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Isolation and Functional Characterization of Single Domain Antibody Modulators of Caspase-3 and Apoptosis

Katrina McGonigal · Jamshid Tanha · Elitza Palazov · Shenghua Li · Deyzi Gueorguieva-Owens · Siyaram Pandey

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Abstract Apoptosis, or programmed cell death, is an essential process affecting homeostasis of cell growth, development, and the elimination of damaged or dangerous cells. Inappropriate cell death caused by oxidative stress has been implicated in the development of neurodegenerative diseases such as Alzheimer's, Parkinson's, and stroke. On the other hand, a defect in the cell death process leads to the development of cancer. For example, the main player of apoptosis, p53, is defective in many of the human cancers. Apoptosis is regulated by the interplay of pro-apoptotic and anti-apoptotic proteins from the Bcl-2 family and caspases. In particular, specific modulators of the activity of Caspase 3 could be very important for the development of therapies for diseases such as neurodegeneration and cancer. In this study, two V_HHs specific to Caspase 3 (VhhCasp31 and VhhCasp32) were isolated from a heavy chain antibody variable domain (V_HH) phage display library and tested for their apoptosis-modulating effects. While VhhCasp31 was found to be antagonistic towards Caspase 3, VhhCasp32 was agonistic. Furthermore, when expressed as intrabodies in SHSY-5Y neuroblastoma cells, VhhCasp31 rendered cells resistant to oxidative-stress-induced apoptosis, whereas VhhCasp32 resulted in apoptosis. These V_HH antagonist and agonist of apoptosis could have potential for the development of therapeutics for neurodegenerative diseases and cancer, respectively.

Keywords Caspase 3 · Apoptosis · V_HH · Intrabodies · Oxidative stress · Neurodegenerative Diseases · Cancer

K. McGonigal · D. Gueorguieva-Owens · S. Pandey (✉)
Department of Chemistry and Biochemistry, University of Windsor, Windsor, ON, Canada N9B 3P4
e-mail: spandey@uwindsor.ca

J. Tanha · S. Li
Institute for Biological Sciences, 100 Sussex Drive, National Research Council of Canada, Ottawa, ON, Canada K1A 0R6

E. Palazov
Oncology Division, Bureau of Metabolisms, Oncology and Reproductive Sciences, 101 Tunney's Pasture Driveway, Health Canada, Ottawa, ON, Canada K1A 0K9

Introduction

Apoptosis, or programmed cell death, is an important, crucial process in the normal development of an organism through establishment of tissue homeostasis and deletion of harmful or damaged cells. It is an active, physiological process that is characterized by specific morphological and biochemical changes that distinguish it from necrotic cell death [1]. Apoptosis occurs in response to conditions such as DNA damage, oxidative stress, deprivation of growth factors, and stimulation of specific death receptors, and is characterized by cell shrinkage, membrane blebbing, and chromatin condensation [2].

Important proteases involved in the apoptotic pathways are known as caspases. Caspases are cysteine aspartic acid proteases which are present in an inactive form in the cytoplasm of the cell until they are proteolytically cleaved to become activated. Caspases are synthesized in the cell as single-chain precursors and are activated by cleavage at the conserved Asp_{297} residue, where a conformational change occurs bringing it into the correct alignment for catalysis. Some caspases, e.g., Caspase 8/9, play an essential role in apoptosis as initiator proteins, by activating other caspases. Some others (Caspase 3/7) act as executioner proteases causing proteolysis of key proteins leading to DNA fragmentation and apoptosis. Caspase 3 in particular is one of the main executioner caspases of apoptosis and has been found to be activated in nearly every model of apoptosis with substrates including proteins involved in cell maintenance and/or repair. Specifically, the activity of caspase-activated deoxyribonuclease (CAD) is affected by the Caspase 3-mediated cleavage of the CAD inhibitor, ICAD [3].

Reactive oxygen species (ROS) such as superoxide anions, hydrogen and organic peroxides, and free radicals are produced in all aerobic cells as by-products of several metabolic reactions [4]. In particular, ROS are produced in the mitochondria by the partial reduction of molecular oxygen, where they play an important role as regulatory mediators in signalling processes [5]. Normally, these compounds are detoxified by the cells, but when produced in excessive amounts, the detoxification mechanisms fail, resulting in oxidative stress. Oxidative stress induced by excessive production of ROS can result from both external and internal factors. Oxidative stress has been implicated as a factor in cell death [2] and has been linked to diseases such as cancer, diabetes mellitus, neurodegenerative diseases and ischemia/reperfusion injury [5]. Neuronal cell death induced by oxidative stress has been linked with several neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, and stroke [6]. Apoptosis is a physiological event, but when it occurs in excess it can be especially detrimental to post-mitotic cells such as neuronal cells. Since these cells do not divide, they are not replaced after death, leading to a compromise in brain function [7]. Thus, protecting post-mitotic cells from oxidative-stress-induced apoptosis is an important area of research in the treatment of neurodegenerative diseases. Intracellularly, the ratio of the amount of anti-apoptotic to pro-apoptotic proteins determines the vulnerability of a cell to apoptotic stimuli [8]. In cases of oxidative stress, pro-apoptotic proteins such as Caspase 3 may become activated in excessive amounts resulting in inappropriate induction of apoptosis. This could be especially damaging to the organism if occurring in neuronal cells and as a result, this protein is an important target for the prevention of apoptosis in post-mitotic cells.

Intrabodies (intracellular antibodies) are antibody fragments which are expressed intracellularly and directed to specific subcellular compartments for target modulation [9]. Intrabodies can have therapeutic effects by inhibiting (e.g., by sterically preventing interactions of the target protein with its interacting partners), restoring or enhancing the

function of a molecule, or by shuttling the target molecule out of its natural intracellular location [10]. The intrabody approach allows the cell to use its own machinery to produce the therapeutic agent and since the intrabodies are produced only in the cells, it gives the advantages of safety and efficacy [9].

An attractive approach to modulate Caspase 3 *in vivo* is to use single domain antibody-based intrabodies with specificity for Caspase 3. Such intrabodies would have potential for the therapy of neurodegenerative diseases and cancers [10, 11]. Single-domain antibodies (sdAbs) can be obtained from variable domains of camelid heavy chain antibodies (V_HHs), shark new antigen receptors ($V_{NAR}s$) or conventional antibodies (V_Hs and V_Ls) [12]. There are several examples showing the feasibility of sdAbs in intrabody applications [13–19]. Characteristics such as high stability, high solubility, high expression levels, and ability to express in functional forms in the reducing environment of cytoplasm make sdAbs preferable over other antibody fragments in intrabodies applications (see references in [19]). Moreover, because of their small size, single domain intrabodies can pass through intracellular membrane pores into compartments such as nucleus and exert their effects [18]. In a previous study, we identified several sdAbs from a V_HH phage display library [19] which were specific to Bax, a pro-apoptotic protein of Bcl-2 family. These sdAbs were shown to be antagonistic towards Bax and when expressed as intrabodies inhibited apoptosis. Here, we extend the same approach to Caspase 3 and report V_HHs which modulate Caspase 3 in vitro and apoptosis as intrabodies.

Materials and Methods

Expression and Purification of Recombinant Caspase 3

Recombinant His_6-tagged Caspase 3 was expressed in an *E. coli* harbouring the respective gene on a plasmid (ATCC, Manassas, VA). The *E. coli* was grown in LB media [20] with expression of Caspase 3 induced with IPTG. The bacteria were collected by centrifugation at 10,000×*g* for 15 min, and the pellet was collected and frozen overnight. This pellet was then resuspended, sonicated on ice, and centrifuged again at 12,500×*g* for 15 min. The supernatant was collected, as it contained the expressed protein (Caspase 3), and loaded onto a Hi-Trap nickel chelating affinity column. The Caspase 3 was eluted using a 0.5 M imidazole buffer as per manufacturer's protocol (GE Healthcare, Baie d'Urfé, QC, Canada) and dialyzed to remove salts. The purified sample was lyophilized and subsequently resuspended in 0.05 mM EDTA. SDS-PAGE and Western blot were performed to confirm the presence of Caspase 3.

Isolation, Cloning and Production of V_HHs

Panning, phage enzyme-linked immunosorbent assays (ELISA), and protein expression/purification were performed as described [19].

Measurement of the Effect of the V_HHs on the Activity of Caspase 3

A fluorescence assay was used to evaluate the activity of active Caspase 3 in the presence or absence of the different V_HHs. DEVD-AFC (MP-Biomedicals, Aurora, OH) was used as the fluorescent substrate in this assay. This substrate, in the presence of reaction buffer

(0.1 M HEPES, 2 mM DTT, 0.1% CHAPS, 1% sucrose, pH 7.4) and active Caspase 3, was incubated at 37°C for 60 min and fluorescence was measured at 400 nm excitation and 505 nm emission using the SpectraMax Gemini XS (Molecular Devices, Sunnyvale, CA). Caspase 3 activity was measured as relative to the level of fluorescence. For inhibition assays, the V_HHs were incubated with the active Caspase 3 for 30 min at 37°C prior to the addition of the DEVD-AFC in the reaction buffer. Following further incubation for 60 min, in a 96-well microtiter plate, fluorescence was measured as described above.

Cell Line/Cell Culture

SHSY-5Y neuroblastoma cell line (ATCC, Manassas, VA, USA) was grown in DMEM Ham's F12 media (Sigma, Oakville, ON, Canada) supplemented with 10% (*v*/*v*) fetal bovine serum (FBS) (Sigma, Canada), 2 mM L-glutamine (Sigma, Canada), and 10 mg/mL gentamycin (Sigma Canada). The cells were incubated at 37°C with 5% CO_2 and 95% humidity.

Transient Transfection of Mammalian Cells with V_HH Genes

Vector constructs containing V_HH-fluorescent protein fusion genes were prepared for mammalian cell transfections [19]. FuGENE 6 Transfection Reagent (Roche, Laval, QC, Canada) was used to introduce the constructs into SHSY-5Y cells that had been grown in 6-well plates using the ratio of 3 μL of FuGENE reagent per 1 μg of DNA as per manufacturer's protocol. The FuGENE was incubated for 10 min in 100 μL of serum-free media, following which, 1 μg of DNA was added to the solution and incubated for 45 min. The total mixture was then added to each well of a 6-well plate and incubated at 37°C with 5% CO_2 and 95% humidity for 24 h.

Induction of Oxidative Stress in Transfected SHSY-5Y Cells

The transfected SHSY-5Y cells were treated with 50 μM H_2O_2 (Sigma, Canada) for 1 h, following which the media was replaced with fresh media. Three hours following the H_2O_2 treatment, the cells were examined microscopically for signs of apoptosis and effects of the V_HHs.

Cellular Staining and Microscopy

After induction of oxidative stress as described above, transiently transfected cells were stained with Alexa Fluor 594-Annexin V (Molecular Probes, Eugene, OR) and Hoechst Dye 33342 at a final concentration of 10 μM (Molecular Probes). The cells were removed from the plates using 0.15% trypsin (Sigma, Canada) and spun down at 6,250×*g*. Following this, the pellet was washed and resuspended in PBS and spun down again at 6,250×*g*. This pellet was resuspended in Annexin V binding buffer (Molecular Probes). Annexin V Binding Dye was added to this suspension 1 μL/50 μL suspension and incubated at room temperature for 10 min. Hoechst Dye was then added at a concentration of 1 μL/mL of cell suspension and incubated for 5 min in the dark. After the total 15-min incubation, 10 μL of the cell suspension was placed on a slide and observed under a fluorescence microscope for nuclear morphology. The images were taken using a fluorescent camera (QImaging, Regita, Burnabay, BC, Canada). The images were then processed using Improvision Open*Lab* v3.1.2, and Adobe Photoshop v7.0.

Results

Purification of Recombinant Caspase 3

Recombinant Caspase 3 was expressed in *E. coli*, in fusion with a His_6 tag and subsequently purified by immobilized metal affinity chromatography. Purified protein from two preparations eluted by an imidazole buffer was confirmed by Western blotting to be Caspase 3. It was shown that purified Caspase 3 was present in both active (15 kDa) and inactive forms (35 kDa) (Fig. 1). This was the case for both examined protein preparations; however, the active form is the predominant species in both preparations. The recombinant Caspase 3 was subsequently used for panning experiments against a naïve llama V_HH phage display library [21] and for Caspase 3 activity assays.

Identification, Binding, and in vitro Functional Analyses of anti-Caspase 3 V_HHs

Pro/anti-apoptotic proteins are prime targets for selective modulation of apoptosis in treating neurodegeneration and cancer. Caspase 3 is a known universal executioner protein of apoptotic pathway and, thus, a key protein to modulate. In order to obtain apoptosis-modulating antibodies, we panned a naïve llama V_HH phage display library [21] against recombinant Caspase 3. Screening of 22 colonies gave two different V_HH sequences, VhhCasp31 and VhhCasp32, occurring at frequencies of 19 and 3, respectively (Fig. 2a). The V_HHs had the marker amino acids described previously [22]. Both V_HHs bound strongly to Caspase 3 but not to a control bovine serum albumin in phage ELISAs (Fig. 2b). V_HHs were expressed in fusion with C-terminal c-Myc-His_5 tag in *E. coli* and purified to homogeneity for subsequent functional studies.

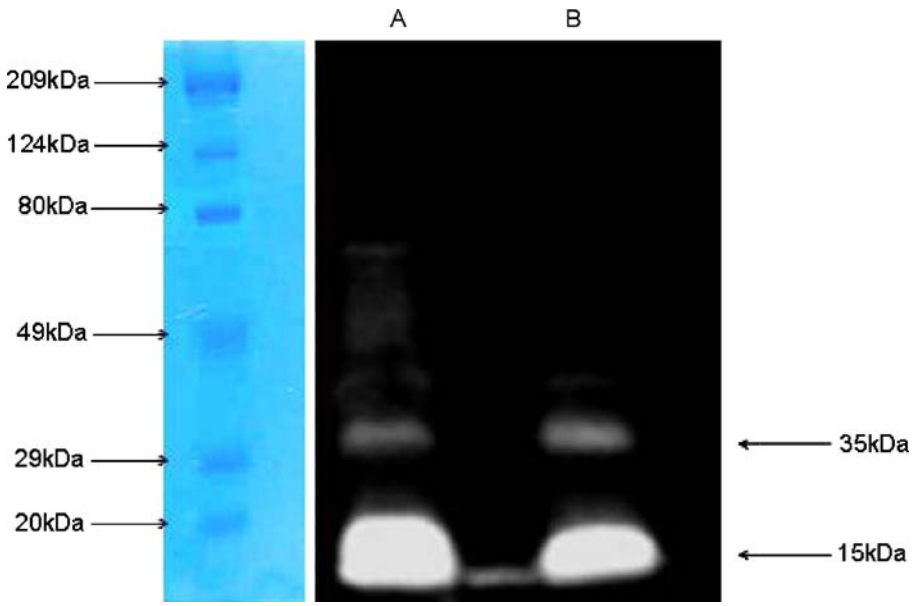

Fig. 1 Western blot of purified recombinant Caspase 3 Western blots of two different protein preparations (*A* and *B*) show bands reacting with anti-Caspase 3 antibodies of the expected size of active (15 kDa) and inactive (35 kDa) forms

a

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VhhCasp31  DVQLQASGGGLVQPGGSLRLSCAASGSLSR
VhhCasp32  EVQLQASGGGLVQAGGSLRLSCAASTNIFR

VhhCasp31  ITVMGWYRQAPGKQRELVAIITSSGG-TNY
VhhCasp32  DKFMAWYRQAPGKQRELVASITTGGR-TDY

VhhCasp31  ADSVKGRFTISRDNAKNTVYLQMNSLKPED
VhhCasp32  ADSVKGRFTISRDNTKDTMYLQMNSLKPED

VhhCasp31  TAVYYCLAARGYDRYWGRGTQVTVSS
VhhCasp32  TAVYYCAGFLG-RTYWGQGTQVTVSS
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b

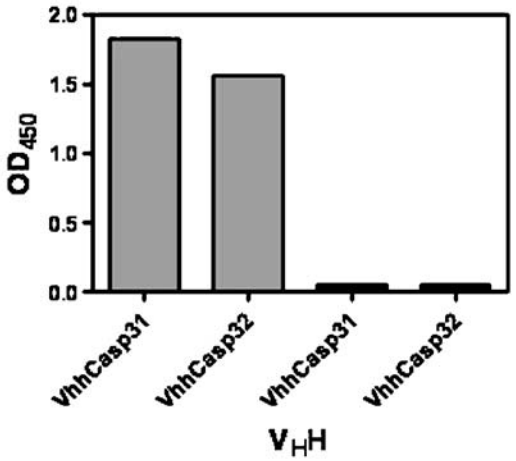


Fig. 2 Amino acid sequence (**a**) and binding analysis (**b**) of VhhCasp31 and VhhCasp32. **a** Complementarity determining regions 1 (CDR1), CDR2 and CDR3 are underlined and appear sequentially. Dashes are included for sequence alignment. **b** The graph shows the binding, by ELISA, of V_HH-displayed phages to immobilized Caspase 3 (*first two columns*). The V_HH-phages did not bind to bovine serum albumin (*last two columns*)

Next, we performed Caspase 3 activity assays to determine if VhhCasp31 and VhhCasp32 alter the activity of Caspase 3. A tetra-peptide substrate (DEVD) conjugated to AFC was used in this assay. In the presence of active Caspase 3 the substrate is cleaved, releasing the fluorescent AFC which then gives a measure of activity of Caspase 3. Caspase 3 was pre-incubated with V_HHs prior to the addition of the reaction buffer and DEVD-AFC substrate and the activity of Caspase 3 was monitored for decreases or increases in comparison to the reaction without any V_HH or with an irrelevant V_HH. As can be seen in Fig. 3, in the presence of an equimolar concentration of VhhCasp31 (4.7 μM), the activity of Caspase 3 (4.7 μM) decreases to 61% compared to a control (non-treated; 100%). Conversely, VhhCasp32 treatment at the same concentration resulted in an increase in Caspase 3 activity of 22.5%. The effect of the V_HHs on Caspase 3 was also found to be concentration dependent. At VhhCasp31 concentrations three times that of Caspase 3 (14.1 μM), Caspase 3 activity was decreased to as low as 14.5%. At the same concentration, VhhCasp32 treatment led to a 38% increase in Caspase 3 activity. When treated with an irrelevant V_HH, Vhh5.2, under both concentration conditions, Caspase 3 demonstrated negligible changes in activity (Fig. 3), ruling out the possibility that the modulating effects of VhhCasp31 and VhhCasp32 might be non-specific.

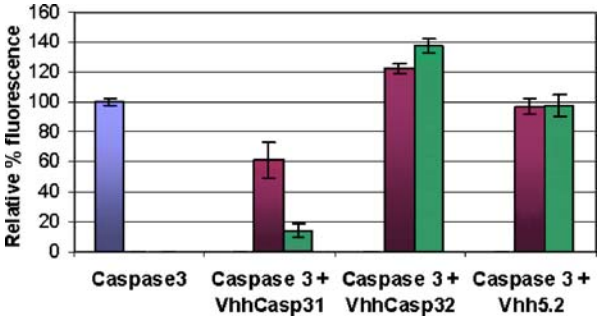


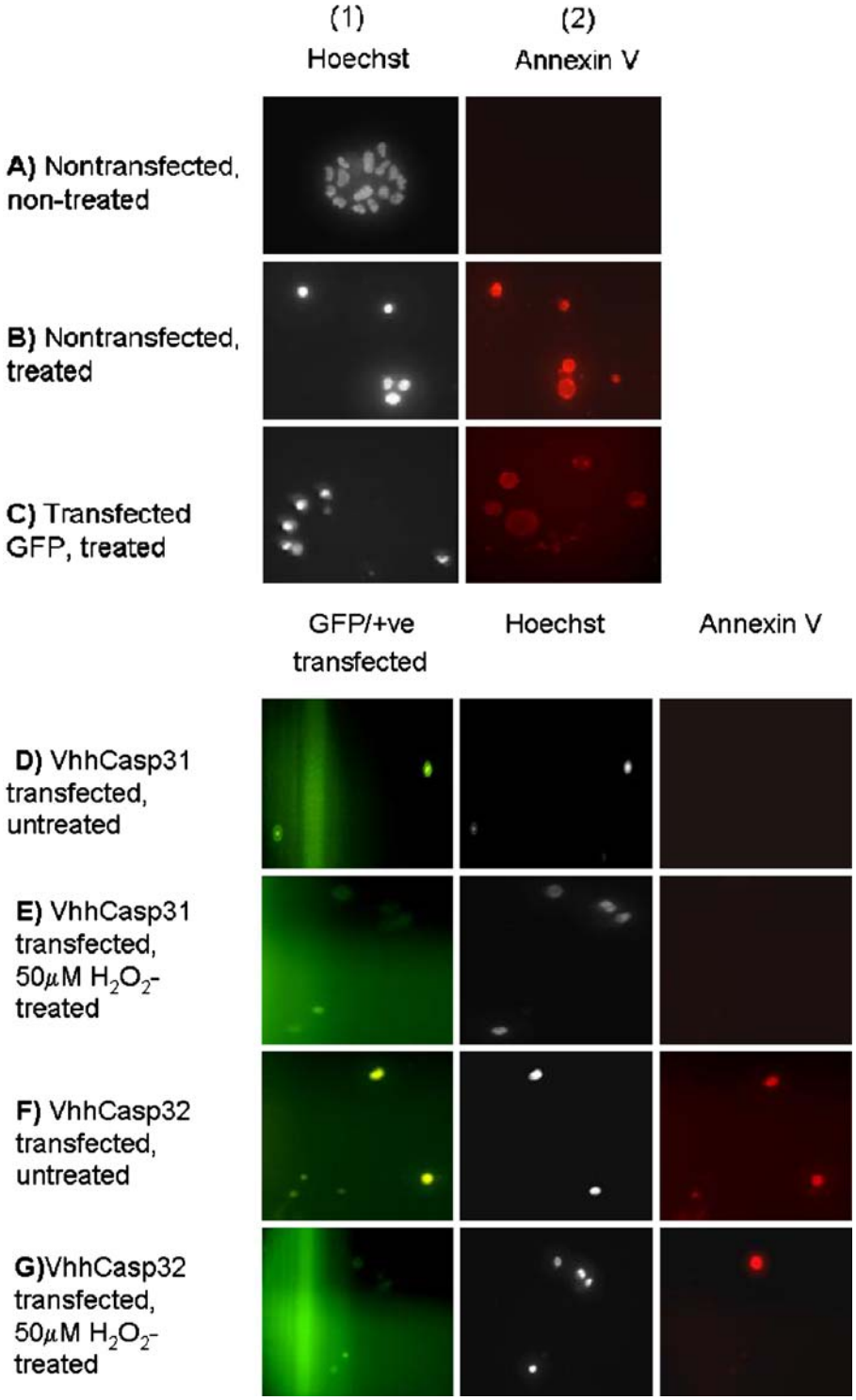
Fig. 3 Modulating effect of VhhCasp31 and VhhCasp32 on Caspase 3 activity 4.7 μM of active recombinant Caspase 3 was treated with an equal (*purple*) or 3-fold (*green*) concentration of VhhCasp31, VhhCasp32 or Vhh5.2 V_HH control. The activity of the Caspase 3 was measured in terms of fluorescence release into solution relative to the fluorescence release observed with Caspase 3 alone as described in "Materials and Methods". Experiments were performed in triplicates and standard errors were calculated using Microsoft Excel v6.0 software

Effects of Transiently-expressed VhhCasp31 and VhhCasp32 Intrabodies on Oxidative-Stress-induced Apoptosis in SHSY-5Y cells

Although, the results described above indicated that V_HHs were capable of binding as well as modulating the in vitro activity of Caspase-3, it remains to be determined if they would affect Caspase 3 when expressed inside mammalian cells, and, thus, modulate apoptosis. We, therefore set out to determine the effects of the V_HHs expressed intracellularly as intrabodies on apoptosis. In order to accomplish this, vectors for expression of VhhCasp31 and VhhCasp32 V_HHs as intrabodies in SHSY-5Y mammalian cells were constructed [19]. Following transfection with VhhCasp31- and VhhCasp32-containing expression vectors, the cells were challenged with 50 μM H_2O_2 and observed for physiological changes associated with apoptotic cell death by cellular staining with Hoecsht and Alexa Fluor 594-Annexin V. Hoechst dye intercalates with the DNA and thus, highly condensed nuclei like those associated with apoptosis, will fluoresce brightly when viewed under the fluorescent microscope. The Annexin V conjugate binds to phosphatidylserine, a marker for early apoptosis. Thus, cells that fluoresce red will be indicative of apoptosis. The V_HHs were expressed as fusion protein conjugated to green fluorescent protein, thus, a successful V_HH expression can be "visualized" as green fluorescence. It can be seen in Fig. 4A that non-transfected SHSY-5Y cells that are not challenged with H_2O_2 do not show apoptotic nuclei upon Hoechst staining. Also, no positive Annexin V staining indicated healthy, non-apoptotic cells.

Signs of apoptosis can be seen in non-transfected cells challenged with 50 μM H_2O_2, indicated by brightly stained, condensed nuclei with Hoechst staining and positive red Annexin V staining (Fig. 4B). SHSY-5Y cells were also transfected with a vector expressing green fluorescent protein (GFP) alone. These cells were also challenged with

Fig. 4 Cellular and nuclear morphology of VhhCasp31- and VhhCasp32-transfected SHSY-5Y cells. ▶ Control (non-transfected) SHSY-5Y cells were passaged at the same point as the transfected SHSY-5Y cells and stained with cell permeable Hoechst dye to visualize healthy viable cells. Annexin V Fluor conjugate 594 was also used to visualize any apoptotic cells as described in Materials and Methods. The concentration of H_2O_2 for the H_2O_2-treated cells was 50 μM. As seen in panels *D–E*, column "GFP", all the V_HH-transfected cells are green indicating a successful expression of V_HH-GFP fusion proteins. All fluorescent pictures were taken using an inverted stage fluorescence microscope (Magnification: 400×)


(1)
Hoechst
(2)
Annexin V
A) Nontransfected, non-treated
B) Nontransfected, treated
C) Transfected GFP, treated
GFP/+ve transfected
Hoechst
Annexin V
D) VhhCasp31 transfected, untreated
E) VhhCasp31 transfected, 50μM H_2O_2-treated
F) VhhCasp32 transfected, untreated
G)VhhCasp32 transfected, 50μM H_2O_2-treated

50 μM H_2O_2 and upon staining with Annexin V (red) it can be seen that the GFP does not offer any protection from apoptosis (Fig. 4C). In contrast, apoptosis-modulating effects of VhhCasp31 and VhhCasp32 can be seen in cells expressing these V_HH intrabodies. Cells transfected with VhhCasp31 and challenged with 50 μM H_2O_2 do not display morphology and staining indicative of apoptosis (Fig. 4E) just like the unchallenged VhhCasp31-transfected cells (Fig. 4D). Lastly, it can be noted in Fig. 4F and Fig. 4G that cells transfected with VhhCasp32 display signs of apoptosis even without H_2O_2 treatment. These results indicate that VhhCasp31 is an antagonist and VhhCasp32 is an agonist of apoptosis.

Discussion

The results obtained here show a novel way of both quenching and increasing the activity of Caspase 3. This study considered the ability of two Caspase 3-specfic V_HHs to modulate the activity of isolated Caspase 3 as well as within SHSY-5Y cells via transient transfections. The VhhCasp31 was capable of inhibiting the activity of Caspase 3, whereas VhhCasp32 enhanced Caspase 3 activity. Moreover, VhhCasp31 and VhhCasp32, respectively, inhibited and accelerated apoptosis when expressed as intrabodies in mammalian cells.

SHSY-5Y cells were used as a model in this study to show prevention of cell death in regards to neurodegenerative disease. SHSY-5Y cells have the ability to be differentiated into neuronal cells, making them a good choice for qualitatively observing the effects of oxidative stress, and the abilities of the V_HHs to affect cell death via transient transfections [6]; this "transient" model, however, provides only qualitative data. Using stably transfected, SHSY-5Y cell lines which permanently express the V_HH genes one could obtain quantitative data such as apoptotic index. Additionally, stably transfected, V_HH-expressing SHSY-5Y cells could be differentiated into neuronal cells which are closer a model to neurons in neurodegenerative disease.

For the purposes of this study, transient transfections were performed to evaluate the ability of the V_HHs to quench the activity of Caspase 3 (activated by oxidative stress) by VhhCasp31 and to increase the activity of Caspase 3 by VhhCasp32 within the cells. Applying the V_HHs directly to active Caspase 3 isolated from cellular lysate does allow for observation into the modulating abilities of the V_HHs, however, expression of the V_HHs intracellularly takes into account factors present in the cell that may affect the ability of the V_HHs to modulate Caspase 3 activity. The V_HHs may have different effects within the cells when compared with their actions outside of the cell. For example, the V_HHs could prove to be toxic to the cell, may not be expressed at all, and may either show prevention of apoptosis or no protection from apoptosis. Even though the V_HHs may show specificity toward Caspase 3, it may not show beneficial alterations within the cell, or even cause problems with other metabolic pathways within the cell.

Caspase 3 was chosen as a target for to modulate apoptosis, as it is one of the most common executioners of apoptosis [23]. Caspase 3 is also activated by both intrinsic and extrinsic pathways of apoptosis, with the ability to be cleaved by both Caspase 8 and the Caspase 9-APAF-1 complex, meaning that it can become activated by ligand signalling from the plasma membrane as well as signalling from the mitochondria. Caspase 3, as an executioner caspase, plays a role in mediating the cleavage of ICAD, the inhibitor of CAD, which is responsible for DNA degradation [3]. Caspase 3 is one of the last checkpoints in the pathway toward DNA degradation, and subsequent apoptosis, thus making it an important target for modulating of the apoptotic pathway.

The V_HHs may have altered the activity of Caspase 3 by binding to (1) the active site of Caspase 3, preventing its catalytic function, (2) different subunits, preventing them from dimerizing, hence preventing procaspase 3 from becoming active, (3) a site on procaspase 3 that enhances the cleavage and subsequent dimerization, thus boosting activity and (4) a site on Caspase 3 that results in conformational changes leading to inactivation (by VhhCasp31) or enhancement (by VhhCasp32) of Caspase 3 activity.

The results obtained here show that the V_HH intrabodies, by modulating Caspase 3, stop apoptosis of neuronal cells under oxidative stress or induce apoptosis. Such V_HH antagonist and agonist of apoptosis can be exploited for the therapy of neurodegenerative diseases and cancer, respectively. Indeed, Caspase 3 has been proposed as a target for cancer therapy [24]. The V_HHs can be used directly as drugs in the context of gene therapy [18] or used in competition binding assays for screening from pharmacophore libraries small molecular weight functional mimics of V_HHs. As therapeutics, these compounds may not have the challenging issue of cell entry facing protein therapeutics.

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