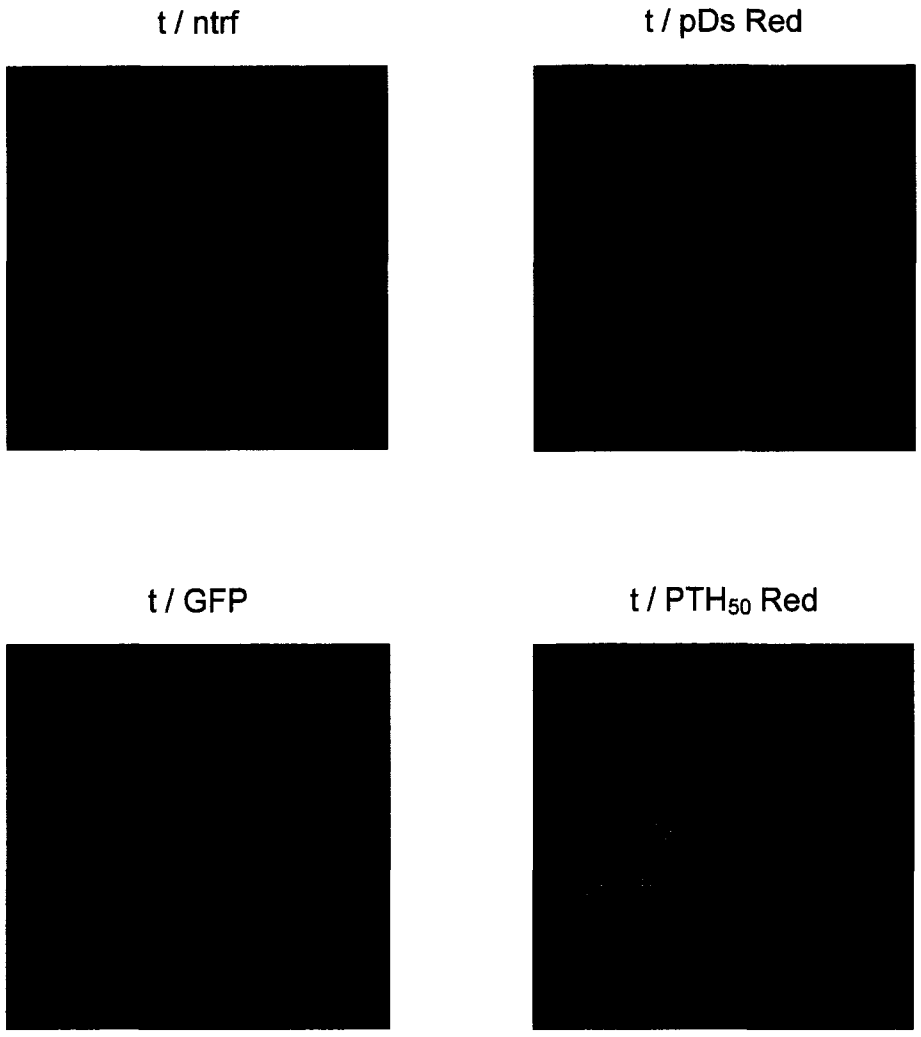

Figure 4.12 Plasma membrane flipping in cells lacking anti-Bax V_HHs

Control cell lines treated with 100μM H_2O_2 for 1h show high levels of plasma membrane flipping through strong Annexin V binding. Cells transfected with the fusion protein alone (GFP or RFP) or a non-specific V_HH (PTH_{50}) behave as the non-transfected/treated cells (t/ntrf) indicating that no oxidative stress protection is offered by these complexes.

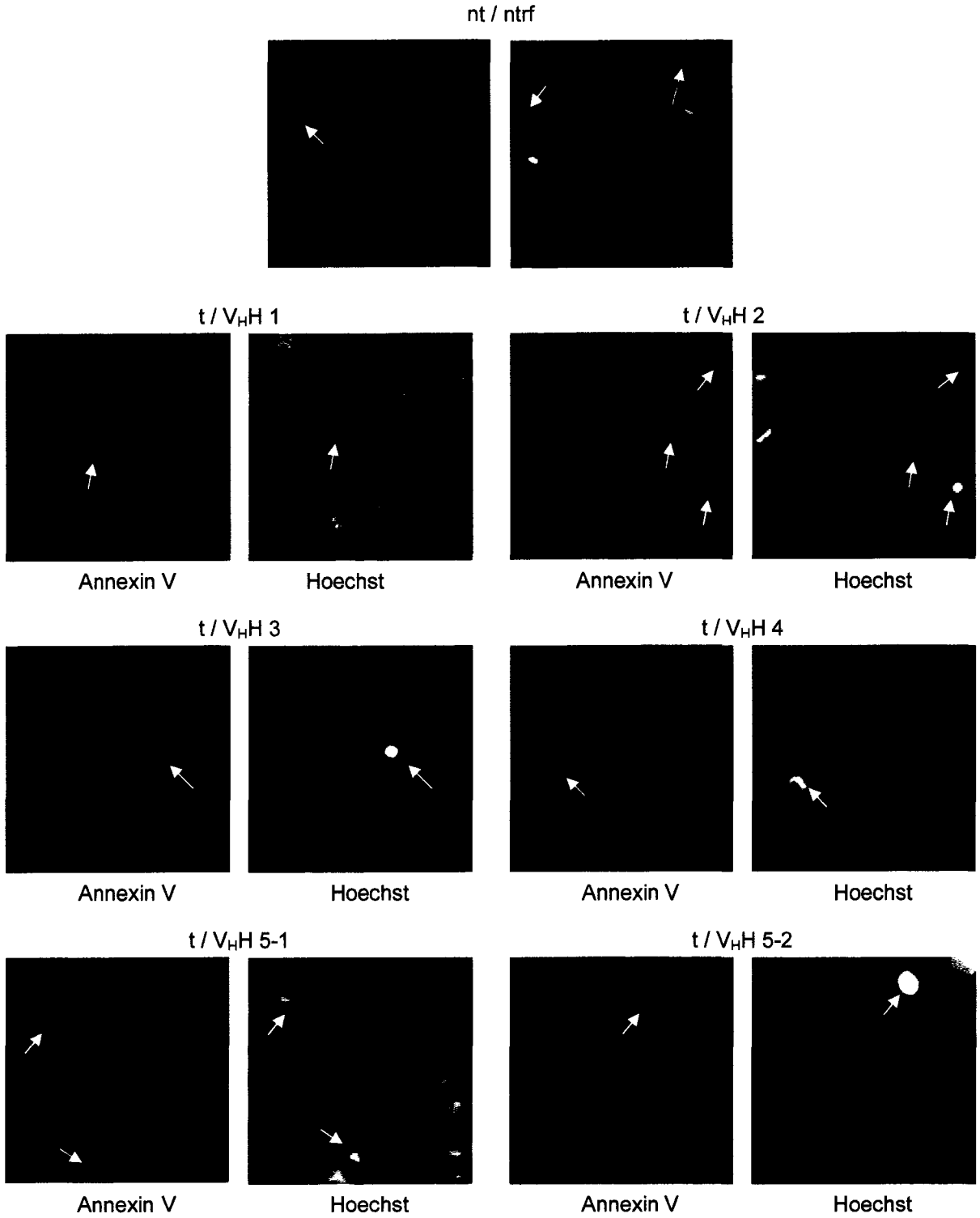

Figure 4.13 Limited plasma membrane flipping detected in cells expressing anti-Bax V_HHs

Cells transfected with any of the six anti-Bax V_HHs were exposed to oxidative stress using 100μM H_2O_2 for 1h and monitored after 24h. These cells show limited Annexin V binding indicating that oxidative stress-induced apoptosis is inhibited due to the inhibition of Bax activity by the anti-Bax intrabodies. Complimentary nuclei images as also shown to confirm the presence of a large population of healthy cells in the respective fields. Overall, cells expressing anti-Bax intrabodies and exposed to the treatment behave similarly to non-treated/non-transfected cells (nt/ntrf). White arrows indicated apoptotic cells, yellow arrow shows a dividing nucleus and therefore it does not appear as Annexin positive.

4.4.2.6 Monitoring mitochondrial membrane potential following 100μM H_2O_2 treatment

Control and anti-Bax V_HH expressing cell lines were monitored using JC-1 dye in order to measure the mitochondrial membrane potential (MMP) 24 hours after induction of oxidative stress using 100μM H_2O_2 as described above. JC-1 is a cationic dye which stains the mitochondria in healthy cells with a punctate red stain which is detected using fluorescence microscopy. Specifically, this lipophilic dye bears a delocalized positive charge which allows it to accumulate in healthy mitochondria (possessing an internal net negative charge), forming J-aggregates which fluoresce red. If the MMP collapses, JC-1 cannot accumulate in the mitochondria and the dye remains in the cytosol as a monomer fluorescing a diffuse green. The green fluorescence is also seen in healthy mitochondria as some dye does remain in the cytosol at all times, therefore the true marker for detecting healthy vs. destabilized MMP is the presence of red staining.

Cells expressing the anti-Bax intrabodies show healthy, punctuate red staining after the oxidative stress similar to the negative control cell line (non-treated/non-transfected) (Fig. 4.14). Hoechst staining was also shown in parallel here to confirm the total cells present in each field and also confirms that virtually all cells expressing anti-Bax intrabodies contain healthy MMP. These results agree with the earlier studies which looked at other apoptotic features such as nuclear fragmentation and plasma membrane flipping, showing that cells expressing anti-Bax intrabodies can tolerate oxidative stress (at the level of 100μM H_2O_2) and essentially behave as normal non-treated SHSY-5Y.

MMP was also monitored through a quantitative method using MitoCasp Assay Kit 6 hours after induction of oxidative stress using 100μM H_2O_2 for 1h, as described in

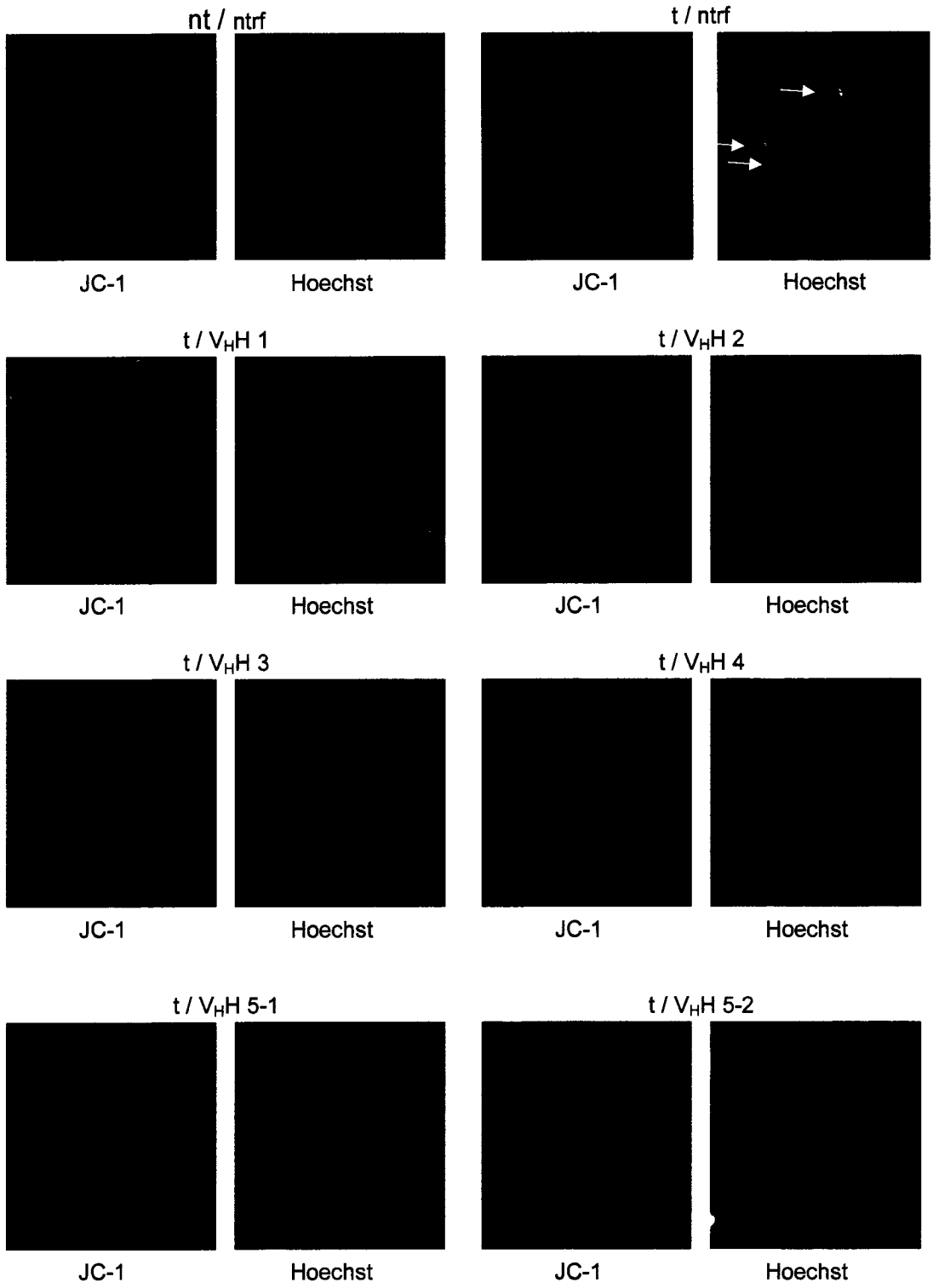

Figure 4.14 Stable MMP detected through JC-1 staining in cells expressing anti-Bax intrabodies

Oxidative stress was induced by 100μM H_2O_2 for 1h and after 24h cells expressing anti-Bax intrabodies (and control non/transfected cells) were stained with JC-1 which fluoresces red as it accumulated in healthy mitochondria. Healthy mitochondria are detected in the majority of anti-Bax expressing cells, comparable to the non-treated/non-transfected cells.

Methods. This assay also uses a cationic dye which accumulates in healthy mitochondria and can be detected using a fluorescence plate reader. The results of this experiment are shown as a measure of fluorescence/μg of protein (Fig. 4.15). Shown here, cells expressing V_HH 5-2 behave like the negative control cell line (non-treated/non-transfected), fluorescing at almost double the intensity of the treated/non-transfected cells. The difference in fluorescence indicating stable MMP, between the non-transfected treated cells compared to both the non-treated/non-transfected and the treated/ V_HH5-2 transfected cells was found to be statistically significant ($p<0.05$). Therefore, these results suggest that by binding to Bax intracellularly, the anti-Bax intrabodies can prevent the permealization of the mitochondrial membrane (leading to MMP collapse) both at 6 and 24 h after treatment, thereby preventing apoptosis.

4.4.2.7 Caspase 3/7 activation is inhibited in cells expressing V_HH 5-2

The activation of executioner caspases 3/7 is one consequence of Bax induced mitochondrial destabilization as described in Chapter 1. The activation of these enzymes in cells expressing anti-Bax intrabody 5-2 was tested as described in Methods using Apo 3/7 HTS kit following the induction of oxidative stress. Specifically, this assay uses a quenched $(z\text{-DEVD})_2$-R110 peptide substrate. Active caspases 3 and 7 are able to cleave away the quenched dye R110 which can be detected fluorometrically when not complexed with the DEVD substrate.

For this study, cells were treated with 100μM H_2O_2 for 1h and tested following 6h at which point sufficient caspase activation has been previously shown to occur, in our laboratory. The results were calculated as fluorescence / μg of protein, and statistically

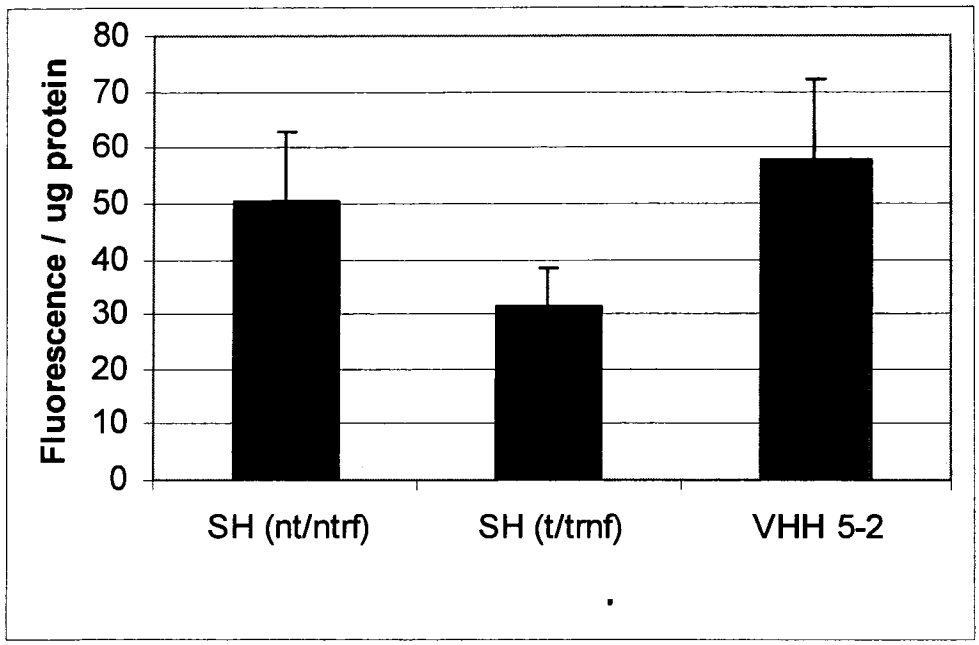

Figure 4.15 Strong MMP detected in anti-Bax intrabody expressing cells following 100μM H_2O_2 treatment

The MMP was monitored in cells 6h after exposure to oxidative stress using 100μM H_2O_2 for 1h, using MitoCasp Assay as described in Methods. In healthy mitochondria with stable MMP, the cationic dye used in this assay is able to aggregate and can be detected using a fluorescence plate reader. Non-transfected/ treated cells (t/ntrf) exhibited significantly lower fluorescence than the non-treated/non-transfected cells (nt/ntrf) and the treated V_HH 5-2 expressing cells ($p<0.05$). This suggests that by binding and inhibiting Bax intracellularly, the anti-bax intrabodies can prevent MMP collapse induced by oxidative stress.

analyzed to show that the treated/non-transfected (t/ntrf) cells exhibited significantly higher caspase 3/7 activation in comparison to the non-treated/non-transfected (nt/ntrf) cells and the treated/ V_HH 5-2 expressing cells ($p<0.05$).

In addition general caspase activation was also analyzed in cells expressing the anti-Bax 5 intrabody using the Caspase detection component of the Dual Sensor: MitoCasp Assay kit previously described. This assay uses a novel approach to detect caspase activation, based in carboxyfluorescein (FAM) labled fluoremethyl ketone (FMK) peptide caspase inhibitor. These inhibitors are cell permeable and non-cytotoxic therefore they can easily bind to active caspases intracellularly and are measured using a fluorescence plate reader. The results are calculated as fluorescence / μg of protein and show a statistically significant decrease ($p<0.05$) of active Caspases 7-9 by approximately 50% in V_HH 5-2 transfected cells, compared to the treated / non-transfected control cells (t/ntrf) (Fig.4.17).

4.4.2.8 Low Levels of Lipid Peroxidation detected in cells expressing anti-Bax V_HHs

Lipid peroxidation is an important marker for oxidative stress induced apoptosis.

Cells transfected with anti-Bax intrabodies were monitored for their ability to resist oxidative damage through the detection of lipid peroxidation as described in Methods. Cells treated with 100μM H_2O_2 for 1h were tested after 24 h, compared to a negative and positive control (non-treated / non-transfected cells and treated/non-transfected cells respectively) (Fig.4.18). The results shown represent a compilation of 5 experiments, in which the lipid peroxidation was measured as μg MDA/μg protein. In

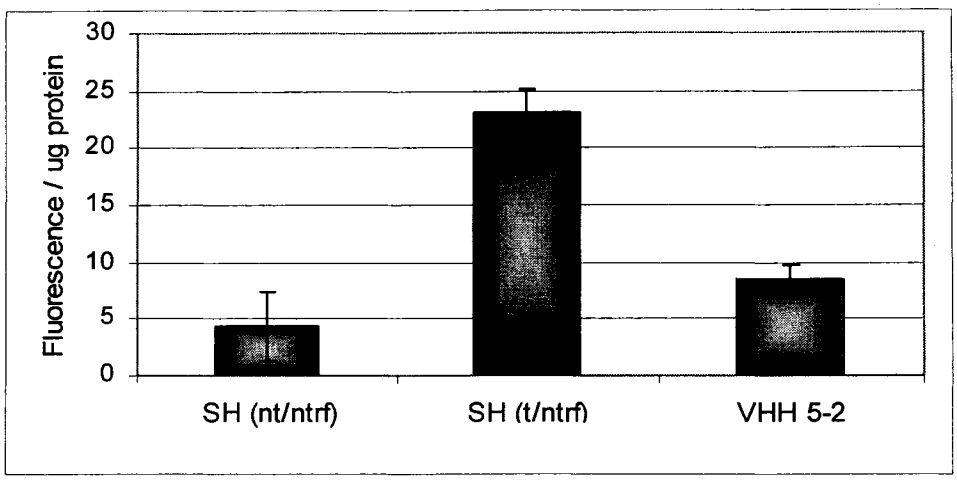

Figure 4.16 Caspase 3/7 activation is inhibited by presence of V_HH 5-2

Cells expressing the anti-Bax intrabody 5-2 show significantly lower Caspase 3/7 activation compared to treated/non-transfected cells (t/ntrf) 6h after oxidative stress induced by 100μM H_2O_2 for 1h ($p<0.05$). In this study was carried out using a Caspase 3/7 HTS Assay as described in Methods. In this assay an increase in fluorescence is indicative of caspases (3/7) activation, since these active proteases are able to release the quenched fluorescent dye linked to caspase substrate (DEVD). In fact the V_HH 5-2 expressing cells behave similar to non-treated/non-transfected cells (nt/ntrf), as these two groups were not statistically different ($p>0.05$).

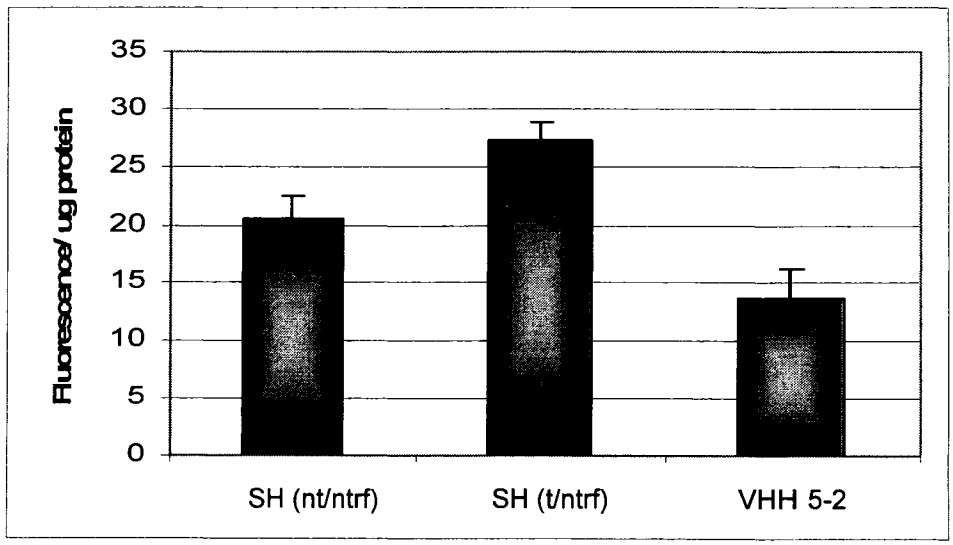

Figure 4.17 Caspase 7-9 activation inhibited in presence of V_HH 5-2

Caspase 7-9 activation was monitored in cells 6h after oxidative stress (100μM H_2O_2 for 1h) using the Caspase component of the MitoCasp Assay. This assay fluorescently labled Caspase 7-9 inhibitors which accumulate in cells where these proteases are activated. Cells expressing the anti-Bax intrabody 5-2 show significantly lower Caspase 7-9 activation compared to non-transfected cells (t/ntrf) ($p<0.05$).

each experiment, the value for the treated/non-transfected cells was taken as 100% and all other values were calculated with respect to this control and represented as a percent lipid peroxidation.

Cells expressing the anti-Bax intrabodies (and exposed to oxidative stress) showed limited lipid peroxidation comparable to the non-treated/non-transfected SHSY-5Y since the peroxidation levels of these groups were not determined to be statistically different ($p>0.05$), further confirming the ability of these intrabodies to prevent oxidative stress induced apoptosis, by inhibiting Bax function and its effect on the mitochondria, including the elevation of ROS.

4.4.2.8.1 Effect of anti-Bax intrabodies on cells treated with 200μM H_2O_2

Cell lines expressing each of the six anti-Bax intrabodies showed strong resistance to apoptosis following oxidative stress induced by hypoxia/hypoglycemia (12h) and 100μM H_2O_2 (1h), which was not exhibited by any of the control cell lines (to the same extent). However, using these levels of oxidative stress, it was difficult to distinguish the most efficient intrabody; therefore cells were exposed to 200μM H_2O_2 (1h) treatment. Cells viabilities (Fig. 4.19) were calculated 24h after treatment, as previously described by counting healthy vs. apoptotic nuclei stained with Hoechst Reagent (Fig. 4.20). Non-treated/non-transfected SHSY-5Y (nt/ntrf) were once again used as a negative control, while all positive control and experiment cell lines (as previously described) were exposed to the treatment. In nt/ntrf cells a 96% viability was seen, which was close to the viability detected for cell lines expressing V_HH 1,2,3 and 5-2 (86%, 87%, 89% and 90% respectively). Cell lines expressing intrabody 4 and 5-1

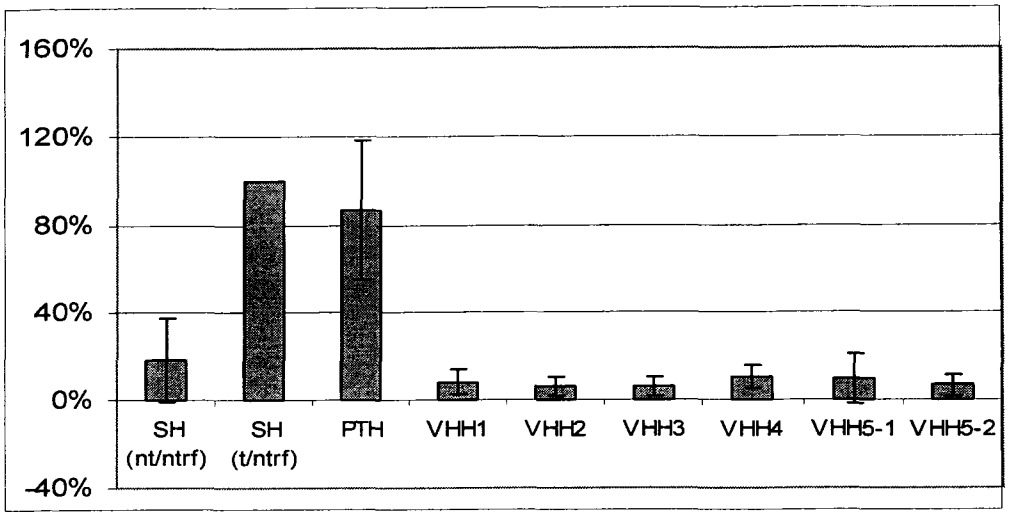

Figure 4.18 Lipid peroxidation reduced by anti-Bax intrabodies following 100μM H_2O_2 treatment

Cells were treated using 100μM H_2O_2 for 1h and monitored for lipid peroxidation following 24h. The presence of all six anti-Bax intrabodies significantly reduces the level of lipid peroxidation as these cells behave similar to the non-treated/non-transfected control SHSY-5Y ($p>0.05$, indicating no statistical difference between the nt/ntrf and all V_HH expressing cells). Lipid peroxidation was not significantly reduced in cells expressing the non-specific V_HH (PTH) in comparison to the t/ntrf cells ($p>0.05$).

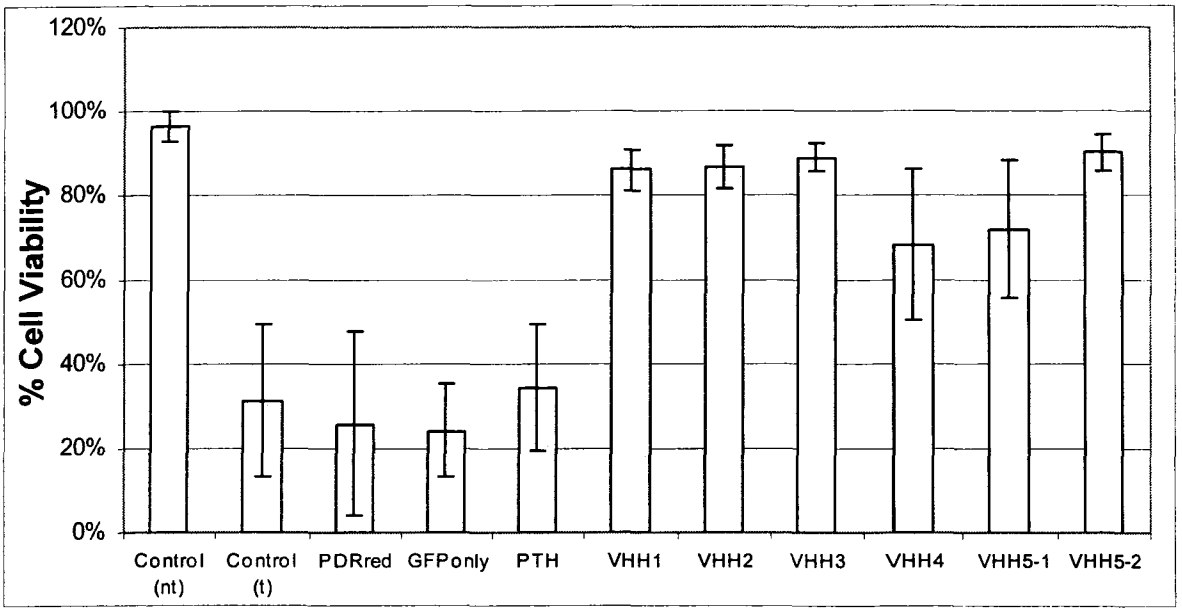

Figure 4.19 Cell viability detected following 200μM H_2O_2

Cells expressing anti-Bax V_HH show strong resistance to apoptosis. V_HH 5-2 proved to be the most efficient at resisting this stress level, exhibiting 90% cell viability (96% shown for non-treated/non-transfected cells). V_HH 4 and 5-1 (68% and 70% respectively) were least efficient of all the anti-Bax intrabodies at resisting apoptosis and yet still showed more promising results than all the positive control cell lines with viabilities less than 35%. The cell viability rate for all cells expressing anti-Bax intrabodies was found to be significantly higher when compared to each treated control ($p<0.05$).

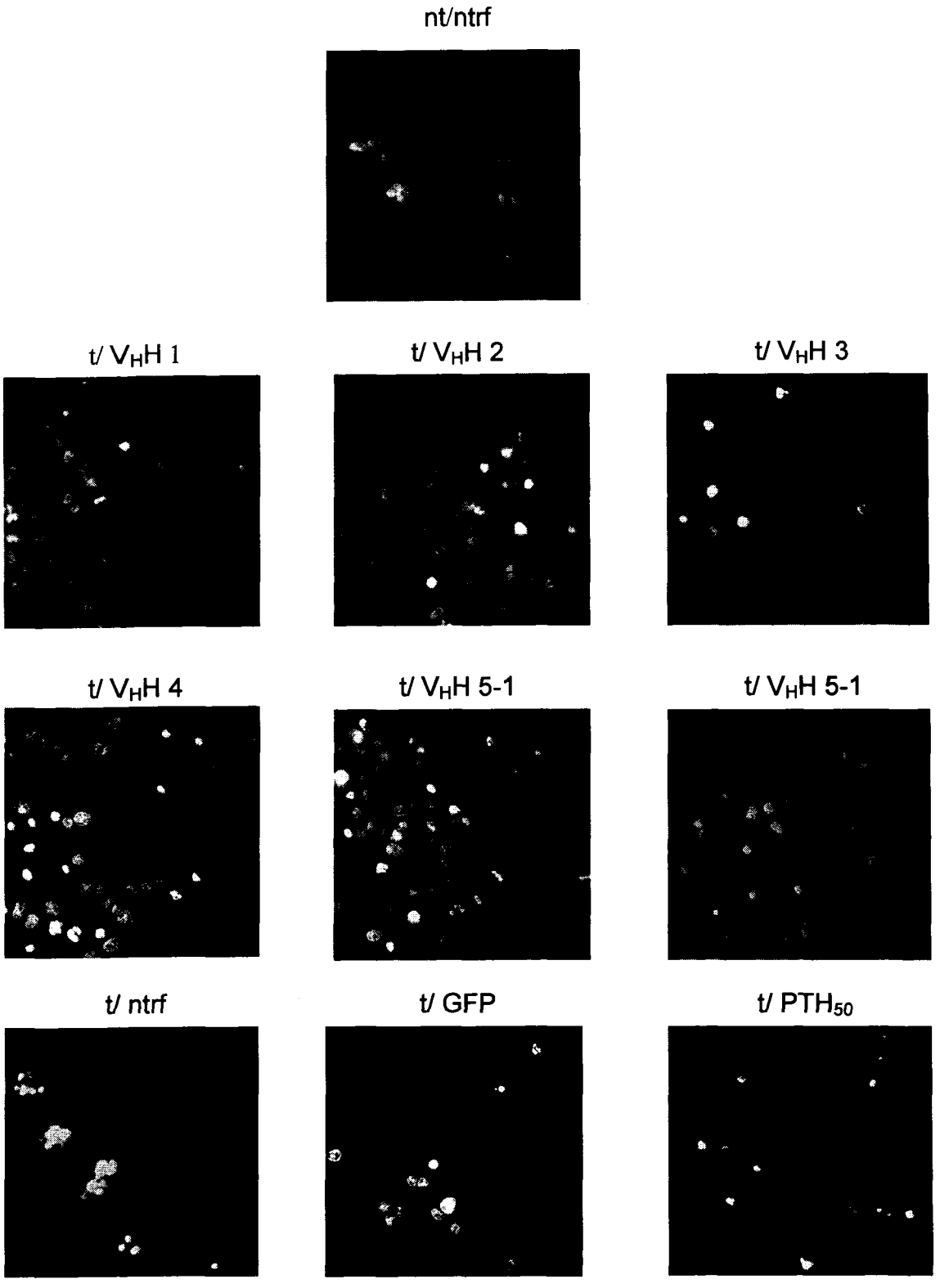

Figure 4.20 Nuclear morphology following 200μM H_2O_2 (1h) treatment

Cells expressing the anti-Bax intrabodies show only few apoptotic nuclei in each field shown following Hoechst staining 24h after oxidative stress (200μM H_2O_2 (1h)). Contrastingly the positive control cell lines (t/ntrf, t/GFP, t/PTH_{50}) show a substantial proportion of apoptotic nuclei. At this time point many of these cells were floating (completely dead) and thus not visualized in the images selected.

showed a decrease in viability (68%, 70% respectively) however, this was still an improvement over the positive control cell lines. Cells which were not-transfected (t/ntrf) and those expressing only GFP (t/GFP), RFP (t/pDs-Red) or a non-specific intrabody (t/PTH_{50}) showed poor cell viability rates (which were all significantly lower that the anti-Bax V_HH expressing cells, $p<0.05$) following this extreme level oxidative stress (32%, 26%, 24%, 35% respectively). Interestingly, these results are in general agreement with the *in vitro* ROS data presented earlier, confirming that these specific anti-Bax intrabodies can bind and inhibit Bax activity, thereby protecting the mitochondria and cell death.

4.4.2.9 MMP following 200μM H_2O_2 treatment

JC-1 staining was also used to monitor the mitochondrial membrane potential in cells 24h after exposure to high levels of oxidative stress using 200μM H_2O_2 (1h) (Fig.4.21). As expected, cells expressing the anti-Bax intrabodies exhibited red fluorescence indicating the presence of healthy, non-permealized mitochondria (V_HH 1, 3 and 5-2 shown here), similar to the non- treated /non-transfected control cells shown. Furthermore, non-transfected cells (which did undergo the described treatment) were not resistant to mitochondrial permealization and thus show no punctate red fluorescence. The respective nuclei for each JC-1 image are also shown to confirm the number of cells in each field and to show the healthy nuclei of V_HH transfected cells.

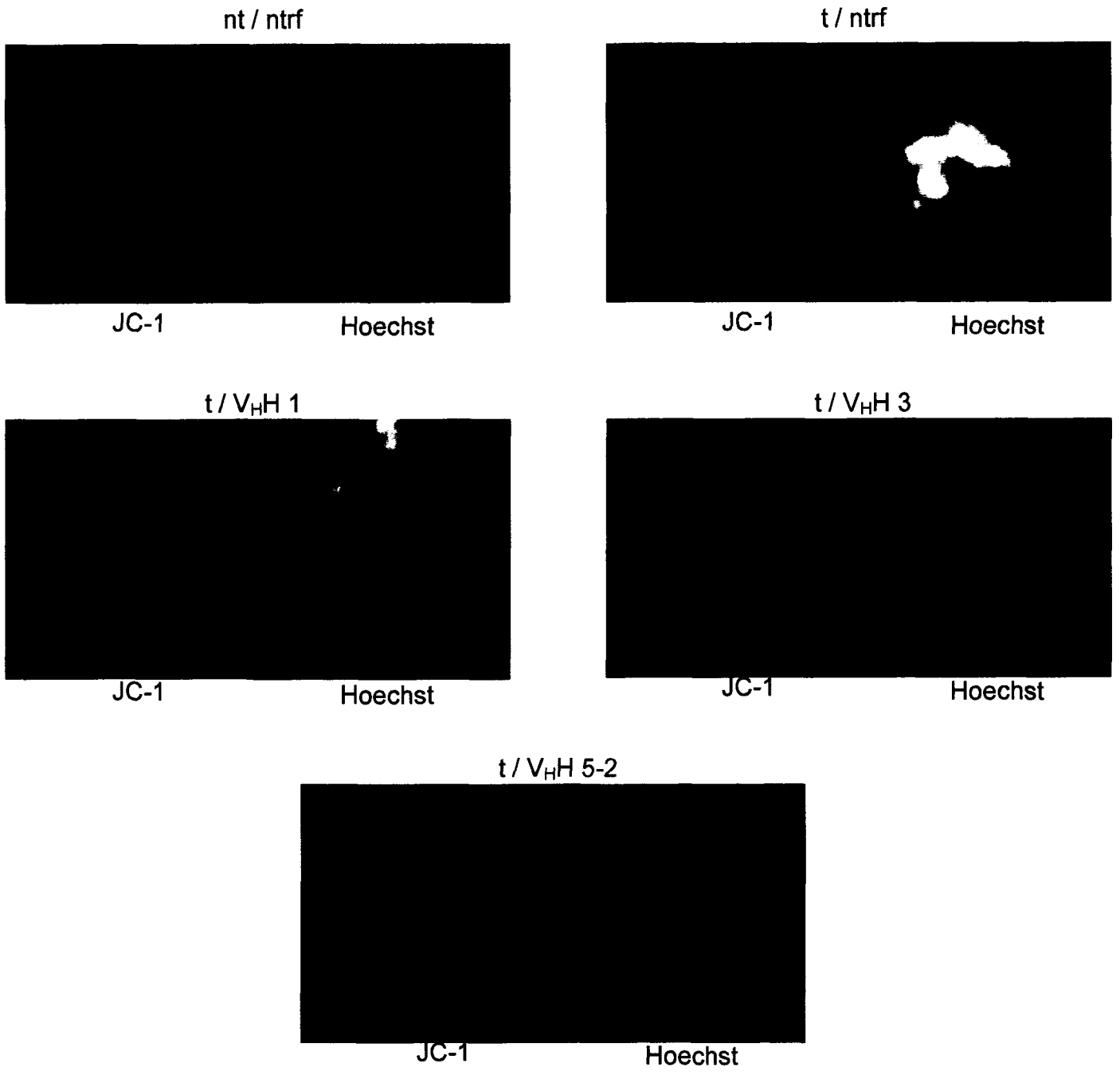

Figure 4.21 Monitoring MMP through JC-1 staining

Following oxidative stress treatment using 200μM H_2O_2 (1h), red JC-1 aggregates are detected in cells expressing anti-Bax intrabodies (1, 3, 5-2 shown) indicating healthy mitochondria in these cells similar to the non-treated/non-transfected controls (nt/ntrf). In cells not expressing any V_HHs, mitochondrial destabilization occurs after the described treatment an no punctuate red fluorescence is detected. Complimentary Hoechst pictures are also given, showing healthy nuclei in each field of anti-Bax expressing cells.

4.4.2.10 Cells protected by anti-Bax V_HH following 200μM H_2O_2 treatment proliferate at a normal rate

Cells expressing the anti-Bax intrabodies showed strong resistance to apoptosis even after exposure to very high levels of H_2O_2 (200μM), as shown through cellular staining techniques and low levels of caspase activation. To ensure that this resistance was not at the expense of various other cellular mechanisms (e.g. cell division), we monitored these cells following treatment for 10 days. Cells expressing anti-Bax intrabodies were treated with 200μM H_2O_2 (1), while non-transfected SHSY-5Y were treated with only 100 μM H_2O_2 (since no growth was detected following the higher level of stress). After 48h the cells were trypsinized and counted using Trypan Blue (as described in Methods). This step was repeated after 5 and 10 days in order to record a growth curve for each cell line. All cell lines showed similar growth curves, and V_HH5-2 is shown in Figure 4.22, along with the growth curve for treated/non-transfected control cells.

Normally, SHSY-5Y populations double approximately every 48h, however the V_HH expressing cells were not counted more frequently (e.g. every other day) since the growth may be negatively affected by the frequent exposure to trypsin. Cells expressing V_HH 5-2 are shown to undergo just under two cycles of division from day 5 to day 10, which is in general agreement with the doubling rate of untreated SHSY-5Y (Fig. 4.22). At this level of oxidative stress, any surviving non-transfected SHSY-5Y at 24h after treatment did not survive following replating.

4.4.2.11 Stable cell lines of ant-Bax V_HHs in absence of marker proteins (GFP/RFP)

Stable cell lines using the anti-Bax V_HH without the presence of fluorescent proteins (GFP or RPF) were also created to ensure that the protective properties offered by these intrabodies were not caused by the possible interaction of the V_HH with the marker proteins. We were able to successfully transfect and select all V_HHs except V_HH4 due to a suspected problem with the antibiotic resistance gene. However, the remaining five stable cell lines expressing the anti-Bax intrabodies, showed strong resistance to 200μM H_2O_2 (1h), and in the case of V_HH 5-1, we actually detected an increase in cell viability percentage proving that there is no advantage to the presence of a marker protein (Fig. 4.23). The cell viability rates of cells transfected in anti-Bax intrabodies alone, were significantly higher than all control treated cell lines ($p<0.05$).

4.4.2.12 Anti-Bax intrabodies show limited protection against serum deprivation

The specificity of the anti-Bax intrabodies was tested by depriving cell (expressing the V_HHs) from serum in the media. Serum derivation causes cell death due to the absence of numerous essential growth factors required to sustain a healthy cell, which are not directly associated with the Bax associated apoptosis pathway. The specificity of the anti-Bax intrabodies to protect against only Bax associated apoptosis was tested by serum deprivation of the transfected cells. The cells were incubated in serum free media for 24h and 72h after which point nuclear morphology was monitored using Hoechst reagent (Fig.4.24). After 72h the transfected cells were undistinguishable

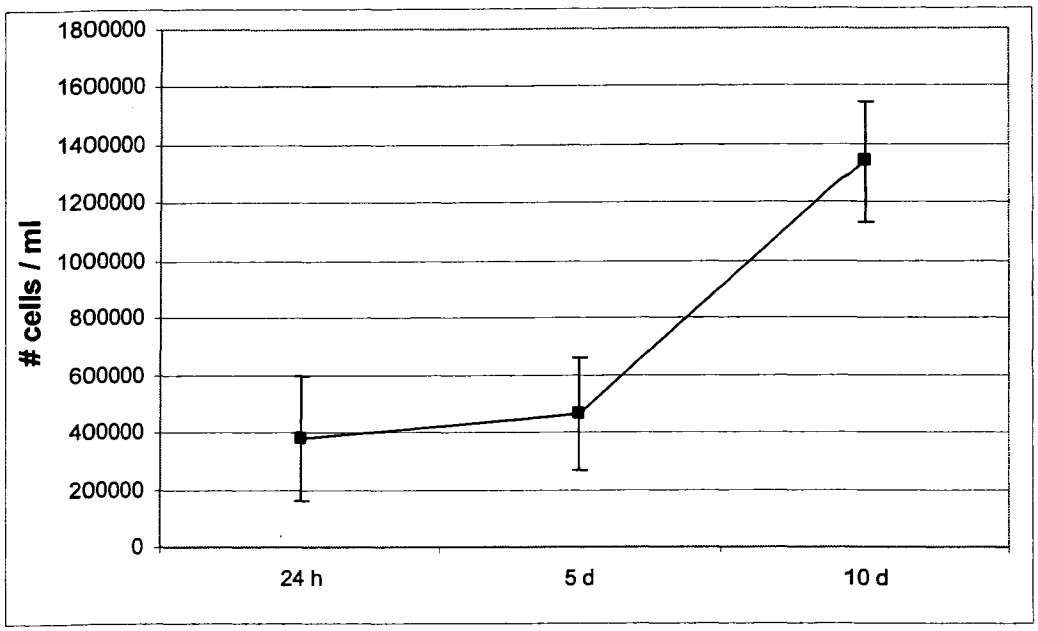

Figure 4.22 Cell division is not affected following oxidative stress in cells expressing anti-Bax V_HHs

Cell division was monitored by counting live cells (vs.dead dead cells) using Trypan Blue, starting at 24h after 200μM H_2O_2 (1h) treatment in cells expressing the anti-Bax intrabodies (V_HH 5-2 shown here). Cells expressing V_HH 5-2 underwent just under two division cycles from 5d to 10d, which is in general agreement with the doubling rate of SHSY-5Y.

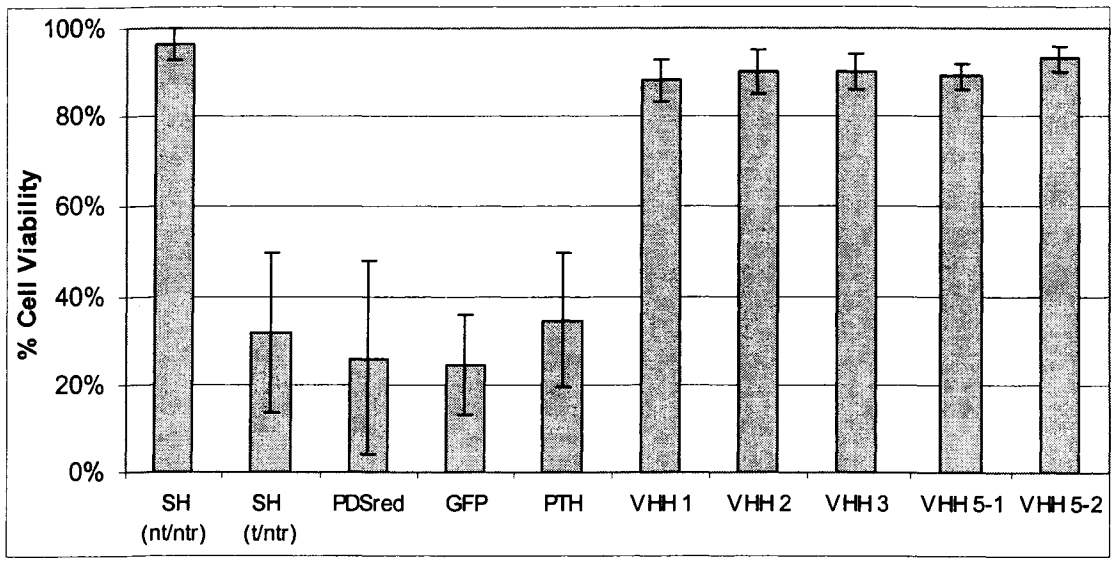

Figure 4.23 Fusion proteins (GFP or RFP) do not offer advantage in protection from oxidative stress

Cells transfected with anti-Bax V_HHs without any fluorescent marker proteins showed strong resistance to apoptosis 24h after intense oxidative stress (200μM H_2O_2 for 1h). In fact for V_HH 5-1 we detected an increase in cell viability when the intrabody was expressed without a marker protein. Overall, the cell viability of V_HH transfected cells was found to be significantly higher than all the control treated cells ($p<0.05$).

from the treated/non-transfected cell lines ($p>0.05$). This indicated that the anti-Bax intrabodies exhibit their effect specifically on the Bax induced apoptosis pathway.

4.4.2.13 Preliminary investigation for the possible interaction between anti-Bax V_HHs and VDAC

Since the anti-bax V_HH studied here were able to protect the mitochondria from Bax associated permealization, we wanted to understand if the intrabodies were possibly binding to Bax at its VDAC interaction site, thus preventing the Bax induced opening of the mitochondrial PTP. Using both V_HH 5-2 expressing cells and non-transfected SHSY-5Y, we performed an immunoprecipitation using anti-VDAC antibodies and protein G-Sepharose-beads (as described in Methods). From previous work in our laboratory and other published works (Adachi M *et al.*, 2004), Bax is understood to interact with VDAC and can be co-precipitated using anti-VDAC antibody attached to protein G-Sepharose resin. Using this information, we hypothesized that if the anti-Bax intrabodies were interacting with Bax at the VDAC binding site, the presence of the intrabodies would prevent Bax-VDAC interaction which can be monitored through immunoprecipitation followed by SDS-PAGE and Western Blot. After performing this experiment three times, a decrease in Bax was shown, for fractions extracted from cells expressing anti-Bax V_HHs, compared to non-transfected SHSY-5Y fractions (Fig. 4.25). From this we suspect that the intrabodies may be preventing the Bax-VDAC interaction resulting in the inhibition of mitochondrial destabilization which has been shown in works throughout this study. However, since the ratio of anti-Bax V_HH to Bax expressed in the transfected

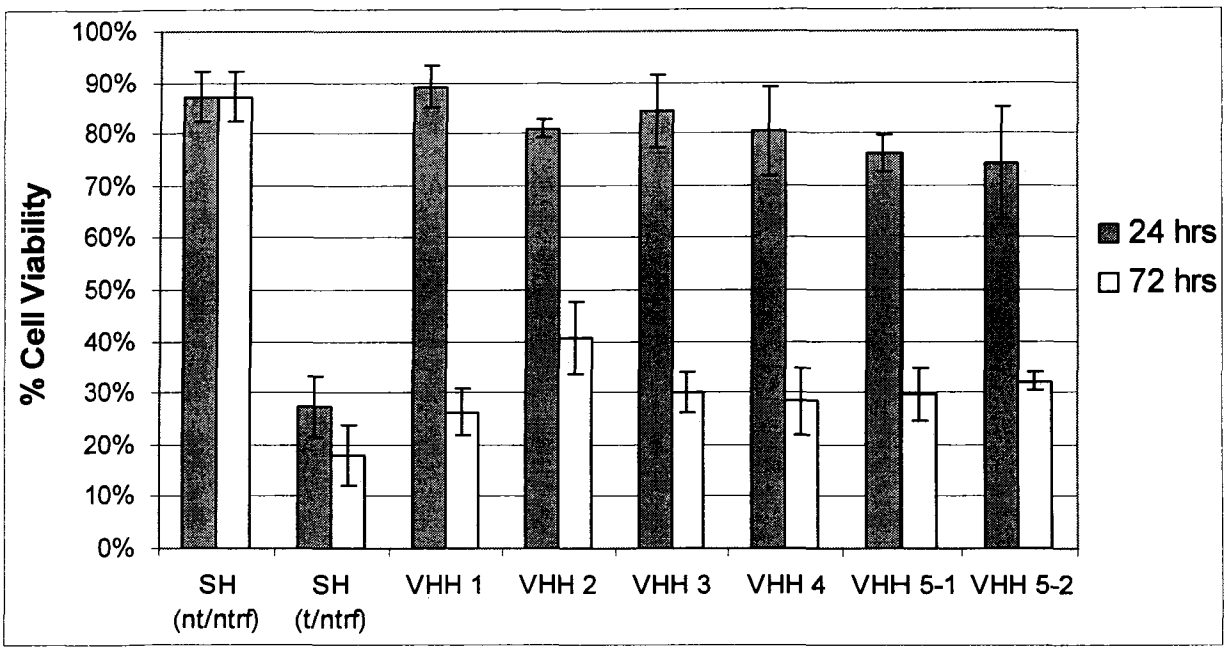

Figure 4.24 Anti-Bax V_HHs offer limited protection against serum deprivation

The specificity of the anti-Bax intrabodies to only protect against Bax associated apoptosis (e.g. oxidative stress induced) was tested by depriving cells from serum. Cells expressing the V_HHs showed no significant protection from this treatment after 72h indicating that their activity is Bax specific ($p>0.05$ at 72h for all V_HH transfected cells compared to t/ntrf).

cell lines is unknown, this experiment may provide more conclusive results if we were to incubate a known quantity of anti-Bax single domain antibody and recombinant Bax in presence of isolated mitochondria.

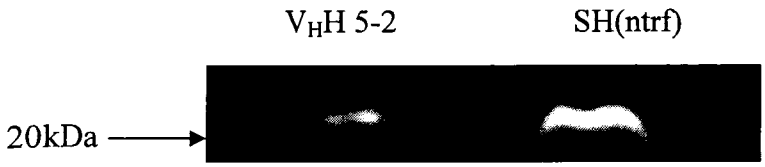

Figure 4.25 Anti-Bax V_HH 5-2 limits Bax – VDAC interaction *in vivo*

Bax proteins was co-precipitated with VDAC in cells expressing V_HH 5-2 and non-transfected SHSY-5Y (SH(ntrf)) using anti-VDAC linked to Proten G-Sepharose beads and resolved using SDS-PAGE and Western Blot. Approximately 60μg of mitochondrial protein loaded for each fraction and the blot was probed with monoclonal anti-Bax primary antibody. In mitochondrial fractions isolated from cells expressing V_HH 5-2, a decrease in Bax co-precipitation was seen (compared to non-transfected fractions) indicating that the intrabodies may be protecting the mitochondria from permealization by preventing the interaction of Bax and VDAC, which are associated with the permealization of the outer mitochondrial membrane and subsequent damage to this organelle.

Chapter 5: Discussion

In this study we have identified six unique single domain (V_HH) antibodies against the pro-apoptotic protein Bax. When expressed intracellularly these intrabodies did not show any toxicity to the host cells, and rendered the cells resistant to damage from intense oxidative stress, by blocking Bax function. These intrabodies were able to protect cells from oxidative stress induced-apoptosis by inhibiting a key protein in this pathway, Bax. Therefore, the anti-Bax intrabodies have important potential as models for identifying chemical blockers which can mimic the action of the V_HHs described here, for possible treatment of neurodegenerative diseases which involve Bax associated oxidative stress induced-cell death.

5.1 Targeting Bax as a means to inhibit oxidative stress induced apoptosis

In order to prevent oxidative stress induced-cell death in post-mitotic cells, we chose to target Bax protein due to its central role and its ability to behave as a check point in this cell death pathway (Korsmeyer, S *et al.*, 1998). Specifically, Bax is understood to translocate from the cytosol to the MOM where it likely associates with VDAC a component of the mitochondrial permeability transition pore, causing the opening of the mitochondrial outer membrane (MOM) and release of various pro-apoptotic proteins such as cytochrome *c*, which lead to subsequent cell death. Bax is understood to play an early role in the intrinsic apoptosis pathway and thus an important target for efficiently inhibiting this process in post-mitotic cells.

The increase in Bax activity in the presence of reactive oxygen species also makes this protein a valuable target for elucidating treatments for neurodegenerative diseases,

which involve inappropriate induction of apoptosis due to oxidative damage. Specifically, over-expression of Bax is shown in several neurodegenerative diseases such as stroke and Alzheimer's Disease (AD). Specifically, the Bax associated intrinsic apoptosis pathway has been directly linked to neuronal cell death following ischemia/reperfusion injury. Furthermore, by inhibiting Bax function (*in vitro*) using Bax blockers such as the anti-apoptotic drug furosemide, neuronal cell death was shown to be significantly reduced (Chi-Hsin, L *et al.*, 2005). In addition, elevated levels of Bax have also been shown to co-localize with amyloid–beta plaques, which are believed to contribute to AD progression and neuronal cell death through an apoptotic mechanism involving Bax (MacGibbon, G *et al.*, 1997). Therefore, the central role of Bax in the induction of neuronal cell death makes it an important target for protecting these post-mitotic cells from apoptosis in the treatment of neurodegenerative diseases.

5.2 Using single domain antibodies to inhibit Bax activity

Single domain antibodies (V_HH) are derived from llama species which possess only heavy chain antibodies. These antibodies are particularly advantageous for *in vivo* studies, compared to small antibody fragments derived from conventional human IgGs (scFvs), due to their smaller size and greater solubility. By isolating and amplifying only the specific antigen binding regions, we can select low molecular weight peptide fragment with very specific and strong binding affinities for an antigen of interest, such as Bax. In a llama phage display library, the specific antibody gene against almost any potential antigen (isolated from the genome of naïve llama lymphocytes) is individually cloned into a phage which expresses the anti-body of choice on its surface. Therefore

through a panning assay we can both isolate the specific V_HH for any antigen and more importantly its gene. Overall, through a panning technique we have isolated six unique single domain antibodies and their genes. This allowed us to study these sdAbs in various ways: *in vitro* to test their efficacy at preventing Bax induced mitochondrial permealization and *in vivo* through transfection, thereby expressing the V_HHs as intrabodies to ensure that they can prevent oxidative stress induced apoptosis while remaining non-toxic to the host cell.

5.3 Specificity and efficacy of anti-Bax V_HHs in preventing Bax function

Though the exact mechanism of Bax induced mitochondrial membrane permealization is still debated, it is well documented that the presence of Bax does indeed cause mitochondrial membrane potential (MMP) collapse resulting in generation and release of more ROS and other pro-apoptotic proteins such as cytochrome *c* (from the inner membrane space (IMS)) (Korsmeyer, S *et al.*, 1998; Brenner, C, 2000; Halestrap, A, 2002; Adachi M *et al.*, 2004). By incubating isolated mitochondria in presence of anti-Bax V_HHs and Bax, we have shown the ability of these antibodies to protect the mitochondria by both decreasing ROS generation and limiting the release of cytochrome *c* from the IMS. When incubated with mitochondria alone (in absence of Bax) the anti-Bax V_HHs showed even lower ROS production than fractions containing mitochondria in buffer (in absence of Bax and sdAbs). This suggests that the anti-Bax V_HHs are possibly inhibiting endogenous Bax expressed in SHSY-5Y which may be present in the isolated mitochondrial fraction. A decrease in ROS was not detected in fractions containing a non-specific single domain antibody (raised against caspase 3), indicating that the anti-

Bax sdAbs were able to both bind to Bax and more importantly inhibits its destructive function on the mitochondria. Since, Bax induced MOMP was limited in presence of anti-Bax V_HHs, it is likely that these antibodies may be interacting with the transmembrane (α 9) region of Bax and thereby preventing its interaction with the MOM which can lead to elevation in ROS production and increased cytochrome *c* release.

By using a llama phage display library we were able to select six sdAbs and their respective genes. By inserting each of the genes into a mammalian expression vector, we were able to analyze the activity of the antibodies inside pre-neuronal cells through transient and stable transfection. This investigation was particularly important since the intracellular environment is substantially different than working in the more controlled *in vitro* conditions.

Transient transfection of each of the six V_HHs provided important results showing the potential of the V_HHs to block Bax activity and apoptosis due to oxidative stress. After treatment with 100μM H_2O_2 (1h), which was shown to induce approximately 50% cell death in non-transfected SHSY-5Y, positively transfected cells showed good resistance to this treatment. This was indicated by both Hoechst staining (showing healthy, non-condensed nuclei) and JC-1 staining (showing strong red fluorescence indicative of healthy mitochondrial membrane potential).

Although we were encouraged by the findings of the transient transfection work, in order to provide more conclusive and quantified results we created stable cell lines expressing each of the six anti-Bax V_HHs and three control stable cell lines expressing the fluorescent marker proteins (GFP and RFP alone) and a non-specific V_HH (PTH_{50}).

Cells expressing the anti-Bax V_HHs showed strong apoptosis resistance when exposed to various levels of oxidative stress, including hypoxia/hypoglycemia (12h), 100μM and 200μM H_2O_2 (1h) treatments. These results were in general agreement with the *in vitro* ROS results, with the exception of anti-Bax V_HH 1 and 2 which were not as efficient at decreasing ROS production, but still showed comparable cell viabilities when expressed *in vivo*. This is not very surprising since the extracellular and intracellular environments are quite distinct. Specifically, it is possible that when expressed in the mammalian neuroblastoma cells, intrabodies 1 and 2 may experience some conformational modification in their quaternary structure increasing their ability to bind Bax. In addition, although anti-Bax V_HH 1 and 2 may bind Bax less efficiently *in vitro*, it is possible that these intrabodies may be weakly interacting with multiple pro-apoptotic Bcl-2 proteins (e.g. Bax and Bak), rendering their host cells more resistant to apoptosis. Alternatively, the Bax binding efficacy of intrabodies 3 and 5-2 can be slightly reduced *in vivo* due to interference of various cytosolic proteins, thereby resulting in cell viability rates comparable to the less efficient anti-Bax V_HH 1 and 2. Anti-Bax V_HH 4 and 5-1 exhibited lower poteintial for Bax inhibition in both environments as demonstrated by higher ROS production and lower cell viability (compared to the remaining four anti-Bax V_HH clones) *in vitro* and *in vivo*, respectively.

Following these treatments, the nuclei of anti-Bax V_HH transfected cells showed few brightly stained condensing nuclei (indicating apoptotic cells) even after the harsh 200μM H_2O_2 treatment, with V_HH 1,2,3 and 5-2 exhibiting cell viabilities close to non-treated/non-transfected cells. In contrast, the control cell lines showed poor cell viability

rates under the same conditions, indicating that the protection offered by the anti-Bax intrabodies did not result from non-specific interactions.

These results were further confirmed using other cellular staining techniques including Annexin V and JC-1. Annexin V is a specific marker for early phase apoptosis, which measures plasma membrane flipping. Specifically Annexin V binds to phosphatidyl serine complexes which translocate from the inner to the outer leaflet of the plasma membrane, and are detected by a fluorescent ring formation around cells in early phase apoptosis. Limited Annexin V binding was detected in cells expressing the anti-Bax intrabodies, exposed to oxidative stress (100μM H_2O_2 (1h)), unlike the control cell lines which exhibited strong Annexin V binding after the above treatment.

JC-1 staining was also used to confirm the positive effect of the anti-Bax intrabodies following oxidative stress (100μM and 200μM H_2O_2 (1h)), by monitoring the mitochondrial membrane potential (MMP) of the experimental and control cell lines. In healthy cells, JC-1 aggregates in the mitochondria and fluoresces red, while the dye remaining in the more dilute cytosol environment fluoresces green. In apoptotic cells, where the mitochondria have been damaged, JC-1 cannot accumulate in this organelle and thus remains in the cytosol where only the diffuse green fluorescence is detected. As expected, cells expressing the anti-Bax intrabodies exhibited healthy mitochondria indicated by strong red fluorescence, not detected in treated/non-transfected cell lines.

In addition, caspase activation a downstream consequence of mitochondrial destabilization was also shown to be inhibited in cells expressing anti-Bax intrabodies. Low levels of activation of both executioner caspases (8 and 9) and executioner caspases (3 and 7) were shown in the anti-Bax V_HH expressing cells, after oxidative stress (100μM

H_2O_2 (1h) or hypoxia/hypoglycemia (12h)), while control non-transfected cells showed a doubling of the activation levels of both types of caspases after the same treatments.

Furthermore, we have also been able to show that the protection from oxidative stress offered to cells was solely due to the presence of the specific anti-Bax V_HHs. This was proven by showing that cells expressing only fluorescent proteins (GFP or RFP) or a non-specific V_HH (PTH_{50}) showed poor survival rates following oxidative stress which had little effect on the anti-Bax V_HH transfected cells. Finally, when stable cell lines were formed without the fluorescent marker proteins, the cell viability rates remained high and even increased for cells expressing V_HH 5-1, which indicated that only the anti-Bax V_HHs are responsible for the resistance to oxidative damage, both *in vivo* and *in vitro*.

5.4 Anti-Bax V_HH are specific inhibitors of the Bax associated apoptosis pathway

The specificity of the anti-Bax intrabodies against Bax associated apoptosis was tested using serum deprivation treatment for up to 72h. Cells transfected with anti-Bax intrabodies showed resistance to apoptosis after 24h, however this protective effect was decreased significantly after 72h, at which point the anti-bax V_HHs were rendered ineffective. The minimal protection offered by the anti-Bax intrabodies can be explained since the mechanism of serum deprivation induced cell death is not completely Bax dependent.

Although the exact mechanism leading to apoptosis induced through serum deprivation is under debate, it is believed that components of both the intrinsic and extrinsic apoptosis pathway may be involved. One consequence of serum deprivation is

the inactivation of cAMP-dependent protein kinase (protein kinase A, PKA) which is responsible for proper regulation of numerous cell pathways, including but not limited to glycogen synthesis and breakdown, glycolysis, DNA condensation (Huang, N *et al.*, 2001). In addition, the elimination of necessary growth factors such as IL-3, which normally stimulates the phosphorylation and inactivation of Bad (a pro-apoptotic member of the Bcl-2 family) also plays a role. In its phosphorylated form Bad is rendered inactive and cannot heterodimerize with Bcl-X_L, an anti-apoptotic member of the Bcl-2 family (Zha, J *et al.*, 1996), resulting in cell survival. Absence of IL-3 during serum deprivation prevents Bad phosphorylation, allowing it to interact and inhibit the anti-apoptotic function of Bcl-X_L, resulting in apoptosis. This can be further aggravated by presence of Bax which has also been shown to interact with multiple anti-apoptotic proteins including Bcl-X_L (Sattler, M *et al.*, 1997).

In a more resent study by Charles *et al.* 2005, serum deprivation was associated with the increase in Bax expression and decrease in Bcl-2 expression, which leads to the induction of apoptosis (Charles, I *et al.*, 2005). However, unlike the intrinsic apoptosis pathway in which Bax seems to play a central role, other factors must also be taken into account in serum deprivation. Another factor leading to apoptosis through serum deprivation is activation of caspase 8 which is independent of the mitochondrial and Bax associated cell death pathway (Akihiro, H *et al.*, 2006).

Therefore, by inhibiting Bax activity, anti-Bax intrabodies can maintain strong cell viabilities 24h following serum deprivation, however this effect is overwhelmed by other cellular process such as caspase 8 activation, whose expression is reported to increase after 72hrs in cells undergoing serum deprivation.

5.1 Elucidation of possible binding site for anti-Bax V_HHs

Bax permealization of the outer mitochondrial membrane (OMM) has been shown to occur through the formation of the permeability transition pore (PTP) when Bax binds to the MOM component of the PTP, VDAC (Narita, M *et al.*, 1998). Since mitochondria membrane potential was not destroyed in cells expressing the anti-Bax intrabodies following oxidative stress, we believed that the Bax-VDAC interaction may be prevented in these cells. This theory was tested by comparing the level of Bax which is co-precipitated with VDAC in control (non-transfected) and anti-Bax V_HH expressing cells. We showed that the level of Bax binding to VDAC was indeed decreased in the transfected cells, which suggests that the antibodies may be binding to the VDAC binding site on Bax (specifically the transmembrane, α9 helix). However, since the level of expression of the anti-Bax intrabodies is not known, we plan to retest this theory using anti-Bax single domain antibodies and recombinant Bax (*in vitro*).

Due to the strong similarities in all Bcl-2 type proteins, we cannot eliminate the possibility that the anti-Bax V_HH may also be binding to other Bcl-2 type proteins. The intrinsic apoptosis pathway is regulated by the pro- and anti-apoptotic members of the Bcl-2 family. Specifically, a major determining factor for induction of apoptosis is the change in the ratio between these protein groups. Therefore if, we limit the activity of pro-apoptotic proteins such as Bax, the ratio will shift in favour of the anti-apoptotic proteins, resulting in inhibition of apoptosis as seen in cells expressing the anti-Bax intrabodies. If the intrabodies also inhibited the anti-apoptotic Bcl-2 proteins, this ratio would not be affected, and we would then see no major change in cell viabilities compared to the treated/non-transfected cells. Since this effect was not seen, this

suggests that the anti-Bax intrabodies are probably not functionally interfering with the activity of the anti-apoptotic Bcl-2 type proteins.

Finally, due to the functional redundancy of pro-apoptotic members of the Bcl-2 family, specifically Bax and Bak it is possible that the intrabodies are binding to common sites such as the BH3 domain (which is inaccessible in anti-apoptotic Bcl-2 type proteins), thereby preventing dimerization and subsequent activation of these proteins.

Chapter 6: Conclusions & Future Work

In this study we have identified several non-toxic, V_HH clones which have be identified for their ability to bind recombinant Bax specifically. Through *in* vitro studies involving direct Bax induced MOMP, (characterized by increased ROS) these antibodies exhibited potential to both bind and more importantly inhibit Bax function, thereby limiting mitochondrial damage brought on by this pro-apoptotic protein. In addition when these V_HHs were expressed intracellularly, as intrabodies, they rendered their host cells resistant to oxidative-stress-induced apoptosis, by preventing the Bax permealization of the mitochondria (during oxidative stress).

This work has important implication for studying cellular mechanisms *in vivo*, by phenotypically inhibiting protein functions. Although, protein inhibition at the mRNA level, through RNAi, is a common means to study protein functions, intrabodies against specific intracellular targets may offer greater precision and feasibility, since they can be designed to target specific components of a particular protein of interest.

In order to determine how the anti-Bax V_HHs inhibit Bax activity, it would be important to further confirm their ability to bind on the VDAC site of Bax (the transmembrane c-terminus, α9). In addition it would also be useful to identify other proteins which may be interacting with the anti-Bax V_HHs. This can be accomplished by carrying out an immunoprecipitation assay using anti-GFP bound to protein-G linked sepharose, since the anti-Bax intrabodies are expressed in fusion with GFP. Any proteins in addition to Bax which may be interacting with the anti-bax intabodies can then be further analyzed and identified through mass spectroscopy.

In addition this work also opens new opportunities for treating neurodegenerative diseases which involve cell death induced by oxidative stress and Bax activation. Beside their direct use as intrabodies in the context of gene therapy, the present V_HHs can also be used as models to identify specific and non-toxic small molecular weight inhibitors of Bax from commercially available pharmacophore libraries.

The application of the anti-bax intrabodies as direct therapeutic agents against neurodegenerative diseases is limited largely by the difficulty in crossing the blood brain barrier. As a result a future goal is to use one of the more efficient V_HH (5-2), which has shown strong anti-Bax activity both *in vitro* and *in vivo*, as a model for identifying a small chemical compound. This chemical compound can be selected from a pharmacophore library for its ability to compete for the binding of Bax *in vitro*.

Specifically, a binding assay can be carried out using radiolabeled (C^{14}) V_HH bound to recombinant Bax. The pharmacophore library can be screened to identify a chemical which can competitively disrupt this interaction by binding at the same site on Bax as the V_HH. In this way a low molecular weight mimic of the V_HH can be identified which will be more likely to cross the tightly regulated blood brain barrier in order to prevent oxidative stress induced apoptosis. In addition, as opposed to gene therapy, by using a chemical compound the administration level (frequency and dosage) can be properly controlled to prevent any possibility of uncontrolled cell growth leading to cancer.

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