IDENTIFICATION OF ALLOSTERIC INHIBITORS AGAINST CASPASE-6 ACTIVITY IN ALZHEIMER'S DISEASE

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ABBREVIATIONS

AAM	Alternatively Activated Macrophage
Aβ	Amyloid β Peptide
AD	Alzheimer Disease
AFC	7-amino-4-trifluoromethylcoumarin
Akt	Protein Kinase B
ALS	Amyotrophic Lateral Sclerosis
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid
Apaf-1	Apoptotic Protease Activating Factor 1
APP	Amyloid Precursor Protein
ARK5	AMPK-related Kinase 5
Asp	Aspartate
Atg3	Autophagy-related Protein 3
ATP	Adenosine Triphosphate
BAC	Bacterial Artificial Chromosome
BACE	Beta-site Amyloid Precursor Protein Cleaving Enzyme
Bcl-2	B-Cell Lymphoma 2
BIR	Baculoviral Inhibitor of Apoptosis Protein Repeat
C31	31 Amino Acid Peptide of C-terminal Cleaved APP
C. elegans	*Caenorhabditis elegans*
c-FLIP	Cellular FLICE-like Inhibitor Protein
c-IAP1	Cellular Inhibitor of Apoptosis Protein 1
c-IAP2	Cellular Inhibitor of Apoptosis Protein 2
c-maf	Cellular Muscular Aponeurotic Fibrosarcoma
CA1	Cornus Ammonis 1
CAD	Caspase-activated Deoxyribonuclease
CARD	Caspase Activation Recruitment Domain
Casp1	Caspase-1
Casp2	Caspase-2
Casp3	Caspase-3

Casp4	Caspase-4
Casp5	Caspase-5
Casp6	Caspase-6
Casp7	Caspase-7
Casp8	Caspase-8
Casp9	Caspase-9
Casp10	Caspase-10
Casp11	Caspase-11
Casp12	Caspase-12
Casp13	Caspase-13
Casp14	Caspase-14
Casp15	Caspase-15
Casp16	Caspase-16
Casp17	Caspase-17
Casp18	Caspase-18
CBP	CREB Binding Protein
CED-3	Cell Death Protein 3
CDK1	Cyclin Dependent Kinase 1
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CHO	Chinese Hamster Ovary
CNS	Central Nervous System
CREB	cAMP-Responsive Element Binding Protein
CSF	Cerebrospinal Fluid
DED	Death Effector Domain
DIABLO	Direct IAP Binding Protein with Low pI
DISC	Death Inducing Signaling Complex
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DR6	Death Receptor 6
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid

FAD	Familial Alzheimer Disease
FADD	Fas-associated Protein with Death Domain
FasL	First Apoptosis Signal Ligand
FBS	Fetal Bovine Serum
FLICE	FADD-like IL-1β-converting Enzyme
FLIP	FLICE-like Inhibitor Protein
$FLIP_L$	FLICE-like Inhibitor Protein Long
$FLIP_S$	FLICE-like Inhibitor Protein Short
fmk	Fluoromethylketone
GFP	Green Fluorescent Protein
GGA3	Golgi Associated, Gamma Adaptin Ear Containing, ARF Binding Protein 3
HAL	Hexaminolevulinate
HAUSP	Herpesvirus-associated Ubiquitin-specific Protease
HCT116	Human Colon Carcinoma Cells
HD	Huntington Disease
HEK293T	Human Embryonic Kidney Fibroblasts
HEPES	N-2-hydroxyethylpiperazine-N-2-ethane Sulfonic Acid
HIV	Human Immunodeficiency Virus
HIVE	Human Immunodeficiency Virus Encephalitis
Hsp90α	Heat Shock Protein 90 α
hTERT	Human Telomerase Reverse Transcriptase
Htt	Huntingtin
IAP	Inhibitor of Apoptosis Protein
ICAD	Inhibitor of Caspase-activated Deoxyribonuclease
ICE	Interleukin-1β-Converting Enzyme
IFNγ	Interferon γ
IL-1β	Interleukin-1β
IL-18	Interleukin-18
IL-33	Interleukin-33
IPTG	Isopropyl β-D-1-thiogalactopyranoside

IRAK-M	Interleukin-1 Associated Kinase-M
KO	Knockout
LPS	Lipopolysaccharide
LTD	Long Term Depression
MAPK	Mitogen-activated Protein Kinase
MCAO	Middle Cerebral Artery Occlusion
Mch2β	Mammalian Ced-3 Homologue 2β
MCI	Mild Cognitive Impairment
MEM	Minimal Essential Media
mHtt	Mutant Huntingtin
miRNA	Microribonucleic Acid
MMP-2	Matrix Metalloproteinase 2
MMP-9	Matrix Metalloproteinase 9
MPP^+	1-methyl-4-phenylpyridinium Ion
mTOR	Raptor
mTORC1	Mammalian Target of Rapamycin Complex 1
NCI	Non Cognitive Impairment
NFT	Neurofibrillary Tangle
NGF	Nerve Growth Factor
NK	Natural Killer
NLR	Nucleotide-binding Oligomerization Domain-like Receptor
NLRP1	Nod-like Receptor Protein 1
NMDA	N-methyl-D-Aspartate
NP	Neuritic Plaque
NT	Neuropil Thread
NuMa	Nuclear Mitotic Apparatus Protein
PAINS	Pan Assay Interference Compounds
PAK2	p21-activated Kinase 2
PANX1	Pannexin 1
PARD3	Par-3 Family Cell Polarity Regulator
PARP	Poly (ADP-ribose) Polymerase

PD	Parkinson Disease
PDT	Photodynamic Therapy
PEG	Polyethylene Glycol
PIDD	p53-induced Protein with a Death Domain
PINK1	PTEN-induced Putative Kinase
PIPES	Piperazine-N, N-bis 2-ethanesulfonic Acid
PKC	Protein Kinase C
PP2	Protein Phosphatase 2
PRR	Pattern Recognition Receptor
PSEN1	Presenilin 1
PSEN2	Presenilin 2
PTEN	Phosphatase and Tensin Homolog
RAIDD	Receptor-interacting Protein Associated CED-3 Homologous Death Protein with a Death Domain
RCasp6	Recombinant Caspase-6
RIPK1	Receptor Interacting Protein Kinase 1
RIPK3	Receptor Interacting Protein Kinase 3
ROCK I	Serine/threonine Kinase Rho-associated Kinase I
ROS	Reactive Oxygen Species
SAD	Sporadic Alzheimer Disease
SATB1	Special AT-rich Sequence Binding Protein
SB	Stennicke's Buffer
SDCCAG3	Serologically Defined Colon Cancer Antigen 3
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SGK	Serine/threonine Protein Kinase
Smac	Second Mitochondria-derived Activator of Caspase
SOD1	Copper/zinc Superoxide Dismutase
SRBP	Sterol Regulatory Binding Protein
STS	Staurosporine
TauΔCasp6	Tau Cleaved by Caspase-6
TNFα	Tumor Necrosis Factor α

TRADD	Tumor Necrosis Factor Receptor type 1-Associated Death Domain Protein
UPS	Ubiquitin Proteasomal System
VCP	Valosin Containing Protein
v-FLIP	Viral FLICE-like Inhibitor Protein
WT	Wild Type
XIAP	X-linked inhibitor of Apoptosis Protein
YAC	Yeast Artificial Chromosome
Zn^{2+}	Zinc Ion

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ABSTRACT

Knock-in transgenic mouse models and human post-mortem studies have demonstrated Caspase-6 (Casp6) activity to be involved in both age-dependent cognitive impairment and Alzheimer disease, suggesting Casp6 as a potential therapeutic target. Although Casp6 inhibitors have been identified, many of them are non-selective. Here, we show that by using a novel human genetic approach, we identified a non-conserved allosteric pocket that bears favorable tertiary architecture for accommodating small molecules. An *in silico* screen of 77,000 diverse molecules against this pocket identified fifty-four potential Casp6 inhibitors. This thesis will focus on the fifteen hits identified from the Sigma-Aldrich commercial library. *In vitro* screening of these compounds identified compound **10** and its analogues as the most potent and selective Casp6 inhibitors. In addition, kinetic analyses show that these compounds inhibit Casp6 through a non-competitive mode of inhibition. Furthermore, these compounds are non-toxic and reduce Casp6 activity in HCT116 cells, whereas they induce cytotoxicity in HEK293T cells. Finally, extensive compound **10** analogue screens have identified compound **10P** as our hit compound that could potentially be used as a starting point for medicinal chemistry for the optimization of a more potent, selective and non-toxic allosteric inhibitor. Together, not only do these findings identify a novel class of Casp6 inhibitors, but also validate a novel approach for the discovery of allosteric sites for other drug targets.

RÉSUMÉ

Des études réalisées à partir de modèles murins transgéniques et de tissus humains post-mortem ont montré que l'activité de la caspase-6 est impliquée dans les troubles cognitifs liés à l'âge et la maladie d'Alzheimer, suggérant que la caspase-6 pourrait être une cible thérapeutique potentielle pour ces pathologies. Bien que plusieurs inhibiteurs de caspase-6 aient été identifiés, la plupart d'entre eux ne sont pas sélectifs. Dans cette étude, en utilisant une nouvelle approche génétique, nous avons identifié une poche allostérique non conservée pouvant lier des petites molécules. Un criblage *in silico* de 77 000 molécules contre cette poche allostérique a permis d'identifier 54 inhibiteurs potentiels de caspase-6. Ce travail de thèse se concentre sur 15 de ces composés, appartenant à banque de composés Sigma-Aldrich. Des analyses *in vitro* ont identifié le composé 10 et ses analogues comme étant les inhibiteurs de caspase-6 les plus puissants et sélectifs. Des analyses cinétiques ont montré que ces composés inhibent la caspase-6 de manière non-compétitive. De plus, ces composés sont non toxiques et diminuent l'activité de la caspase-6 dans les cellules HCT116, mais sont cytotoxiques dans les cellules HEK293T. Enfin, nos études ont révélé que le composé 10P serait la molécule qui pourrait potentiellement servir de base pour l'optimisation de composés allostériques plus puissants, sélectifs et non toxiques. En conclusion, en plus d'avoir identifié de nouveaux inhibiteurs de caspase-6, nous avons validé une nouvelle approche pour la découverte d'inhibiteurs allostériques.

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This thesis is dedicated in loving memory to ***Wilson Vicenté***.

INTRODUCTION: RATIONALE AND OBJECTIVES

Alzheimer disease (AD) is the most common cause of dementia accounting for an estimated 60% to 80% of cases (Alzheimer's Association, 2016). The etiology of AD is poorly defined as scientists have been debating over the tau and amyloid hypotheses for over twenty-five years. As the social and economic burden of AD continues to grow, it is important to explore other avenues of AD treatments at this time. Caspase-6 (Casp6) has recently gained considerable attention as a novel therapeutic target because of its involvement in AD progression. Therefore, my objective was to identify a Casp6 inhibitor.

Our lab discovered that Casp6 plays an integral role in both AD and age-dependent cognitive impairment. Casp6 activity causes the accumulation of amyloid β peptide in stressed primary human neurons (LeBlanc 1995, LeBlanc et al. 1999). In addition, Casp6 is present in neurofibrillary tangles, neuropil threads and in senile plaques of degenerating neurites in all stages of sporadic AD and in severe familial AD (Guo et al. 2004, Albrecht et al. 2007, Albrecht et al. 2009). More recently, we demonstrated that Casp6 levels in the entorhinal cortex and the Cornus Ammonis 1 (CA1) region of the hippocampus, the first areas of the brain to be affected by AD (Braak et al. 1997), correlate with episodic memory in aged individuals within normal cognitive range (Ramcharitar et al. 2013). Furthermore, we developed a mouse model expressing a self-activated form of human Casp6 in the CA1 region and this mouse develops age-dependent episodic and spatial memory impairments in the absence of neuritic plaques and neurofibrillary tangles (LeBlanc et al. 2014). Collectively, these results suggest that the elimination of plaques and tangles would have an insignificant effect on AD progression, and that these lesions are an effect rather than a cause of AD. Targeting early events that lead to the accumulation of these lesions would be a more effective approach. In this regard, we believe Casp6 represents a therapeutic target against age-dependent cognitive impairment and AD.

Several studies indicate Casp6 to be a viable drug target. The first Casp6 knockout mice developed normally without exhibiting any major apoptotic defects (Zheng et al. 1999). More recent Casp6 knockout mouse models have demonstrated a role for Casp6 in inhibiting B-cell differentiation; therefore, Casp6 inhibition in the elderly could sensitize their immune response (Watanabe et al. 2008). In humans, Casp6 is expressed in all peripheral tissues with the highest expression levels in the colon, suggesting that its inhibition could cause colon cancer (Godefroy et al. 2013). However, inflammation induced tumorigenesis in the mouse colon is Casp6 independent (Foveau et al. 2014). Furthermore, some rare variants of human Casp6 have no catalytic activity (unpublished data). Taken together, these findings suggest that Casp6 activity is dispensable for normal physiology.

Several Casp6 inhibitors have been identified, each with their own advantages and disadvantages. Methylene blue inhibits Casp6 activity through the active site; however, since the active site is highly conserved, methylene blue also inhibits caspase-1 and -3 activity (Pakavathkumar et al. 2015). Zinc inhibits Casp6 through a non-competitive allosteric mechanism (Velazquez-Delgado et al. 2012), but it also inhibits caspase-3, and -9 (Perry et al. 1997, Huber et al. 2012). Mammalian ced-3 homologue 2β is the only selective endogenous Casp6 inhibitor, but it blocks Casp6 activation and has no effect on activated Casp6 (Lee et al. 2010). Moreover, a synthetic allosteric peptide was reported to selectively downregulate Casp6 activity in neuronal cells; however, it had poor cell penetration properties (Stanger et al. 2012). To our knowledge, no selective small molecule allosteric inhibitors that target active Casp6 have been reported.

We hypothesized that targeting an allosteric site of active Casp6 would selectivity inhibit its activity. I test this hypothesis by screening various compounds against purified recombinant Casp6 and other caspases. Through a novel human genetic approach, an allosteric pocket of low sequence conservation amongst different caspases was identified (unpublished data). The rationale was that binding of a small molecule within this pocket would affect Casp6 activity. Our collaborators at the IRIC ran an *in silico* screen of 77,000 diverse molecules from commercial chemical libraries and their own medicinal

chemistry platform. Fifty-four compounds were identified: fourteen from IRIC, twenty-five from Chembridge Corporation and fifteen from Sigma-Aldrich. This thesis will focus on the work I have conducted on the compounds identified from the Sigma-Aldrich commercial library.

I. LITERATURE REVIEW

1.1. CASPASES

1.1.1. Discovery of Caspases

Caspases belong to a family of highly conserved cysteine proteases that have a strict specificity to cleave their substrates after aspartate (Asp) residues. The first member of this family to be described was Caspase-1 (Casp1) (Black et al. 1989, Kostura et al. 1989), which was purified and cloned by two independent groups in 1992 (Cerretti et al. 1992, Thornberry et al. 1992). Based on its ability to proteolytically process interleukin-1β (IL-1β), it was originally named interleukin-1β-converting enzyme (ICE). In 1993, it was reported that ICE shared greater than 24% identity with *Ced-3*, an essential gene for programmed cell death during *Caenorhabditis elegans* (*C. elegans*) development (Yuan et al. 1993). This finding, predicting for the first time that cysteine protease activity could be linked to apoptosis, prompted the rapid identification and characterization of novel caspases. In 1994, Caspase-3 (Casp3) was discovered to be the corresponding human protein of *Ced-3* (Fernandes-Alnemri et al. 1994). By 1996, ten different caspases had been characterized and the official caspase nomenclature was established (Alnemri et al. 1996). To date, eighteen members of the mammalian caspase family have been identified (Eckhart et al. 2008), eleven of which are found in humans (Casp1-10 and -14).

1.1.2. Caspase Structure

Caspases are synthesized as inactive zymogens that contain three distinct domains: an N-terminal prodomain, a large central subunit (p20) containing the active site and a small C-terminal subunit (p10) (Fig. 1). A single Asp cleavage site separates the N-terminal prodomain from the large subunit and an intersubunit linker sequence containing two Asp cleavage sites separates the p20 subunit from the p10 subunit. Within the cell, procaspases exist either as monomers or dimers, a crucial property that defines their activation mechanism. To become active, all procaspases must dimerize followed by cleavage within the intersubunit linker to enable intimate interactions between the p20 subunit and the p10 subunit. This cleavage results in the formation of two chains that help form the active site (Walker et al. 1994, Wilson et al. 1994). The p20 subunit contains the

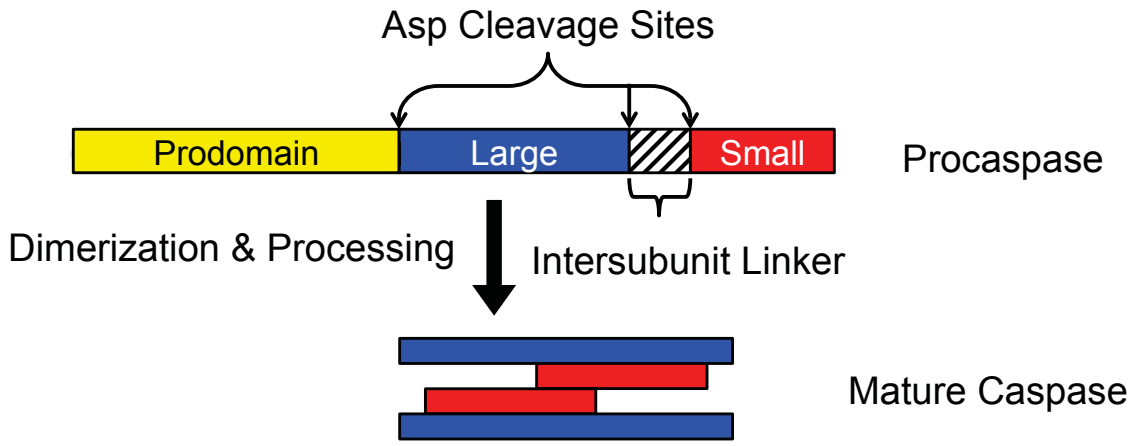

Figure 1 | Schematic representation of procaspase and mature caspase domains.

catalytic dyad residues Cys285 (Casp1 numbering), which is part of a highly conserved QACXG pentapeptide motif (X can be R, Q or D) and His237. Both the p10 and p20 subunits contain residues that help form the substrate-binding pocket. Upon maturation, two additional cleavage events occur to remove the prodomain and intersubunit linker.

Based on the crystal structure of Casp1 (Walker et al. 1994, Wilson et al. 1994), Caspase-2 (Casp2) (Schweizer et al. 2003), Casp3 (Rotonda et al. 1996), Casp6 (Sattar et al. 2003), Caspase-7 (Casp7) (Wei et al. 2000), Caspase-8 (Casp8) (Blanchard et al. 1999) and Caspase-9 (Casp9) (Renatus et al. 2001), active caspases exist as homodimers which consist of two p20 subunits and two p10 subunits (Fig. 2). The secondary structure of each monomer (p20–p10) consists of six parallel and antiparallel β-sheets intertwined with five α–helices positioned on opposing sides of the plane formed by the β-sheets. The first four β strands and helices 1-3 form the p20 subunit whereas the last two β strands and helices 4-5 form the p10 subunit. The dimer interface consists of the β6 strands from both monomers contacting each other in an antiparallel manner. The monomers are arranged through a C2 axis of symmetry such that one monomer is inverted relative to the other.

Each caspase has two active sites found on opposite ends. Five loops are important for active site formation. Within each active site, four loops (L1 – L4) come from one monomer. During activation, the intersubunit linker is cleaved and the two halves of the

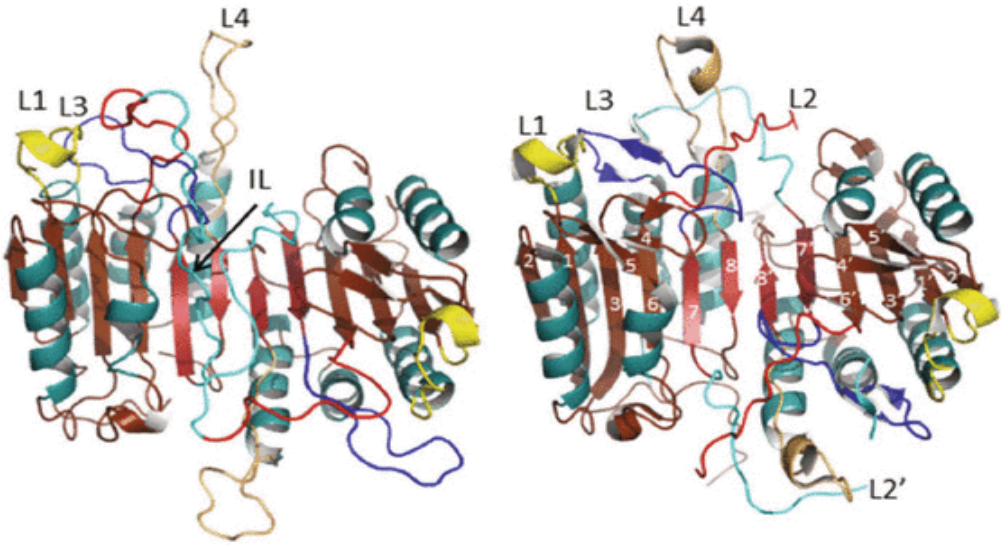

Figure 2 | Crystal structure of procaspase-3 and mature caspase-3. Active site loop coloring: yellow = L1, red = L2, cyan = L2', blue = L3, tan = L4. Reprinted from (Cade et al. 2015) with permission from Springer International Publishing.

linker form L2 and L2'. Within the caspase family, the length of L1 and L3 is highly conserved whereas L2 and L4 are more variable which permits for differences in substrate preferences (Shi 2004).

1.1.3. Caspase Classifications

Based on their function, caspases are mainly classified as either inflammatory or apoptotic. The apoptotic caspases can be further subdivided as either initiator or executioner caspases. Caspases can also be classified based on the length of their prodomain, which coincides with their mode of activation. Other less commonly used classification criteria include substrate specificity and phylogenetic clades.

1.1.3.1. Classification Based on Function

Caspases have essential roles in both inflammation and apoptosis. Casp1, Caspase-4 (Casp4) and Caspase-5 (Casp5) are involved in cytokine maturation whereas Casp2, Casp3, Casp7, Casp8, Casp9 and Caspase-10 (Casp10) are involved in apoptosis. The apoptotic caspases can be further subdivided into caspases that initiate the apoptotic cascade (Casp2, Casp8, Casp9, and Casp10) and those that are involved in the execution

phases of apoptosis (Casp3 and Casp7). The apoptotic initiator caspases can be further subdivided as being involved in the intrinsic pathway (Casp2 and Casp9) or the extrinsic pathway (Casp8 and Casp10). Casp6 is traditionally classified as an apoptotic effector caspase; however, mounting evidence suggests that it has a role outside of apoptosis since its activation in HEK293T cells does not cause cell death (Klaiman et al. 2009, Gray et al. 2010). An exception to this classification is Casp14, which is involved in keratinocyte differentiation (Lippens et al. 2000).

1.1.3.2. Classification Based on Procaspase Structure / Mode of Activation

Caspases can also be classified based on the length and composition of their N-terminal prodomain (Fig. 3). The inflammatory and initiator caspases have long N-terminal prodomains (~100 amino acids) that contain either a caspase activation recruitment domain (CARD) (Casp1, Casp2, Casp4, Casp5, and Casp9) or two tandem death effector domains (DEDs) (Casp8, and Casp10). These caspases exist as stable monomers until they are activated by dimerization through the induced proximity model. In this model, high local concentrations and favorable orientation of procaspases within large multiprotein complexes favors dimerization followed by autoprocessing (Muzio et al. 1998). By contrast, the effector caspases have short N-terminal prodomains (20 – 30 amino acids) that lack adaptor protein binding regions. These caspases exist as inactive dimers that are often dependent on apoptotic initiator caspases for processing and activation. Therefore, caspase activation occurs in a hierarchical manner such that initiator caspase activation precedes and facilitates effector caspase activation.

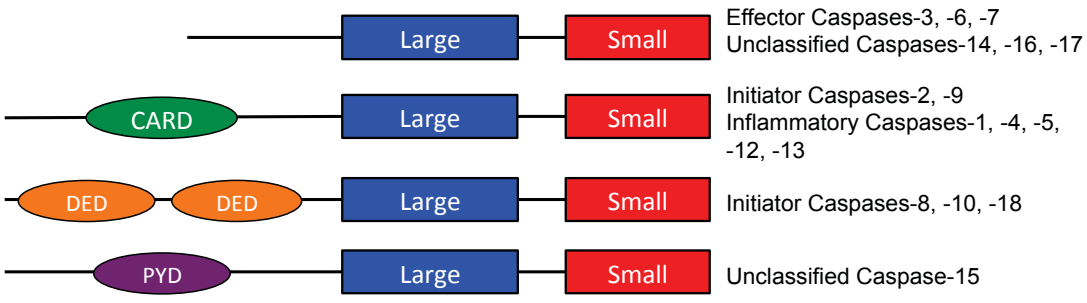

Figure 3 | Domain arrangement of mammalian caspases. Adapted from (Cade et al. 2015) with permission from Springer International Publishing.

1.1.3.3. Classification Based on Substrate Specificity

Substrate specificity is determined by a series of four substrate residues, P4-P3-P2-P1, which bind to the active site in four substrate-binding sites, S4-S3-S2-S1, respectively (Fig. 4). The active site of each caspase contains a positively charged S1 subsite that binds the negatively charged P1 aspartate within the substrate. Traditionally, caspases were thought to cleave strictly after aspartate residues; however, there have been many studies demonstrating that caspases can also cleave substrates after glutamate residues (Krippner-Heidenreich et al. 2001, Srinivasula et al. 2001, Moretti et al. 2002, Checinska et al. 2009, Soares et al. 2011). More recently, caspases have been shown to cleave after phosphoserine residues as well (Seaman et al. 2016). Despite an absolute requirement for aspartate, glutamate or phosphorylated serine in the P1 position, the substrate specificity in the P2-P4 positions can widely vary due to structural differences within the S2-S4 substrate-binding sites.

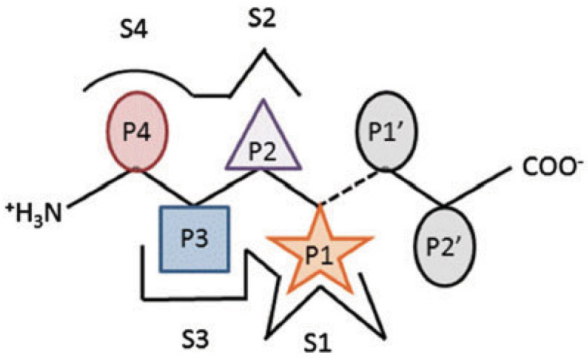

Figure 4 | Caspase substrate binding site. Reprinted from (Cade et al. 2015) with permission from Springer International Publishing.

Thornberry et al. developed a positional scanning combinatorial library approach to define the optimal tetrapeptide substrate for ten caspases (Thornberry et al. 1997). The results of this study divide the caspases into three distinct groups based on the identity of the residue in the P4 position of the substrate. Group I, which includes Casp1, Casp4 and Casp5, prefers large aromatic / hydrophobic side chains in the P4 position, with the optimal sequence of WEHD. Group II, which includes Casp2, Casp3 and Casp7, require Asp in the P4 position for efficient catalysis with the optimal sequence of DEXD, where X is V, T or H. Group III, which includes Casp6, Casp8 and Casp9, prefer large aliphatic side chains in the P4 position with the optimal sequence of (L/V) EXD. With the exception of Casp2 and Casp6, this classification is consistent with the above-mentioned functional subdivisions of the caspase family.

1.1.3.4. Classification Based on Phylogenetic Clades

Eckhart et al. proposed another classification system of caspases based on phylogenetic clades (Eckhart et al. 2008). In this study, the phylogenetic relationships of mammalian caspases were re-evaluated. Using the positions of introns as stable characters during recent vertebrate evolution, three clades of caspase genes were identified: Clade I (Casp1, Casp2, Casp4, Casp5, Casp9, Casp12, Casp14, Casp15, and Casp16), Clade II (Casp3, Casp6, Casp7, and Casp17) and Clade III (Casp8, Casp10, and Casp18). Previously, this group identified Casp15 (Eckhart et al. 2005), and in this study, three new mammalian caspases (Casp16, Casp17, and Casp18) were identified. Within its prodomain, Casp15 contains a unique pyrin domain, which is similar to Caspy and Caspy2 found in zebrafish (Masumoto et al. 2003). Similar to effector caspases, Casp16 and Casp17 have short prodomains. In addition, Casp18 is similar to the apoptotic initiators involved in the extrinsic pathway since it has two DEDs. With the exceptions of Casp14, Casp15, and Casp16, this new classification system is consistent with the prodomain classification system in which Clade I caspases have a CARD domain, Clade II caspases have a short prodomain and Clade III caspases have two DEDs.

1.1.4. Caspase Functions

Caspases are important for signaling during immune responses. In response to viruses, bacteria or cell damage, caspases process cytokines that mediate inflammation. In addition, caspases are involved in apoptotic signaling. In response to death signals, caspases are activated and cleave vital substrates to induce apoptosis. Although caspases are known for having major roles in inflammation and apoptosis, they are not limited to these two functions. Other lesser-known roles of caspases include proliferation, differentiation, neural development and axon guidance.

1.1.4.1. Inflammation

1.1.4.1.1. Inflammatory Caspase Activation

Inflammatory caspases exist as stable monomers until they are activated by dimerization through the induced proximity model. These caspases contain a long prodomain that contain a CARD that promotes their interaction with an activation complex known as the

inflammasome. The inflammasome consists of nucleotide-binding oligomerization domain-like receptor (NLR) proteins, which are a family of pattern recognition receptors (PRRs), adaptor proteins containing a CARD, and inflammatory procaspases (Martinon et al. 2002). NLR proteins are held in an inactive conformation until a stimulus promotes its interaction with adaptor proteins and subsequent inflammasome formation. In some cases, an adaptor protein is not required but serves to enhance procaspase activation (Faustin et al. 2007).

1.1.4.1.2. Inflammatory Signaling Pathways: Caspase-1

The inflammatory caspases consist of Casp1, Casp4, Casp5, Casp11, Casp12, and Casp13. Among these, Casp1 is the most intensively studied member. Pro-IL-1β is processed by Casp1, which subsequently permits its secretion from monocytes and macrophages in response to pathogens and other pro-inflammatory stimuli (Thornberry et al. 1992). Casp1 is also involved in the processing of interleukin-18 (IL-18), a cytokine involved in interferon γ (IFNγ) secretion (Ghayur et al. 1997, Gu et al. 1997), and interleukin-33 (IL-33), a cytokine involved in T helper cell type II polarization (Schmitz et al. 2005). Since Casp1 was identified as the first homologue of *C. elegans* Cell Death Protein 3 (CED-3), it was initially thought to have a role in cell death; however, Casp1 KO mice did not support this observation (Kuida et al. 1995, Li et al. 1995). These mice were resistant to endotoxic shock and had altered cytokine production and secretion, suggesting an inflammatory role rather than an apoptotic role. Casp1 also mediates the activation of the pro-inflammatory transcription factor NF-κB (Lamkanfi et al. 2004). Interestingly, rather than using its catalytic activity, the CARD domain recruits the kinase receptor-interacting protein 2 (RIP2), which is involved in NF-κB activation (Lamkanfi et al. 2004). Additionally, inflammatory caspases can protect injured cells. Bacterial pore forming toxins can induce K^+ efflux, which subsequently leads to inflammasome assembly and Casp1 activation. Casp1 cleaves and activates sterol regulatory binding elements (SRBPs) that promote cell survival by facilitating membrane repair (Gurcel et al. 2006). Furthermore, unlike apoptosis which aims to avoid an immune response through engulfment of apoptotic bodies, Casp1 activity can lead to cell lysis and release

of pro-inflammatory intracellular contents through a programmed cell death process known as pyroptosis (Fink et al. 2006).

1.1.4.1.3. Other Inflammatory Caspases: Caspase-4, -5, -11, -12, and -13

Less is known about the other inflammatory caspases. These caspases are grouped as cytokine processors since they demonstrate a higher degree of sequence similarity to Casp1 than to the apoptotic caspases. Casp11 is found only in mice and shares a high degree of homology with human Casp4 and Casp5 (Wang et al. 1996). In mice, Casp11 is essential for Casp1 activation (Wang et al. 1998). Similar to Casp1 null mice, Casp11 null mice do not process pro-IL-1β to IL-1β and are resistant to endotoxic shock induced by lipopolysaccharide (LPS) (Wang et al. 1998). Casp1 expression is modulated by IFNγ, whereas Casp5 and Casp11 expression is modulated by LPS, which suggests that inflammatory caspases are differentially regulated and have distinct functions (Lin et al. 2000, Schauvliege et al. 2002). In addition, Casp4, Casp5, and Casp11 can bind LPS through their CARD domains to cause oligomerization and cell death (Shi et al. 2014). Casp12 has acquired a frame shift mutation resulting in a truncated protein because of a premature stop codon (Fischer et al. 2002). In some people of African descent, a read through single nucleotide polymorphism encodes full length Casp12, making them more susceptible to sepsis because of an attenuated inflammatory and innate immune response to endotoxins (Saleh et al. 2004). Casp13 was originally reported to be a member of the human caspase family (Humke et al. 1998), however it was later found to be a bovine ortholog of human Casp4 (Koenig et al. 2001).

1.1.4.2. Apoptosis

1.1.4.2.1. Initiator Caspases

Similar to the inflammatory caspases, apoptotic initiator caspases exist as stable monomers and contain a long prodomain that functions as a signal integrator for apoptotic signals. The prodomain may contain a single CARD (Casp2 and Casp9) or two DEDs (Casp8 and Casp10). These motifs also enable initiator caspases to be recruited to activation complexes to enable their dimerization and subsequent autoprocessing. The initiator caspases can be further subdivided as those involved in the intrinsic pathway

(Casp2 and Casp9) and those involved in the extrinsic pathway (Casp8 and Casp10). The intrinsic pathway, also known as the mitochondrial pathway, involves the release of cytochrome *c* from the intermembrane space of the mitochondria into the cytoplasm. The extrinsic pathway, also known as the death receptor pathway, involves cell death signals that originate at the plasma membrane where an extracellular ligand binds to its receptor to induce receptor oligomerization.

1.1.4.2.1.1. Caspases in the Intrinsic Pathway: Caspase-2 and -9

The intrinsic apoptotic pathway involves stimuli such as oxidative stress, heat shock and deoxyribonucleic acid (DNA) damage. These stimuli alter the intracellular balance of pro- and anti-apoptotic members of the B-cell lymphoma 2 (Bcl-2) protein family, which controls cytochrome *c* release from the intermembrane space within mitochondria (Fan et al. 2001, Finkel 2001). In the presence of cytochrome *c* and the cofactor adenosine triphosphate (ATP), the adaptor protein apoptotic protease activating factor 1 (Apaf-1) oligomerizes to form a heptameric signaling complex known as the apoptosome (Acehan et al. 2002). Zymogen Casp9 is recruited to the complex through its CARD domain, which results in its activation through an apoptosome induced conformational change (Hu et al. 1998, Jiang et al. 2000). Active Casp9 propagates the apoptotic signal by processing Casp3 and Casp7 (Slee et al. 1999).

Casp2 also has a role in the intrinsic pathway. In response to DNA damage, p53 induces the expression of p53-induced protein with a death domain (PIDD) and its adaptor receptor-interacting protein associated ICH-1/CED-3 homologous death protein with a death domain (RAIDD) (Tinel et al. 2004). Together with Casp2, RAIDD and PIDD form another oligomerization platform known as the PIDDosome to promote Casp2 activation (Tinel et al. 2004). Casp2 subsequently causes the release of cytochrome *c* from the mitochondria to induce apoptosis (Guo et al. 2002, Lassus et al. 2002). In addition to PIDDosome formation, alternative mechanisms for Casp2 activation may exist since processing of Casp2 is detected in the absence of the PIDDosome complex (Manzl et al. 2009).

1.1.4.2.1.2. Caspases in the Extrinsic Pathway: Caspase-8 and -10

The extrinsic pathway is triggered by the ligation of transmembrane death receptors to ligands such as first apoptosis signal ligand (FasL). Ligand binding initiates receptor trimerization followed by recruitment of adaptor proteins to initiate the formation of the death-inducing signaling complex (DISC) by recruiting adaptor proteins such as Fas-associated protein with Death Domain (FADD) and tumor necrosis factor receptor type 1-associated death domain protein (TRADD). Through DED interactions on FADD or TRADD, the DISC recruits zymogen Casp8 or Casp10. Similar to the apoptosome, the DISC functions as a caspase activation platform. Activated Casp8 transmits the apoptotic signal by proteolytically processing zymogen Casp3. Two pathways of Fas-mediated apoptosis have been described, leading to the classification of cells as either type-I or -II (Scaffidi et al. 1998). Type I cells have sufficient quantities of active Casp8 to activate Casp3 to execute apoptosis, whereas type II cells induce apoptosis through generating truncated Bid to aid in the release of cytochrome *c* from the mitochondria (Li et al. 1998, Luo et al. 1998). Casp10 is also activated by the DISC and propagates the apoptotic signal by cleaving Bid (Milhas et al. 2005).

1.1.4.2.2. Effector Caspases: Caspase-3, -6 and -7

In contrast to initiator caspases, effector caspases (Casp3, Casp6 and Casp7) exist as inactive dimers in the cell that require cleavage within the intersubunit linker to become active. They have short N-terminal prodomains that lack CARD and DED domains and do not require death scaffolds for dimer formation (Pop et al. 2001, Milam et al. 2009). Apoptotic effector caspases are cleaved by apoptotic initiator caspases and the lymphocyte specific serine protease granzyme B. Casp3 and Casp7 are both processed by Casp9 (Pan et al. 1998, Slee et al. 1999). Active Casp3 can subsequently activate Casp2 and Casp6, followed by Casp6-mediated processing of Casp8 and Casp10 (Slee et al. 1999). Since Casp8 can process Casp3 through type I signaling, a positive feedback loop is formed through the extrinsic pathway. Active Casp3 can also process Casp9 to generate an additional feedback loop through the intrinsic pathway (Slee et al. 1999).

Effector caspase activity has been implicated in several apoptotic processes. Apoptosis occurs in two stages: formation of apoptotic bodies followed by phagocytosis and lysosomal degradation. During the first stage, apoptotic cell death is characterized by specific morphology, which includes nuclear fragmentation, chromatin condensation and blebbing of the plasma membrane to form apoptotic bodies (Kerr et al. 1972). The morphology is a consequence of the cleavage of over 400 different cellular substrates by caspases (Luthi et al. 2007). Only a small subset of these substrates has been well characterized and linked to apoptotic cell morphology. Effector caspases cleave and activate caspase-activated deoxyribonuclease (CAD) which allows it to translocate to the nucleus and degrade chromosomal DNA (Enari et al. 1998). Effector caspases also break the inhibitory association of inhibitor of caspase-activated deoxyribonuclease (ICAD) with CAD (Enari et al. 1998). In addition, cleavage of the intermediate filament protein vimentin disrupts the cytoplasmic network and amplifies the cell death signal through a proapoptotic cleavage product (Byun et al. 2001). Furthermore, the serine/threonine kinase rho-associated kinase I (ROCK I), which contributes to the coupling of actin-myosin filaments to the plasma membrane, is activated through caspase cleavage, which subsequently causes plasma membrane blebbing and nuclear fragmentation (Coleman et al. 2001, Croft et al. 2005). Although Casp6 activity alone is not sufficient to induce apoptosis (Klaiman et al. 2009, Gray et al. 2010), Casp6 contributes to the apoptotic phenotype through being the only caspase that cleaves nuclear filament protein lamin A (Orth et al. 1996, Takahashi et al. 1996, Ruchaud et al. 2002). During the second stage of apoptosis, caspases are involved in signaling pathways that cause chemotaxis of phagocytes to apoptotic bodies. For example, Casp3-mediated activation of phospholipase A2 results in the release of a lipid signal that attracts monocytes and macrophages (Lauber et al. 2003). In addition, Casp3 and Casp7 activate the plasma membrane channel Pannexin1 (PANX1), which mediates the release of chemotactic signals (Chekeni et al. 2010). Phagocyte recruitment and engulfment is essential for preventing apoptotic bodies from releasing their contents into the extracellular space and causing inflammation since secondary necrosis may occur when there are too many apoptotic bodies for the phagocytes to engulf.

1.1.4.3. Unclassified Caspases: Caspase-14, -15, -16, -17 and -18

Caspases that are not classified as inflammatory or apoptotic include Casp14, Casp15, Casp16, Casp17 and Casp18. Casp14 expression is restricted to epidermal keratinocytes and is involved in differentiation (Lippens et al. 2000). Similar to the apoptotic effectors, Casp14 has a short prodomain and requires both cleavage and dimerization for *in vitro* activation (Mikolajczyk et al. 2004). A natural activator of Casp14 has not been identified. Casp15 is expressed in various mammalian species including pig, cattle and dog (Eckhart et al. 2005). The prodomain of Casp15 contains a pyrin domain, which is unique among mammalian caspases (Eckhart et al. 2005). Casp15 has a role in initiating apoptosis since its overexpression in mammalian cells induces the activation of Casp3 (Eckhart et al. 2005). Casp16 is most similar to Casp14 and has been conserved in marsupials and placental mammals, including humans (Eckhart et al. 2008). Casp17 is most similar to Casp3 and is conserved among fish, frog, chicken, lizard and platypus but is absent in marsupials and placental mammals (Eckhart et al. 2008). Both Casp16 and Casp17 contain short prodomains lacking adaptor regions. Casp18 is most similar to Casp8 and is found in the platypus, opossums, and chickens but is absent from placental mammals (Eckhart et al. 2008). Similar to Casp8 and Casp10, Casp18 contains two DED regions in its prodomain, indicating that it is likely an initiator caspase.

1.1.4.4. Differentiation

As mentioned above, Casp14 has a role in keratinocyte differentiation (Lippens et al. 2000); however, other caspases are involved in the differentiation of other cell types. Casp6 and Casp7 are involved in the enucleation of lens cells, megakaryocytes, erythrocytes, platelets, and keratinocytes (Dahm 1999, De Botton et al. 2002). During enucleation of erythroblasts, Casp2, Casp3, Casp6 and Casp9 are involved in the cleavage of nuclear proteins such as lamin B (Zermati et al. 2001). Rather than inducing apoptosis, Casp3 can also activate protein kinase C-d, (PKC-d) which is positive regulator of keratinocyte differentiation (Fernando et al. 2002). In addition, Casp3 is important for the differentiation of bone marrow stromal stem cells since Casp3 knockout (KO) mice display attenuated osteogenic differentiation leading to decreased bone mineral density (Miura et al. 2004). Furthermore, an increase in Casp1 and Casp3 activity

is observed during neural stem cell differentiation (Fernando et al. 2005). The mechanism of Casp3-mediated differentiation of a wide variety of cell types may involve the activation of caspase-activated DNase (CAD) and subsequent CAD-mediated modifications in chromatin structure (Larsen et al. 2010).

1.1.4.5. Proliferation

Many studies have shown an essential role for Casp8 in the proliferation of lymphocytes. Patients with homozygous mutations in Casp8 frequently succumb to microbial infections because of their inability to activate T, B or Natural Killer (NK) cells (Chun et al. 2002). Mice with a conditional Casp8 KO in peripheral T cells show a similar lymphoproliferative phenotype characterized by lymphadenopathy and splenomegaly (Salmena et al. 2005). In proliferating cells, Casp8 is largely unprocessed and becomes weakly activated (Su et al. 2005), whereas during apoptosis Casp8 is mostly processed and strongly activated (Peter et al. 2003). This pathway is regulated by FADD-like IL-1β-converting enzyme (FLICE)-like inhibitor protein long ($FLIP_L$). Casp8 forms a heterodimer with $FLIP_L$, a protein similar to Casp8 that lacks the catalytic site. This complex prevents Casp8 catalytic activity while at the same time, promoting proliferation through NF-kB signaling (Kataoka et al. 2004). In addition, this complex prevents receptor interacting serine/threonine kinase 3 (RIPK3)-dependent necrosis without inducing apoptosis (Oberst et al. 2011). Taken together, these studies demonstrate that Casp8 can also have an anti-apoptotic role in immune cells.

1.1.4.6. Neuronal Function

Caspases have several non-apoptotic functions in nerve cells. For instance, caspases are involved in axonal guidance and synaptogenesis. Mice deficient for Casp9 and Apaf-1 exhibit misrouted axons, impaired synaptic formation and defects in the maturation of their olfactory sensory neurons in the absence of cell death (Ohsawa et al. 2010). The underlying mechanism involves Casp9-mediated cleavage of Semaphorin 7A, which is crucial for the proper projection of axons (Ohsawa et al. 2010). In addition, Casp6 has been demonstrated to play an integral role in neuronal degeneration in the absence of apoptosis in mouse and primary human neurons (Nikolaev et al. 2009, Sivananthan et al.

2010). Mouse neurons subjected to nerve growth factor deprivation shed the N-terminal fragment of APP, which subsequently binds to DR6 to induce Casp6 activation and axon degeneration (Nikolaev et al. 2009). Primary human neurons that overexpress wild type APP or express mutant APP undergo amyloid β peptide (Aβ) independent, but Casp6-dependent neuritic degeneration (Sivananthan et al. 2010). Furthermore, Casp3 and Casp6 have been implicated in axonal degeneration that occurs as a normal part of development (Simon et al. 2012). Mice deficient for Casp3 and Casp6 show delays in developmental pruning of retinocollicular axons (Simon et al. 2012). Furthermore, Casp3 activity is essential for long-term depression (LTD) in the hippocampus region (Li et al. 2010). During LTD, the removal of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors from the postsynaptic membrane is facilitated by Casp3 activity without causing apoptosis (Li et al. 2010).

1.1.5. Regulation of Caspase Activity

1.1.5.1. Inhibitors of Apoptosis Proteins

The inhibitors of apoptosis proteins (IAPs) include eight mammalian family members that function as intrinsic regulators of caspase activity (Deveraux et al. 1999). The overexpression of IAP family proteins is sufficient to inhibit apoptosis induced by proapoptotic Bcl-2 family proteins (Deveraux et al. 1999). Each IAP contains one to three 70-80-residue Zn^{2+} binding modules known as a baculoviral IAP repeats (BIRs). Among this family, only X-linked inhibitor of apoptosis protein (XIAP), cellular inhibitor of apoptosis protein 1 (c-IAP1) and cellular inhibitor of apoptosis protein 2 (c-IAP2) directly inhibit the catalytic activity of caspases. These three proteins inhibit both initiator (Casp9) and effector caspases (Casp3 and Casp7) (Deveraux et al. 1997, Roy et al. 1997, Deveraux et al. 1998). The structure of XIAP consists of three consecutive BIR domains followed by a RING zinc finger domain. The BIR2 domain of XIAP inhibits Casp3 and Casp7 with a K_i of 2-5 nM (Takahashi et al. 1998), whereas the BIR3 domain inhibits Casp9 with a K_i of 50 nM (Sun et al. 2000). XIAP inhibits Casp3 and Casp7 by making extensive contacts with residues that are essential for catalytic activity (Chai et al. 2001, Huang et al. 2001, Riedl et al. 2001) and inhibits Casp9 by binding to residues that are essential for dimerization (Shiozaki et al. 2003). The structure of c-IAP1 and c-IAP2

consists of three consecutive BIR domains followed by a CARD domain and a RING zinc finger domain. Similar to XIAP, c-IAP1 and c-IAP2 bind directly to Casp3 and Casp7 to inhibit catalytic activity (Roy et al. 1997). The BIR containing region of c-IAP1 and c-IAP2 is sufficient to induce apoptosis while the RING domain has a role in enhancing c-IAP1 and c-IAP2 potency (Roy et al. 1997).

1.1.5.2. FLICE-like Inhibitor Proteins

FLICE-like inhibitor proteins (FLIPs) are well known inhibitors of death receptor induced apoptosis (Thome et al. 1997, Scaffidi et al. 1999). FLIPs are catalytically inactive proteins with close sequence homology to Casp8. Two major groups of FLIPs exist: viral FLIPs (v-FLIPs), encoded by the gamma-herpes virus, and cellular FLIPs (c-FLIPs), the mammalian homologue of v-FLIP. Both groups possess two DEDs in their N-terminal domains to facilitate their recruitment to the DISC. Two splice variants of c-FLIP have been shown to inhibit Casp8 activation. FLICE-like inhibitor protein short ($FLIP_S$) is the homologue of the Casp8 prodomain only and directly blocks Casp8 recruitment to the DISC (Kirchhoff et al. 2000). By contrast, $FLIP_L$ does not prevent recruitment to the DISC; rather, it blocks zymogen Casp8 processing at the DISC (Krueger et al. 2001). A third isoform of c-FLIP, c-$FLIP_R$, has also been described and demonstrated to have a similar role to c-$FLIP_S$ in the regulation of death receptor-induced apoptosis (Golks et al. 2005).

1.1.5.3. Virally Encoded Inhibitors

In addition to v-FLIP, other viral inhibitors exist to target caspase activity within their host cells to survive or counter an immune response. CrmA is a suicide inhibitor which is cleaved and then covalently binds to the caspase so that it undergoes a conformational change (Gettins 2002). Although CrmA is typically a serine protease inhibitor, it also potently inhibits Casp1, Casp4, Casp5, Casp8, Casp9 and Casp10 (Komiyama et al. 1994, Zhou et al. 1997, Garcia-Calvo et al. 1998). CrmA can also inhibit Casp3 and Casp6 to some extent (Garcia-Calvo et al. 1998). Similarly, baculoviral protein p35 covalently attaches to the catalytic cysteine upon cleavage but remains covalently bound to the active site because it blocks the hydrolytic water molecule from completing catalysis (Xu

et al. 2001, Xu et al. 2003). Baculoviral p35 is an effective inhibitor of Casp1, Casp3, Casp6, Casp7, Casp8, and Casp10 (Zhou et al. 1998).

1.1.5.4. Post-translational Modifications

Caspase activity can also be regulated through post-translational modification. The RING domain of XIAP is implicated in ubiquitin-regulated degradation. The anti-apoptotic effect of XIAP is enhanced through ubiquitin-protein ligase activity that promotes its proteosomal degradation of Casp3, Casp7 and Casp9 (Suzuki et al. 2001). Murine cells expressing an E3 ligase deficient form of XIAP have increased levels of Casp3 (Schile et al. 2008), demonstrating the importance of XIAP ubiquitin ligase activity for the regulation of apoptosis. By contrast, polyubiquitylation of Casp8 by cullin3-based E3 ligase has been reported to enhance its activity (Jin et al. 2009). In addition, sumoylation of Casp2, Casp7, and Casp8 has been associated with nuclear localization (Besnault-Mascard et al. 2005, Shirakura et al. 2005, Hayashi et al. 2006); however, it is unclear if this post-translational modification has an effect on their activity. Furthermore, phosphorylation has been shown to affect caspase activity. Casp3 activity is inhibited through Ser150 phosphorylation by p38-mitogen-activated protein kinase (MAPK) (Alvarado-Kristensson et al. 2004) and protein phosphatase 2 (PP2) (Alvarado-Kristensson et al. 2005), but is enhanced through phosphorylation by protein kinase C-δ (PKC-δ) at an unknown site (Voss et al. 2005). Phosphorylation of Casp6 by AMPK-related kinase 5 (ARK5) kinase at Ser257 leads to its inactivation through disruption of the substrate-binding groove (Velazquez-Delgado et al. 2012). p21-activated kinase 2 (PAK2) phosphorylates Casp7 at Ser20, Thr173 and Ser239 to decrease its activity (Li et al. 2011). Finally, phosphorylation by MAPK (Allan et al. 2003), cyclin dependent kinase 1 (CDK1) (Allan et al. 2007), and p38α (Seifert et al. 2009) at Thr125, PKCζ at Ser144 (Brady et al. 2005), and Protein Kinase B (Akt) at Ser196 (Cardone et al. 1998) leads to decreased Casp9 activity, whereas phosphorylation by cellular abelson murine leukemia viral oncogene homolog (c-Abl) at Tyr153 enhances activity (Raina et al. 2005).

1.1.6. Synthetic Caspase Inhibitors

1.1.6.1. Active Site Inhibitors

1.1.6.1.1. Peptide Inhibitors

Peptide inhibitors generally have a reactive group replacing the P1' residues. Peptides linked to a fluoromethylketone (fmk) group bind irreversibly whereas peptides linked to aldehydes are reversible. The electrophilic carbonyl of the ketone or aldehyde binds to the catalytic cysteine, thereby inhibiting it. Peptide inhibitors can range from a single *O*-methyl-aspartate residue (e.g. Boc-Asp-fmk), to tripeptides (e.g. z-Val-Ala-Asp-fmk; zVAD-fmk) and tetrapeptides (e.g.z-Val-Glu-Ile-Asp-fmk; zVEID-fmk). Specific caspase inhibitors have been designed based on preferred tetrapeptide motifs.

More complex peptide mimetic inhibitors have also been designed. Several classes of compounds including 1-(2-acylhydrazinocarbonyl)-cycloalkyl carboxamides, 8, 5-fused bicyclic compounds and caprolactam ring containing peptidomimetics have been described to competitively inhibit Casp1 activity (Soper et al. 2006, Soper et al. 2006, Wang et al. 2007). A new class of compounds known as urazolopyrazine-based β-strand peptidomimetics has been shown to inhibit Casp3 and Casp8 (Wang et al. 2010). In addition, hydantoin-based peptidomimetics inhibit Casp3 by occupying the S3 pocket with basic groups (Vazquez et al. 2008). Pan-caspase inhibitors have also been described. For example, dipeptidyl aspartyl fluoromethylketones are broad-spectrum inhibitors that have IC_{50} values of 6 – 60 nM for different caspases (Wang et al. 2007).

1.1.6.1.2. Small Molecule Inhibitors

Several classes of non-peptide inhibitors have also been designed. 5-diakylaminosulfonylisatins inhibit Casp3 and Casp7 in the 2-6 nM range and exhibit 1000-fold selectivity relative to Casp1, Casp2, Casp4, Casp6, and Casp8 (Lee et al. 2000, Lee et al. 2001). In addition, pyrrolo [3,4, -c] quinolone-1,3-diones have been shown to potently inhibit Casp3 with IC_{50} values in the range of 3-10 nM (Kravchenko et al. 2006). Furthermore, tetrafluorophenoxymethylktone-based small molecules have been identified and shown to inhibit Casp3 activity with IC_{50} values ranging from 0.11 to 10 μM (Samiulla et al. 2012).

1.1.6.2. Allosteric Inhibitors

Unlike competitive inhibitors, allosteric inhibitors act away from the active site. These inhibitors can block the activation of caspases or downregulate their activity through conformational changes affecting the active site. Several allosteric inhibitors have been synthesized. Two allosteric inhibitors known as FICA and DICA bind at the dimer interface of Casp3 and Casp7 (Hardy et al. 2004). These inhibitors form disulfide bridges within the interface and cause the enzyme to be trapped in a zymogen-like conformation (Hardy et al. 2004). High throughput screening has recently identified another class of allosteric inhibitors for Casp3, Casp7, Casp8, and Casp9 (Feldman et al. 2012). These inhibitors also bind near the dimer interface; however, unlike FICA and DICA, they bind non-covalently (Feldman et al. 2012). Allosteric inhibitors that bind near the dimer interface of Casp1 have also been described (Datta et al. 2008). These inhibitors disrupt and prevent a conserved salt bridge from forming, thereby reducing catalytic efficiency of Casp1 100- to 200-fold (Datta et al. 2008). A Casp2 allosteric inhibitor known as AR_F8, a designed ankyrin repeat protein, stabilizes a conformation distinct from the inactive zymogen conformation (Schweizer et al. 2007). A Casp6 allosteric inhibitor has recently been described to downregulate its activity through tetramerization of both the active and zymogen conformations (Stanger et al. 2012).

1.2. CASPASE-6

The Casp6 gene was originally identified in human Jurkat T lymphocytes as *MCH-2*, a mammalian *Ced-3* homologue (Fernandes-Alnemri et al. 1995). Casp6 has been classified as an apoptotic effector caspase since it has a short prodomain and shares 41% and 37% sequence identity with Casp3 and Casp7, respectively. Despite this, there are some significant differences in Casp6 compared to the other effector caspases. One major difference is that unlike Casp3 and Casp7, Casp6 activity does not always induce apoptosis. The depletion of Casp6 in human embryonic kidney (HEK293T) cell extracts has minimal impact on chromatin margination, DNA fragmentation and nuclear condensation (Slee et al. 2001). In addition, overexpression of Casp6 in HEK293 cells results in high Casp6 activity in the absence of cell death (Klaiman et al. 2009). Furthermore, in a study that used an optimized orthogonal protease to activate each executioner caspase, it was found that activation of Casp3 or Casp7, but not Casp6, was

sufficient to induce apoptosis in HEK293 cells (Gray et al. 2010). Lastly, primary human neurons microinjected with active recombinant Casp6 do not undergo rapid cell death (Zhang et al. 2000). When compared with other caspases, differences in Casp6 structure, activation, regulation and substrates suggest that Casp6 has a non-redundant role within the cell.

1.2.1. Structure

Similar to the other effector caspases, Casp6 is synthesized as a mostly inactive homodimer that must undergo proteolytic processing within the intersubunit linker to become fully activated (Orth et al. 1996, Klaiman et al. 2009). Consistent with all other caspases, the first crystal structure of active Casp6 in an unliganded state showed that the dimeric structure consists of a central 12-stranded β sheet flanked by 10 α helices (Baumgartner et al. 2009). In contrast to the structure of other caspases, the initial Casp6 crystal showed that it adopts a latent conformation with its catalytic machinery misaligned (Baumgartner et al. 2009). The latent conformation has two extended helices that block access to the active site (Baumgartner et al. 2009). It was determined through pre-steady state kinetics that the latent conformation does not lead to latency in substrate turnover, suggesting that this conformation may not have a role in regulating enzymatic activity (Baumgartner et al. 2009). However, a more detailed kinetic analysis of latent Casp6 activity was not performed to confirm this hypothesis. More importantly, *in vitro* enzymology is an insufficient tool to predict intracellular proteolysis. Follow-up studies showed that the latent conformation is dependent on helical propensity of the two extended helices and not dependent on removal of the prodomain and intersubunit linker (Vaidya et al. 2011a, Vaidya et al. 2011b). Interestingly, the prodomain and intersubunit linker maintain enzyme stability since cleaved Casp6 with the prodomain and intersubunit linker represents the most stable form of the latent conformation (Vaidya et al. 2011b). Although the latent conformation represents an inactive form of the enzyme, binding of an active site ligand induces a conformational change that converts the two extended helices to strands, subsequently causing the enzyme to adopt a canonical conformation that is similar to other caspases (Vaidya et al. 2011a, Vaidya et al. 2011b). The latent conformation represents a pH-inactivated form since it had been crystallized at

pH 4.5 and Casp6 activity is almost entirely lost at pH 5 and below (Stennicke et al. 1997). When Casp6 was crystallized at physiological pH in an unliganded active conformation, it was determined that it adopts a canonical caspase structure after all (Muller et al. 2011). The binding of the reversible inhibitor Ac-VEID-CHO to active Casp6 crystallized at pH 6.5 showed that substrate binding induces the formation of the active site loop bundle (Wang et al. 2010). Furthermore, the first zymogen structure of Casp6 crystallized at pH 6.5 showed that it has a relatively long L2 loop compared to Casp3 and Casp7 (Wang et al. 2010). As discussed below, the longer L2 loop allows for intramolecular cleavage and subsequent self-activation, a mechanism not observed in any other caspase (Wang et al. 2010).

1.2.2. Activation

Unlike Casp3 and Casp7, apoptotic initiator activity is not required for Casp6 activation. Casp6 can be activated by the serine protease granzyme B and by effector Casp3 (Orth et al. 1996, Srinivasula et al. 1996) in both death receptor and cytochrome *c* initiated apoptosis (Hirata et al. 1998, Slee et al. 1999). Casp6 activity can also take place in the absence of Casp3 activity (LeBlanc et al. 1999, Doostzadeh-Cizeron et al. 2000, Cowling et al. 2002) and can even mediate Casp3 activation (Allsopp et al. 2000). In addition, inflammatory Casp1 can mediate Casp6 activation in primary human neurons (Guo et al. 2006). This was confirmed in human central nervous system (CNS) AD neurons where active Casp6 was found to co-localize with Nod-like Receptor Protein 1 (NLRP1), a component of the inflammasome (Kaushal et al. 2015), suggesting that NLRP1 inflammasome activation of Casp1 subsequently causes Casp6 activation. Furthermore, Casp6 has a unique intramolecular self-cleavage mechanism to become active *in vivo* and *in vitro* (Klaiman et al. 2009, Wang et al. 2010). Overexpression of Casp6 is sufficient to induce self-activation in cells (Klaiman et al. 2009). To become activated and fully mature, zymogen Casp6 is cleaved at three sites: $TETD_{23}$, $DVVD_{179}$ and $TEVD_{193}$. The $TETD_{23}$ site demarcates the prodomain from the p20 subunit and the $DVVD_{179}$ and $TEVD_{193}$ sites span the intersubunit linker region. When these three sites are mutated, Casp6 exhibits low activity (Klaiman et al. 2009). This finding indicates that Casp6 has the ability to self-process since Casp6 activity is not dependent on cleavage from

upstream caspases. The prodomain of Casp6 plays a role in regulating self-cleavage and activation *in vivo* but not *in vitro* (Klaiman et al. 2009). During self-activation, zymogen Casp6 is first cleaved at $TETD_{23}$ to remove the prodomain followed by a second cleavage at $TEVD_{193}$ and a final cleavage at $DVVD_{179}$ (Wang et al. 2010). Casp1 and Casp8 facilitate Casp6 self-activation through removal of the prodomain without further processing in the linker region (Lee et al. 2010, Wang et al. 2010). By contrast, Casp3-mediated activation of Casp6 is initiated by a cleavage at $DVVD_{179}$, followed by a second cleavage at $TETD_{23}$ and a final cleavage at $TEVD_{193}$ (Wang et al. 2010). Cleavage at the $TEVD_{193}$ site is essential for initiating the autoactivation mechanism (Wang et al. 2010) and further cleavage at $DVVD_{179}$ is required for full activity (Klaiman et al. 2009). Self-activation occurs only in Casp6, because its longer intersubunit linker permits Asp193 to be easily attacked by Cys163 of the same catalytic unit (Wang et al. 2010). The linkers of Casp3 and Casp7 are too short to place their intersubunit cleavage sites in their own active site (Wang et al. 2010).

1.2.3. Regulation

Casp6 activity can be regulated through several mechanisms including gene expression, inhibition of activation, post-translational modification, protein binding, metal binding and proteasomal degradation. At the level of gene expression, Casp6 transcription is directly upregulated by p53 through transactivation (MacLachlan et al. 2002). Similarly, cellular muscular aponeurotic fibrosarcoma (c-Maf) increases apoptosis in peripheral CD8 cells by transactivating Casp6 (Peng et al. 2009). Casp6 transcription may also be influenced by c-Myb since three binding sites for c-Myb are present in the upstream region of the Casp6 gene (Lang et al. 2005). At the level of activation, Casp6 can regulate its own activation through a self-cleavage mechanism (Wang et al. 2010). Similar to the induced proximity model, the autoactivation mechanism is driven by high concentrations of enzyme (Muzio et al. 1998, Klaiman et al. 2009). At low protein concentrations, the prodomain of Casp6 prevents both intramolecular and intermolecular cleavage of the intersubunit linker at the $TEVD_{193}$ site (Cao et al. 2014). In addition, mammalian ced-3 homologue 2β (Mch2β), an alternatively spliced isoform of Casp6 that does not possess any catalytic activity, prevents Casp6 activation *in vitro* and *in vivo* (Lee et al. 2010).

Mch2β inhibits the self-processing of Casp6 after removal of the prodomain, but has no effect on activated Casp6 (Lee et al. 2010). Furthermore, Casp6 activation is regulated by the transcription factor Sox11 (Waldron-Roby et al. 2015). When co-expressed with Sox11, Casp6 activation in HEK293FT cells is dramatically reduced due to Sox11 preventing Casp6 self-cleavage and through downregulation of active Casp6 (Waldron-Roby et al. 2015). Furthermore, Casp6 activation is regulated through phosphorylation by ARK5 at Ser257 *in vivo* and *in vitro* (Suzuki et al. 2004). This phosphorylation inhibits intramolecular cleavage of the Casp6 zymogen by locking it in a $TEVD_{193}$ bound inhibited conformation (Cao et al. 2012). Interestingly, if Casp6 has already been activated, phosphorylation at Ser257 can downregulate its activity (Cao et al. 2012, Velazquez-Delgado et al. 2012). Phosphorylation inhibits already activated Casp6 through steric hindrance that causes misalignment of the substrate-binding groove (Velazquez-Delgado et al. 2012). Furthermore, Zn^{2+} has a role in modulating caspase activity since Zn^{2+} depletion in cells induces apoptosis through caspase activation (Chai et al. 2000). Zn^{2+} allosterically inhibits Casp6 through binding to an exosite that locks it into an inactive, naturally occurring, latent conformation (Velazquez-Delgado et al. 2012). Finally, Casp6 is targeted for degradation by the proteasome (Tounekti et al. 2004). In primary human neurons, proteasomal inhibitors prevent 17β-E2-mediated RCasp6 degradation (Tounekti et al. 2004). In HEK293T cells, the overexpression and subsequent self-activation of Casp6 is not sufficient to induce apoptosis (Klaiman et al. 2009), but in the presence of proteasome inhibitors, Casp6 becomes proapoptotic (Gray et al. 2010). The mechanism of proteasomal-mediated degradation of Casp6 is different from Casp3 and Casp7. XIAP inhibits Casp3 and Casp7 and targets them for ubiquitin dependent proteasomal degradation (Huang et al. 2001, Suzuki et al. 2001). By contrast, XIAP does not interact with Casp6 (Deveraux et al. 1997). 17β-estradiol-mediated inhibition of Casp6 is consistent with the mechanism of p35 inhibition of caspases in which Casp6 is inhibited by a caspase inhibitory factor (Stennicke et al. 2002, Tounekti et al. 2004).

1.2.4. Substrates

Casp6 has a substrate specificity that is different from the other apoptotic effectors, yet similar to the apoptotic initiators (Thornberry et al. 1997). The preferred tetrapeptide motif for Casp3 and Casp7 is DEXD, whereas for Casp6, Casp8 and Casp9, the preferred tetrapeptide motif is ((I/L/V)EXD) (Thornberry et al. 1997). More recently, through the analysis of 871 Casp6 substrates in cell lysate, Casp6 showed an initiator-like VEVD↓(G/S/A) consensus motif with only a faint preference for aspartate at P4, dissimilar to Casp3 and Casp7 at the P4 position (Julien et al. 2016). In addition, Casp6 also exhibits a small preference for negatively charged residues at the P5 position (Julien et al. 2016). Consistent with these findings, Casp6 displays initiator-like activity since it can activate other caspases such as Casp2, Casp3, Casp8 and Casp10 (Slee et al. 1999, Allsopp et al. 2000, Cowling et al. 2002, Inoue et al. 2009). It has been demonstrated that differences in substrate specificity between Casp6 and Casp7 are due to four key substrate-contacting residues (Hill et al. 2016). Interestingly, the substitution of these residues from Casp6 into Casp7 yielded an inactive enzyme, whereas saturation mutagenesis of these sites yielded Casp7 variants with similar substrate specificities to Casp6 (Hill et al. 2016). Studies of this nature have yet to be performed between Casp3 and Casp6.

The substrate specificity of Casp6 enables it to be the only caspase that can cleave the nuclear filament protein lamin A during apoptosis (Orth et al. 1996, Takahashi et al. 1996, Ruchaud et al. 2002). The strict specificity of lamin A proteolysis by Casp6 allows for its endogenous catalytic activity to be monitored in cells (Ehrnhoefer et al. 2011, Mintzer et al. 2012). In addition, special AT-rich sequence binding protein (SATB1), a nuclear matrix protein, is also a unique Casp6 substrate (Galande et al. 2001). SATB1 recruits chromatin-remodeling factors to regulate chromatin structure and its cleavage by Casp6 causes it to become detached from chromatin early in T-cell apoptosis (Galande et al. 2001). Furthermore, as discussed in more detail below, Casp6 can uniquely cleave proteins involved in neurodegeneration such as APP, tau, presenilin 1 (PSEN1), presenilin 2 (PSEN2), huntingtin (Htt) and DJ-1 (Pellegrini et al. 1999, van de Craen et al. 1999, Guo et al. 2004, Graham et al. 2006, Giaime et al. 2010).

Although Casp6 activity alone may not be sufficient for apoptosis in certain cell types (Klaiman et al. 2009), it can cleave a variety of substrates shared by other caspases that contribute to the apoptotic phenotype. During apoptosis, Casp6 targets and degrades the intermediate filament cytoskeleton by cleaving desmin, vimentin, and cytokeratin (Caulin et al. 1997, Byun et al. 2001, Chen et al. 2003). In the nucleus, Casp6 targets transcriptional regulators such as cAMP responsive element binding (CREB)-binding protein (CBP), NF-κB and AP-2α (Levkau et al. 1999, Nyormoi et al. 2001, Rouaux et al. 2003). In addition, Casp6 can cleave proteins that are important for mediating and regulating DNA replication such as cyclin B1, DNA topoisomerase and telomerase reverse transcriptase (hTERT), the catalytic subunit of telomerase (Samejima et al. 1999, Chan et al. 2009, Soares et al. 2011). Casp6 can also cleave and activate the endonuclease DFF40 to mediate DNA degradation (Liu et al. 1999). Furthermore, in addition to lamin A, Casp6 can cleave substrates important for maintaining nuclear integrity such as lamin B, nuclear mitotic apparatus protein (NuMA) and emerin (Shimizu et al. 1998, Columbaro et al. 2001). Taken together, these studies suggest the Casp6 activity plays a role in blebbing of the plasma membrane, nuclear condensation and fragmentation.

Casp6 may also have more complex roles in apoptosis. Cho et al. carried out several proteomic analyses to identify thirty-four novel candidate Casp6 substrates (Cho et al. 2013). Of these proteins, herpesvirus-associated ubiquitin-specific protease (HAUSP), Kinesin5B, Par-3 Family Cell Polarity Regulator (PARD3), GEP100 and Serologically Defined Colon Cancer Antigen 3 (SDCCAG3) were validated as Casp6 substrates with an *in vitro* and *in vivo* cleavage assay (Cho et al. 2013). The functions of these substrates are quite diverse: HAUSP is a deubiquitinating enzyme that removes ubiquitin from the tumor suppressor protein p53 in order to stabilize it (Li et al. 2002); kinesin5B is a microtubule associated motor protein that transports vesicles along the microtubule (Brady 1985); and PARD3 is a PDZ-domain containing scaffold protein that regulates initial cell polarity cues (Suzuki et al. 2006). Therefore, during apoptosis, Casp6 may have a role in regulating p53 levels, membrane trafficking and cell polarization.

Casp6 also cleaves substrates important for preventing necroptosis, a form of programmed cell death that occurs in the absence of caspase activity and depends on the sequential activation of receptor interacting protein kinase 1 (RIPK1) and receptor interacting protein kinase 3 (RIPK3) (Vandenabeele et al. 2010). When apoptosis is induced through the intrinsic pathway, Casp6 cleaves RIPK1 (van Raam et al. 2013). In the absence of Casp6 activity, RIPK1 mediates the production of pro-inflammatory cytokines that contribute to the necroptotic phenotype (van Raam et al. 2013). Similarly, when apoptosis is induced through the extrinsic pathway, necroptosis is negatively regulated by autophagy that requires Casp6 activation (Ye et al. 2013). Casp6 may regulate autophagy by cleaving Autophagy-Related Protein 3 (Atg3) and p62 (Norman et al. 2010).

1.3. CASPASE-6 AND NEURODEGENERATION

Neurodegeneration is a widespread mechanism that involves the regression of axonal branches without causing cell death and naturally occurs during nervous system development to refine connectivity. Under pathological conditions, neurodegeneration can be induced by injury or stress and underlies the pathophysiology of neurodegenerative diseases such as AD, Huntington disease (HD) and Amyotrophic Lateral Sclerosis (ALS). Several studies demonstrate that Casp6 activity is involved in neurodegeneration.

1.3.1. Physiological Neurodegeneration: Axon Pruning

During nervous system development, the generation of neurons and extension of axons is followed by a regressive phase in which some axonal branches are pruned (Luo et al. 2005). Multiple studies have demonstrated a role for Casp6 in axonal pruning. In response to nerve growth factor (NGF) deprivation, a death receptor 6 (DR6) ligand such as APP is shed and binds to DR6 to trigger axon degeneration through Bax signaling and Casp6 activation (Nikolaev et al. 2009). Similarly, rat spinal neurons treated with prion peptide undergo axonal degeneration due to DR6 signaling and subsequent Casp6 activation (Wang et al. 2015). The APP-DR6-Casp6 pathway may also regulate neural connectivity in the adult brain since DR6 is highly expressed in the adult human

hippocampus and temporal cortex (Iyer et al. 2013). Furthermore, to regulate neural connectivity in the adult brain, Casp6 activation is required for p75 neurotrophin receptor (p75NTR)-mediated axonal degeneration (Park et al. 2010).

It is important to note that axon degeneration during axon specific pruning and apoptosis are two distinct pathways (Cusack et al. 2013). Axon specific pruning is Casp6 dependent while neuronal cell body apoptosis is Casp6 independent, and the specific location of NGF deprivation determines which pathway is activated (Cusack et al. 2013). Neuronal cell body apoptosis requires Bax signaling and Casp3 activity (Kuida et al. 1996, White et al. 1998). Although Casp3 is enriched in cell bodies whereas Casp6 is enriched in both cell bodies and axons following NGF deprivation, Casp3 activity may also be important for axonal pruning since it is a direct activator of Casp6 (Srinivasula et al. 1996, Nikolaev et al. 2009, Simon et al. 2012). Indeed, the genetic deletion of Casp3 in sensory axons is fully protective against NGF-withdrawal induced neurodegeneration whereas deletion of Casp6 only provides partial protection suggesting a more integral role for Casp3 (Simon et al. 2012). In addition, Casp6-dependent axonal pruning requires Casp3 and Casp9 (Cusack et al. 2013). However, axon pruning is delayed to an equal degree in Casp3 and Casp6 KO mice, implying that signaling pathways downstream of Casp3 may be different in different contexts and may not lead to Casp6 activation (Simon et al. 2012). Consistent with these findings, in NGF-deprived mouse dorsal root ganglion neurons, Casp3 activity peaks at twenty hours whereas Casp6 activity peaks at ten hours suggesting two parallel pathways (Schoenmann et al. 2010). Although Casp3 is a substrate for Casp6, NGF deprivation induces Casp3 activity by degrading XIAP bound to already active Casp3 (Allsopp et al. 2000, Schoenmann et al. 2010). By contrast, Casp6 is activated through proteolytic processing, presumably by proteases other than Casp3 (Schoenmann et al. 2010).

1.3.2. Pathological Neurodegeneration Mechanisms

In addition to NGF deprivation that occurs as a normal part of development, axon degeneration can be induced by pathological mechanisms that are also dependent on Casp6 activity. For instance, the overexpression of APP in primary human neurons co-

transfected with green fluorescent protein (GFP) results in a Casp6-dependent neuritic beading phenotype (Sivananthan et al. 2010). The increased levels of APP may be causing excess Casp3 activation and subsequent Casp6 activation to cause β-tubulin disruption and neurodegeneration (Simon et al. 2012). Moreover, mutations in *Dctn1*, a gene that facilitates retrograde transport, causes shedding of APP and subsequent Casp6 activation (Vohra et al. 2010). Defective axonal transport may cause degeneration through decreased availability of trophic factors in neuronal cell bodies (Vohra et al. 2010). In transgenic mouse models, mice that overexpress Casp6 exhibit axonal degeneration whereas Casp6 deficient mice are protected from N-methyl-D-aspartate (NMDA)-mediated excitotoxicity, NGF deprivation, and myelin-dependent axonal degeneration (Uribe et al. 2012, LeBlanc et al. 2014). Furthermore, following a stroke, Casp9 facilitates Casp6 activation, which subsequently induces axonal degeneration and neuronal cell death (Akpan et al. 2011). Taken together, these studies show that axonal degeneration can occur in a variety of contexts and that this process often involves Casp6 activity. However, there are cases where Casp6 does not seem to have a role in neurodegeneration. For instance, during Wallerian axonal degeneration, there are no differences in Casp6 activation in rat neural cells following spinal cord injury (Chu et al. 2007). Moreover, APP and Casp6 are not involved in axotomy induced axonal degeneration (Vohra et al. 2010). Furthermore, Casp6 has a role in preventing the regeneration of injured axons. Casp6 is upregulated in injured retinal ganglion cells and its inhibition promotes some regeneration whereas combined suppression of Casp2 and Casp6 enhances axon regeneration ten-fold (Monnier et al. 2011, Vigneswara et al. 2014). Interestingly, Casp6 is also involved in the transmission of neuropathic pain in the absence of neurodegeneration (Berta et al. 2014). In mice, Casp6 is expressed in C-fiber axonal terminals in the superficial spinal cord dorsal horn (Berta et al. 2014). In addition, extracellular Casp6 induces murine microglial activation and subsequent tumor necrosis factor α (TNF-α) secretion (Berta et al. 2014). These results suggest that Casp6 is released from axonal terminals to regulate neuropathic pain.

1.3.3. Neurodegenerative Diseases

With Casp6 having a role in physiological and induced axonal degeneration, it should come as no surprise that Casp6 activity is present in the pathophysiology of several neurodegenerative diseases. Excess Casp6 activity is present in neurofibrillary tangles (NFTs), neuropil threads (NPTs) and neuritic plaques (NPs) in AD (LeBlanc et al. 1999, Guo et al. 2004). In addition, Casp6 is at least partly responsible for the processing of the mutant Htt (mHtt) protein in HD (Wong et al. 2015). Casp6 is also involved in other neurodegenerative diseases including ALS, Parkinson disease (PD) and human immunodeficiency virus (HIV)-mediated neurodegeneration.

1.3.3.1. Alzheimer Disease

AD is a progressive neurodegenerative disorder characterized by initial mild cognitive impairment followed by deterioration of memory and other cognitive domains. In AD, misfolded proteins accumulate in the aging brain resulting in oxidative inflammatory damage, which subsequently causes energy failure and synaptic dysfunction (Querfurth et al. 2010). With the tau and amyloid hypotheses sparking debates between scientists for more than twenty-five years, the etiology of AD is still controversial. Although amyloid plaques and NFTs indeed contribute to AD pathophysiology, their elimination may not improve neuronal dysfunction and cognitive performance since the degenerating pathways that form these lesions would still be activated (LeBlanc 2013). Thus, identifying the events that occur before the accumulation of these lesions is essential for the development of novel therapeutics. Mounting evidence has implicated Casp6 in the initiation of several parallel pathways of neurodegeneration that are associated with AD.

1.3.3.1.1. Amyloid-β Production

Several experiments have demonstrated a role for Casp6 in the overproduction of Aβ. Serum-deprived primary human neuron cultures exhibit increased levels of Aβ (LeBlanc 1995), which are dependent on Casp6 activity (LeBlanc et al. 1999). Although APP is a substrate for Casp6 (LeBlanc et al. 1999, Pellegrini et al. 1999, Weidemann et al. 1999), its activation increases Aβ generation independently of caspase-mediated APP cleavage (Tesco et al. 2003). In Chinese hamster ovary (CHO) cells overexpressing APP, etoposide-induced apoptosis enhances Aβ production; however, site-directed mutagenesis

of APP at the Casp6 cleavage site did not prevent Aβ production (Tesco et al. 2003). Casp6 increases Aβ production through an indirect mechanism by enhancing beta-site APP cleaving enzyme (BACE) activity (Tesco et al. 2007). Casp6 cleaves golgi associated, gamma adaptin ear containing, ARF binding protein 3 (GGA3), an adaptor protein involved in BACE trafficking to lysosomes (Tesco et al. 2007). Pulse chase analyses revealed that the approximate half-life of BACE in CHO cells was nine hours under normal conditions, but following caspase activation with staurosporine (STS), BACE levels did not significantly decrease (Tesco et al. 2007).

1.3.3.1.2. C31-mediated Axonal Degeneration

In AD, Casp6 and mutant APP are involved in axonal degeneration since the expression of mutant APP co-transfected with GFP results in a Casp6-dependent neuritic beading phenotype (Sivananthan et al. 2010). Surprisingly, neuritic beading was still induced when Aβ production was inhibited through site-directed mutagenesis of the BACE cleavage site, suggesting that axonal degeneration is Aβ independent (Sivananthan et al. 2010). Therefore, an alternative mechanism must exist that may involve the Casp6 APP cleavage product. Cleavage of APP by Casp6 at the $VEVD_{664}$ site produces an intracellular thirty-one amino acid peptide (C31) that does not have a role in Aβ production (LeBlanc et al. 1999, Pellegrini et al. 1999, Weidemann et al. 1999, Tesco et al. 2003, Tesco et al. 2007). In comparison with non-AD age-matched controls, AD brains show a significant increase in caspase cleavage of APP at $VEVD_{664}$ in the entorhinal cortex ERC and hippocampus (Zhao 2003, Banwait et al. 2008). Interestingly, $VEVD_{664}$ cleaved APP was also found to be prominent in non-diseased human brains less than forty-five years of age, suggesting that $VEVD_{664}$ processing may be a part of normal proteolytic processing and that this cleavage is down regulated with aging, but aberrantly increased in AD (Banwait et al. 2008). The C31 peptide may contribute to neurodegeneration since it has been demonstrated to be toxic in cell culture models. C31 is a potent inducer of apoptosis in cultured neuroblastoma cells and in primary cultures of neurons (Lu et al. 2000, McPhie et al. 2001). The mechanism of cytotoxicity involves C31 interacting with APP, since C31 toxicity is absent in cells lacking endogenous APP (Park et al. 2009). C31 complexes with APP to recruit binding partners that initiate

signals related to cellular toxicity (Park et al. 2009). In addition, C31 can also inhibit XIAP by binding through a second mitochondria-derived activator of caspase/direct IAP binding protein with low pI (Smac/DIABLO)-like motif, thereby promoting amplification of the caspase cascade (Hell et al. 2003). Furthermore, transgenic mouse models have also demonstrated the crucial role of cleavage at $VEVD_{664}$ in the development of AD-like pathology. APP transgenic mice with a mutated Casp6 cleavage site have normal synaptic transmission, synaptic plasticity and learning despite the presence of elevated levels of APP, $A\beta_{42}$ and plaques (Saganich et al. 2006). Moreover, synaptic loss, dentate gyral atrophy, astrogliosis and behavioral abnormalities are also completely prevented in these mice (Galvan et al. 2006). Mutations in Asp664 have also been shown to prevent AD-like behavioral deficits in transgenic APP V717F (PDAPP) mice as old as thirteen months, suggesting that it either completely prevents or markedly delays disease onset (Galvan et al. 2008). Memory deficits in these mice are prevented only with early training suggesting that C31 prevents retention of information more than learning itself (Zhang et al. 2010). Finally, PAK signaling, which is involved in the pathophysiology of AD (Zhao et al. 2006), requires cleavage of APP at Asp664 (Nguyen et al. 2008). PDAPP mice have significant increases in PAK signaling in the hippocampus whereas this effect is completely prevented in PDAPP D664A mice (Nguyen et al. 2008).

By contrast, another study has suggested that the C-terminal Casp6 cleavage product does not have a role in AD pathogenesis (Harris et al. 2010). J20 mice (Mucke et al. 2000) were compared to B254 mice (Galvan et al. 2006), which encode the additional D664A mutation that prevents C31 release (Weidemann et al. 1999). When each of these mouse lines were compared to a non-transgenic control, J20 and B254 mice had a similar degree of abnormalities in spatial and non-spatial learning and memory, elevated maze performance, synaptic transmission and levels of synaptic activity-related proteins (Harris et al. 2010). These findings suggest that these abnormalities do not require caspase cleavage of APP at position D664 and are not caused by C31.

. **1.3.3.1.3. Tau Cleavage**

Active Casp6 and tau cleaved by Casp6 (TauΔCasp6) is present in pre-tangles and mature tangles in AD brains (Guo et al. 2004). Using a neoepitope antibody that specifically recognizes the p20 subunit of active Casp6, Guo et al. showed that sporadic AD brains have two- to three-fold more active Casp6 in the temporal and frontal cortex, whereas Casp6 is absent in the cerebellum (Guo et al. 2004). Using an additional neoepitope antibody that recognizes TauΔCasp6 at Asp402, Guo et al. also showed that both active Casp6 and TauΔCasp6 are present in NTs, NPs and NFTs (Guo et al. 2004). Both antisera did not stain young brains (<45 years of age), and non-AD neurons were also not stained in AD affected areas, thereby confirming their specificity for AD pathological neurons (Guo et al. 2004). Active Casp6 also co-localizes with hyperphosphorylated tau in the brainstem of AD patients (Wai et al. 2009). In AD brains, Casp6 can also cleave tau at Asp13 and this cleavage event temporally correlates with Casp3-mediated cleavage at Asp421 (Horowitz et al. 2004).

Interestingly, active Casp6 and TauΔCasp6 is present in the hippocampus and cortex of aged non-cognitively impaired (NCI), mild cognitively impaired (MCI) individuals and in individuals with mild to severe sporadic AD (SAD) (Albrecht et al. 2007). Moreover, active Casp6 and TauΔCasp6 is abundant in individuals with severe familial AD (FAD) involving mutations in APP, PSEN1 and PSEN2 (Albrecht et al. 2009). The presence of TauΔCasp6 in both SAD and FAD suggests that Casp6 activity plays a fundamental role in the pathophysiology of AD. Recently, it was demonstrated that Casp6 activity and tau cleavage are increased in cells treated with hydrogen peroxide in a dose-dependent manner, suggesting that other stresses such as an age-related insult in SAD or mutant proteins in FAD, could also have a synergistic role in Casp6 activation (Zhao et al. 2014).

1.3.3.1.4. Other Caspase-6 Targets in AD

In contrast to apoptotic neurons in which active Casp6 localizes to the nucleus and cytoplasm, active Casp6 in AD neurons localizes to the neurites and NFTs without exhibiting the classical features of apoptosis (Guo et al. 2004). A non-apoptotic role for Casp6 was later confirmed in HEK293T cells. Casp6 overexpression in HEK293T cells results in Casp6 activation in the absence of apoptosis (Klaiman et al. 2009). Similarly,

Casp6 activation induced by an optimized orthogonal protease also did not induce cell death (Gray et al. 2010). In order to elucidate the non-apoptotic role of Casp6 in AD neurons, Klaiman et al. conducted a proteomic analysis of Casp6-mediated cleavage of human neuronal proteins (Klaiman et al. 2008). The Casp6 substrates identified included proteins important for structural integrity, cell signaling, protein synthesis, protein degradation, membrane and lipid binding and metabolism (Klaiman et al. 2008). Among these substrates, 40% were cytoskeleton and cytoskeleton associated proteins. In this study, the cleavage of the cytoskeleton protein α-tubulin, and cytoskeletal-associated proteins actinin-4, spinophilin, and drebrin were confirmed through *in vitro* translation analyses (Klaiman et al. 2008). Similar to TauΔCasp6, a neoepitope antiserum to α-tubulin cleaved by Casp6 immunostained NTs, NFTs and NPs in AD neurons and co-localized with Casp6 activity (Klaiman et al. 2008). Casp6 cleaves α-tubulin at Asp438, the same site cleaved by granzyme B, a serine protease secreted by cytotoxic T lymphocytes and NK cells to mediate apoptosis (Adrain et al. 2005). Cleavage at this site by granzyme B enhances microtubule polymerization, suggesting that α-tubulin cleavage by Casp6 in AD could lead to cytoskeleton disruption through aberrant microtubule dynamics (Adrain et al. 2005). Actinin-4, spinophilin, and drebrin, the other three Casp6 substrates identified from this study, each have roles in regulating postsynaptic densities of dendritic spines through interactions with actin (Allen et al. 1997, Shirao et al. 2001, Otey et al. 2004). Therefore, Casp6-mediated cleavage of these substrates may be responsible for synaptic impairment in AD. Consistent with this hypothesis, Casp6 activity (Ramcharitar et al. 2013) and synaptic loss (Scheff et al. 2003) both show a positive correlation with cognitive impairment, suggesting that Casp6 may cause synaptic loss before the onset of AD. In addition, the level of drebrin is known to decrease in AD hippocampal synapses and knockdown of drebrin in rats causes cognitive impairment (Harigaya et al. 1996, Kobayashi et al. 2004). Similarly, spinophilin KO mice exhibit learning problems (Stafstrom-Davis et al. 2001).

In addition to cytoskeleton and cytoskeletal-associated proteins, Klaiman et al. also identified the chaperone proteins heat shock protein 90 α (Hsp90α), heat shock protein gp96 and valosin containing protein (VCP) as potential Casp6 substrates (Klaiman et al.

2008). These proteins facilitate protein degradation through the ubiquitin proteasomal system (UPS); therefore, Casp6 activity may result in the accumulation of polyubiquitinated proteins into misfolded aggregates. In AD, increased ubiquitination has been associated with NFTs, senile plaque neurites and paired helical filaments (Mori et al. 1987, Perry et al. 1987). A follow-up study confirmed VCP, a chaperone like ATPase, as a substrate for Casp6 (Halawani et al. 2010). When full-length VCP is incubated with Casp6 it generates cleavage products that are not seen with effectors Casp3 and Casp7, suggesting that the Casp6 cleavage sites are distinct (Halawani et al. 2010). Through characterization of a VCP Casp6 cleavage site, a neoepitope antibody was developed and showed that VCP cleaved by Casp6 was more abundant in the cell soma and neurites of AD hippocampal neurons relative to NCI controls (Halawani et al. 2010). These findings further reinforce a role for Casp6 in AD as being involved in multiple parallel neurodegenerative pathways.

1.3.3.1.5. Cognitive Impairment

In addition to AD related pathologies, Casp6 activity has been implicated in cognitive impairment. In contrast to an overall distribution of Casp6 or TauΔCasp6 in the hippocampus and cortex of SAD brains, Casp6 activity was confined mostly to the entorhinal cortex of some non-cognitively impaired individuals (Albrecht et al. 2007). Interestingly, the entorhinal cortex is the first region of the brain to be affected by tau pathology (Braak et al. 1997). In NCI individuals, the levels of TauΔCasp6 in the hippocampus inversely correlated with global cognitive scores, suggesting that Casp6 activity influences cognition in the elderly and precedes the clinical diagnosis of AD (Albrecht et al. 2007). A follow-up study involving a larger cohort of aged NCI individuals showed that higher levels of Casp6 in the CA1 region and the entorhinal cortex were predictive of lower episodic memory ability (Ramcharitar et al. 2013). Furthermore, levels of cerebrospinal fluid (CSF) TauΔCasp6 correlate with AD severity and lower global cognitive scores (Ramcharitar et al. 2013). When global cognitive scores and TauΔCasp6 scores were averaged for NCI, MCI, mild, moderate and severe AD cases, a strong linear regression was achieved (r^2 =0.94) (Ramcharitar et al. 2013). Lastly, a Casp6 mouse model has recently been developed in which these mice express a

self-activated form of human Casp6 in the CA1 region of the hippocampus (LeBlanc et al. 2014). These mice exhibit age-dependent spatial and episodic memory impairment in the absence of amyloid plaques and NFTs, suggesting that Casp6 is responsible for lower cognitive scores in NCI individuals (LeBlanc et al. 2014).

1.3.3.1.6. Neuroinflammation

Although Casp6 is an apoptotic effector caspase, a role for Casp6 in neuroinflammation has recently drawn considerable attention. Neuroinflammation is a feature of AD since IL-1β is overexpressed in AD brains and this results in enhanced microglia activation (Griffin et al. 1989). Neuroinflammation is an early signaling event that contributes to the pathophysiology of AD through several signaling pathways. For example, neuroinflammation may contribute to the formation of Aβ plaques since there is a temporal correlation with activated microglia and neuritic plaque distribution (Sheng et al. 1995). In addition, IL-1β promotes expression and phosphorylation of neurofilament and tau proteins (Sheng et al. 2000). More recently, Kaushal et al. demonstrated a role for Casp6 in neuroinflammation (Kaushal et al. 2015). In serum-deprived primary human neurons, Casp6 activation is dependent on NLRP1 and inflammatory Casp1 activity (LeBlanc et al. 1999, Guo et al. 2006, Kaushal et al. 2015). CNS primary human neurons express NLRP1, a component of the NLRP1 inflammasome complex that mediates the activation of Casp1. In AD neurons, the expression of NLRP1 is increased 25- to 30- fold relative to NCI controls (Kaushal et al. 2015). Following its activation by NLRP1, Casp1 mediates Casp6 activation through removal of the prodomain to facilitate Casp6 self-cleavage within the intersubunit linker (Lee et al. 2010, Wang et al. 2010). Support for this mechanism was demonstrated through immunohistochemical staining in which NLRP1 was shown to co-localize with TauΔCasp6 in AD brains (Kaushal et al. 2015). Interestingly, extracellular Casp6 may also have an important role in neuroinflammation since microglial cultures treated with RCasp6 induced microglia activation and TNF-α secretion (Norman et al. 2010). On the other hand, neuroinflammation may also be a consequence of Casp6 activity since mice that overexpress active Casp6 in the CA1 region have increased glial inflammation (LeBlanc et al. 2014). A positive feedback loop may exist in which Casp1 processes pro-IL-1β, which subsequently induces microglial

activation. The activated microglia would then secrete cytokines to induce NLRP1 signaling and subsequently more Casp1 and Casp6 activity. Therefore, in addition to Casp6, NLRP1 and Casp1 may represent potential targets for the development of novel therapeutics against AD.

1.3.3.2. Huntington Disease

HD is an inherited neurodegenerative disease characterized by progressive motor, cognitive and psychiatric impairment (Novak et al. 2010). It is a single gene disease caused by expanded CAG trinucleotide repeats within the Htt gene (The Huntington's Disease Collaborative Research Group, 1993). The encoded polyglutamine causes progressive neurodegeneration that selectively targets GABAergic medium spiny striatal neurons and glutamatergic cortical neurons that project to the striatum (Albin et al. 1990, Albin et al. 1992). The accumulation of N-terminal Htt fragments in human HD brains suggests either enhanced proteolysis or impaired clearance of Htt underlies the pathophysiology of HD (Gutekunst et al. 1999). Indeed, Casp6 has been confirmed to be involved in both situations. The initial observation of Casp6 being involved in HD was made by Wellington et al. who demonstrated that Htt could be cleaved by Casp6 at Asp586 *in vitro* and *ex vivo* (Wellington et al. 2000). Two Casp3 cleavage sites at positions Asp513 and Asp552 were also identified (Wellington et al. 2000). Following treatment with tamoxifen, neuronal and non-neuronal cells with mHtt that had Casp3 and Casp6 resistant cleavage sites exhibited less cytotoxicity and mHtt aggregation than cells with cleavable mHtt (Wellington et al. 2000). Although this study showed a role for caspases being involved in mHtt-mediated toxicity, the toxicity of caspase-specific mHtt fragments was not compared.

Follow-up studies have demonstrated an essential role for Casp6, but not Casp3, in the pathophysiology of HD. In humans, CAG repeat size inversely correlates with age of HD onset and directly correlates with Casp6 levels and its catalytic activity (Graham et al. 2010). To study the effect of the mHtt protein *in vivo*, yeast artificial chromosome (YAC) mouse models of HD with the human HD gene containing 46 (YAC46), 72 (YAC72) or 128 (YAC128) CAG repeats have been developed (Hodgson et al. 1999, Slow et al.

2003). These mice display cognitive impairment followed by motor deficits and age-dependent striatal neurodegeneration with the severity of symptoms correlating with levels of mHtt (Slow et al. 2003, Van Raamsdonk et al. 2005, Graham et al. 2006). In addition, YAC128 mice resistant to Casp6 cleavage at Asp586 do not display the behavioral and neuropathological features of HD whereas mice resistant to Casp3 cleavage at Asp513 and Asp552 display striatal degeneration (Graham et al. 2006, Pouladi et al. 2009). Similarly, transgenic mice expressing the Casp6 derived N-terminal fragment of mHtt also develop neurologic abnormalities (Tebbenkamp et al. 2011). YAC mice resistant to Casp6 cleavage of Htt also show low of levels of active Casp6 relative to YAC mice controls with cleavable Htt, suggesting that the cleaved Htt fragment may be part of a positive amplification cycle of Casp6 activation (Graham et al. 2010). Treatment of YAC128 primary striatal neurons with NMDA caused an increase in Casp6 activation, but not in Casp6 cleavage site resistant neurons, suggesting that excitotoxicity may cause initial Casp6 activation (Graham et al. 2010). Enhanced excitotoxicity seen in HD mouse models may be due to impaired glutamate uptake by glial cells (Faideau et al. 2010, Huang et al. 2010). Furthermore, elevated extrasynaptic NMDA receptor signaling and expression contributes to the HD phenotype and requires Casp6 cleavage of mHtt (Milnerwood et al. 2010). Lastly, primary striatal neurons expressing mHtt treated with synthetic Casp6 inhibitors or co-expressing a catalytically inactive dominant-negative form of Casp6 are protected from neurodegeneration (Hermel et al. 2004, Graham et al. 2010, Aharony et al. 2015).

These studies have indeed shown a role for Casp6 in HD pathogenesis; however, more recent findings involving other mouse models of HD have suggested that other proteases can also cleave mHtt at the Casp6 cleavage site. The genetic ablation of Casp6 in the HdhQ150 knock-in mouse model showed that loss of Casp6 has no effect on the proteolysis of Htt or the levels of full length Htt protein (Landles et al. 2012). Furthermore, in a bacterial artificial chromosome (BAC) mouse model of HD, Casp6 KO mice also do not show a reduction in the proteolysis of mHtt at Aps586; however, they have reduced levels of both full-length and cleaved htt (Gafni et al. 2012). These findings suggest that Casp6 may have an alternate role in impairing the clearance of the Htt

protein through the UPS (Gafni et al. 2012). In contrast to the BACHD and HdhQ150 mouse models, YAC128 Casp6 KO mice show reduced, but not absent, levels of cleaved mHtt (Wong et al. 2015). Each of these mouse models suggests that Casp6 activity alone cannot account for the accumulation of mHtt fragments. Similar to Casp6, Casp8 and Casp10 can also cleave mHtt at position Asp586; however, full-length Casp8 levels in YAC128 Casp6 KO mice were not significantly different compared to YAC128 control mice, suggesting that other proteases may also be involved in the cleavage of mHtt at position Asp586 (Wong et al. 2015).

The nuclear translocation of Htt and the formation of neuropil aggregates are neuropathological hallmarks of HD, and in YAC128 mice, this occurs earliest and to the greatest extent in the striatum (Davies et al. 1997, Gutekunst et al. 1999, Van Raamsdonk et al. 2005). The specific cleavage of Htt at Asp586 may be responsible for its translocation to the nucleus in striatal neurons. Using neoepitope antibodies specific to Htt cleaved at specific sites, it was demonstrated that Casp6-derived fragments cleaved at Asp586 localized to the nucleus whereas Casp3-derived fragments cleaved at Asp552 localized to the perinuclear region (Warby et al. 2008). In addition, endogenous Casp6 zymogen is localized to both the cytoplasm and nucleus whereas active Casp6 is localized in only the nuclear region suggesting that in response to an apoptotic stress, zymogen Casp6 and full-length Htt are trafficked to the nucleus where Casp6 activation occurs and subsequently cleaves Htt (Warby et al. 2008). The mechanism of how Htt is transported into and out of the nucleus is poorly understood. Phosphorylation of Htt at Ser421 by the pro-survival kinases Akt and serine/threonine protein kinase (SGK) may reduce Htt nuclear accumulation by preventing Casp6-mediated Htt cleavage, by reducing the levels of full-length Htt, and by preventing its nuclear localization (Warby et al. 2009). Under physiological conditions, the lowest levels of phosphorylation occur in the striatum and are further reduced in the presence of the polyglutamine tract (Warby et al. 2005). In addition to phosphorylation, the polyglutamine tract may interact with other proteins that mediate the localization of mHtt to the nuclear region (Preisinger et al. 1999).

1.3.3.3. Parkinson Disease

PD is a movement disorder characterized by a massive loss of dopaminergic neurons of the substantia nigra and the accumulation of intra-cytoplasmic inclusions known as Lewy bodies. Oxidative stress is the primary pathogenic mechanism of substantia nigra dopaminergic cell death and is associated with Casp6 activation (Yoo et al. 2003). Most PD cases are of sporadic origin but about five percent of them are of genetic origin and are linked to mutations in parkin, phosphatase and tensin homolog (PTEN)-induced putative kinase (PINK-1) and DJ-1 (Gasser 2005). Of these genes products, DJ-1 has been demonstrated to be a Casp6 substrate (Giaime et al. 2010). DJ-1 is a highly conserved homodimeric protein expressed in the brain that harbors a protective role against oxidative stress (Bandopadhyay et al. 2004, Martinat et al. 2004, Taira et al. 2004). The Casp6 cleavage resistant D149A mutation seen in some recessive forms of PD abolishes its protective phenotype (Giaime et al. 2010). When Casp6 cleaves DJ-1 in TSM1 neurons and neuroblastoma cell lines after STS treatment, the C-terminal fragment exerts an anti-apoptotic function by inhibiting the p53 pathway (Giaime et al. 2010). By contrast, many studies have implicated Casp6 as having a neurodegenerative role in dopaminergic neurons. For instance, the N-terminal fragment of DJ-1 has a pro-apoptotic function by increasing reactive oxygen species (ROS) production and sensitizing neurons to 1-methyl-4-phenylpyridinium ion (MPP^+)-mediated apoptosis (Robert et al. 2012). Similar to Htt cleaved by Casp6, the N-terminal of DJ-1 localizes to the nucleus and its exclusion from the nucleus abrogates its apoptotic effect (Robert et al. 2012). Furthermore, in dopaminergic neurons treated with the neurotoxin paraquat, Casp6 cleaves p55PIK, a subunit of phosphatidylinositol 3-kinases that have roles in cell proliferation, anti-apoptotic pathways and cell cycle progression (Zhou et al. 2014). Taken together, these studies suggest in response to ROS under physiological conditions, Casp6 has a neuroprotective effect through cleavage of DJ-1, whereas excess ROS under pathological conditions induces multiple parallel degenerative pathways.

1.3.3.4. Amyotrophic Lateral Sclerosis

In contrast to other neurodegenerative diseases, Casp6 may have a protective role in ALS, a motor neuron disease characterized by the selective degeneration of motor

neurons of the spinal cord resulting in muscular atrophy, paralysis and eventually death. Mutations in the copper/zinc superoxide dismutase (SOD1) gene are associated with the onset of familial ALS and mice overexpressing the human mutant $SOD1^{G93A}$ gene develop the pathological hallmarks of ALS (Rosen et al. 1993, Gurney et al. 1994). Surprisingly, $SOD1^{G93A}$ mice with a Casp6 deletion have a significantly exacerbated motor phenotype (Hogg et al. 2016). In addition, the onset of disease occurred much earlier in these mice and they had shorter lifespans relative to SOD1 mice without a Casp6 deletion (Hogg et al. 2016). Therefore, Casp6 may have a protective role in ALS, however its mechanism of action has not been determined.

1.3.3.5. HIV-mediated Neurodegeneration

HIV infection of the brain leads to a neurodegenerative condition known as HIV-associated neurocognitive disorder (Cysique et al. 2009). The most severe form of this disorder is often termed HIV encephalitis (HIVE) due to the presence of reactive astrocytosis, microglial nodules and multinucleated giant cells (Gray et al. 2001). The first high-throughput microribononucleic acid (miRNA) profiling study of HIVE brains showed that Casp6 was differentially regulated (Noorbakhsh et al. 2010). Immunohistochemical analysis of HIVE brain sections revealed that active Casp6 was upregulated in astrocytes but not in neurons (Noorbakhsh et al. 2010). In addition, primary astrocytes exposed to the HIV viral protein have increased levels of p53, loss of mitochondrial permeabilization and increased Casp6 activity (Noorbakhsh et al. 2010).

1.3.3.6. Ischemic Neurodegeneration

Ischemic strokes are most often caused by thromboembolism, which is the physical blockage of blood flow to the brain. Due to a lack of oxygen and neuronal energy depletion, the affected area undergoes apoptosis that may be mediated through Casp6 activation. Active Casp6 is present in apoptotic neurons of fetal and adult ischemic brains (Guo et al. 2004). In addition, active Casp6 is present in the cell soma and neuronal processes after a stroke and is activated by initiator Casp9 (Akpan et al. 2011). Inhibition of Casp6 through intranasal Casp9 inhibitors was neuroprotective against cerebral ischemia (Akpan et al. 2011). Furthermore, in a thromboembolic cerebral stroke mouse

model, Casp6 and Casp8 inhibitors reduced infarct volume, reduced edema, reduced neurological deficits, increased the number of proliferating cells, and increased neurofilament levels after middle cerebral artery occlusion (MCAO) (Shabanzadeh et al. 2015). Lastly, Casp6 KO mice demonstrate improved neuronal retention and neurological function relative to wild type (WT) controls following transient MCAO (Akpan et al. 2011). These findings show that Casp6 plays a role in ischemic neuronal degeneration since its inhibition following an ischemic injury attenuates the neuropathological consequences of cerebral infarct.

1.4. CASPASE-6 OUTSIDE THE BRAIN

During fetal development, Casp6 is ubiquitously expressed in most tissues with the lowest levels in the brain and the highest levels in the gastrointestinal system (Godefroy et al. 2013). Cells expressing active Casp6 in these tissues had morphological features of apoptosis, indicating that Casp6 may be important in developmental cell death (Godefroy et al. 2013). In adult tissues, Casp6 expression is lower relative to fetal tissues, but is still present in the colon, stomach, kidney, lung, liver and skin (Godefroy et al. 2013). Consistent with these findings, numerous reports have demonstrated roles for Casp6 in tissues outside the brain. Interestingly, the low levels of Casp6 in both fetal and adult brain tissues indicates that the increased levels of Casp6 in AD are indeed pathogenic (Godefroy et al. 2013).

1.4.1. Apoptotic Targets

Casp6 has the capacity to induce apoptosis in certain cell types. During development, Casp6 but not Casp3, is involved in the regulation of hypoxia-induced apoptosis in tube-forming endothelial cells (Eguchi et al. 2009). In colon epithelial cells, Casp6 cleaves guanylate cyclase alpha 1 during deoxycholate induced apoptosis (Payne et al. 2003). This cleavage event is absent in deoxycholate resistant cancer cells; therefore, Casp6 has a tumor-suppressing role in the colon (Payne et al. 2003). In intestinal epithelial cells undergoing anoikis, a form of apoptosis that occurs when anchorage-dependent cells detach from the extracellular matrix, Casp6 is activated following detachment suggesting that is may be involved in intestinal epithelium homeostasis (Grossmann et al. 1998). In

human hepatoma cell lines, Casp6 has a regulatory role in the promotion of bile acid induced apoptosis (Rust et al. 2009). Eosinophils undergoing spontaneous or nitric oxide induced apoptosis also require Casp6 activity (Ilmarinen-Salo et al. 2010). In addition, Casp6 is activated under pathological conditions. Human and rat islet cells exposed to glucotoxic conditions results in Casp6 activation and lamin A degradation and subsequent metabolic dysregulation (Khadija et al. 2014). Also, *Streptococcus* pneumonia-induced apoptosis of lung epithelial cells is Casp6 dependent (Schmeck et al. 2004). Furthermore, Casp6 activity is involved in some cancer treatments. Lymphoma cell lines treated with various anticancer drugs induce Casp6 cleavage of the mammalian target of rapamycin complex 1 (mTORC1) component raptor (mTOR) and mutagenesis of the Casp6 cleavage sites confers resistance to cell death (Martin et al. 2016). Photodynamic therapy (PDT) with hexaminolevulinate (HAL) in human B cell lymphoma Ramos and Daudi cell lines requires Casp6 activation and Casp6-mediated cleavage of lamin A (Shahzidi et al. 2013). Treatment of these cell lines with a Casp6 inhibitor abrogated HAL-PDT-mediated cleavage of both Casp6 and lamin A and subsequent apoptosis in both cell lines (Shahzidi et al. 2013). Aloe emodin induces cell cycle arrest and apoptosis in human colon cancer cells through Casp6 and Casp9 activation, rather than Casp3 and Casp7 activation (Suboj et al. 2012). ZBP-89 also induces cell cycle arrest in human colon cancer cells through Casp6 activation (Chen et al. 2009). Taken together, these results suggest that Casp6 in certain cell types is sufficient to induce apoptosis, especially when induced with chemotherapeutics.

1.4.2. Immune System

Casp6 has a role in the adaptive immune system by regulating lymphocyte activation. Following activation with a proliferative stimulus, human B cells have increased Casp6 activity and proliferation of B cells is blocked with Casp6 inhibitors, suggesting that Casp6 has a regulatory role in selectively allowing entry of quiescent G_0 B cells to enter the G_1 cell cycle phase (Olson et al. 2003). Indeed, this was demonstrated in Casp6 null mice in which Casp6 null B cells have more cells in G_1 cell cycle phase than in WT B cells (Watanabe et al. 2008). In addition, Casp6 null mice have enhanced plasma cell formation and antigen specific antibody production (Watanabe et al. 2008). Furthermore,

cleavage of 5-lipoxygenase by Casp6 in the Burkitt lymphoma line BL41 correlates with proliferation, again suggesting that Casp6 activity has a role in B cell activation (Werz et al. 2005). More recently, it has been demonstrated that Casp6 also has a role in the innate immune system through its ability to activate macrophages. Interleukin-1 associated kinase-M (IRAK-M), an inhibitor of the innate immune response expressed in alveolar macrophages, is a Casp6 substrate (Kobayashi et al. 2011). During inflammation, neutrophils are recruited to alveolar macrophages and their interaction promotes Casp6 activation and subsequent IRAK-M cleavage (Kobayashi et al. 2011). Casp6 also promotes the activation of alternatively activated macrophages (AAM) while Casp6 disruption suppresses levels of AAM biomarkers (Yao et al. 2016). Interestingly, Casp6 activity in 4T1-tumor associated macrophages modulates matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) expression, suggesting that Casp6 activity can promote tumor cell invasion (Yao et al. 2016).

1.4.3. Differentiation

Casp6 may have a role in lens fiber cell differentiation, which involves the degradation and elimination of all membrane-bound organelles through a process similar to apoptosis (Foley et al. 2004). Lens extracts from rat embryos showed high VEIDase activity during the period organelles were eliminated while zymogen Casp6 levels decreased suggesting a differentiation role for Casp6 (Foley et al. 2004). However, this suggestion has been disputed because Casp6 null mice have properly formed lenses (Zandy et al. 2005). VEIDase activity observed in the lysates from Casp6 KO lenses was indistinguishable from that measured in WT samples since the proteasome also demonstrates VEIDase activity (Zandy et al. 2005). By contrast, excessive Casp6 activity leads to morphological abnormalities in mouse lens secondary fiber cells suggesting that it may have a redundant regulatory role during lens cell differentiation (Morozov et al. 2006). In addition, Casp6 mediates myeloid progenitor cell differentiation (Leong et al. 2010). Mutant nucleophosmin seen in acute myeloid leukemia inhibits Casp6 and Casp8 and subsequently hinders myeloid differentiation (Leong et al. 2010). Deguelin, a selective silencer of mutant nucleophosmin, induces differentiation and apoptosis of mutant AML cells by relieving the inhibition on Casp6 and Casp8 (Yi et al. 2015).

1.5. CASPASE-6 INHIBITORS

Casp6 is an attractive therapeutic target because it has roles in the pathogenesis of AD. Physiologically, Casp6 signaling may be dispensable since Casp6 null mice develop normally without any noticeable apoptotic defects (Zheng et al. 1999). In fact, B cell differentiation into plasma cells is accelerated in Casp6 KO mice and thus, they have more antigen-stimulated production of antibodies, suggesting that Casp6 inhibition in AD patients may increase their immunity (Watanabe et al. 2008). However, another Casp6 KO mouse model showed that these mice have age-dependent increases in cortical and striatal volume, hypokinetic behavior and learning deficits (Uribe et al. 2012). Therefore, it may be important to downregulate its activity rather than completely inhibit it. In addition, since Casp6 is closely related to apoptotic effector caspases, this suggests that its inhibition may potentially lead to cancer. However, no studies have shown a role for Casp6 in preventing cancer. Casp6 is expressed in peripheral tissues with the highest being in the colon (Godefroy et al. 2013). Inflammation-induced tumorigenesis in the mouse colon is Casp6 independent since there are no noticeable differences in tumor formation between WT and Casp6 KO mice (Foveau et al. 2014). Although Casp6 may not initiate tumor formation, its inhibition has been shown to promote chemoresistance in cancer cells. Survivin-3B increases the resistance of neoplastic cells to chemotherapeutics by inhibiting Casp6 activation (Vegran et al. 2013). Furthermore, some cancer treatments rely on Casp6 activity for apoptosis (Suboj et al. 2012, Shahzidi et al. 2013, Yang et al. 2014, Martin et al. 2016). Taken together, these studies suggest that Casp6 downregulation would not initiate tumor formation, but may promote resistance to apoptosis in tumor cells.

1.5.1. Competitive Peptide Inhibitors

Competitive inhibitors have properties that enable them to interact with the enzyme at the active site. For instance, the competitive inhibitor z-VEID-fmk is derived from the Casp6 cleavage site in lamin A. Since only Casp6 cleaves lamin A, this peptide is selective for Casp6 and is often used as a positive control to inhibit Casp6 in cells. However, this compound is not a viable Casp6 inhibitor to use in humans since it induces hepatotoxicity. Recently, a competitive peptide-based Casp6 inhibitor was designed

based on the Htt Casp6 cleavage site (Aharony et al. 2015). This peptide protects cells from mHtt-induced toxicity, protects pre-symptomatic BAC mice from motor deficit and behavioral abnormalities and partially restores post-symptomatic BAC behavioral and cognitive deficits (Aharony et al. 2015). However, this inhibitor may not be selective for Casp6 since the Htt cleavage site can be cleaved by other proteases (Wong et al. 2015). Similarly, a competitive Casp6 peptide-based inhibitor has also been designed based on the AP-2α Casp6 cleavage site, but this inhibitor is less potent than z-VEID-fmk (Nyormoi et al. 2003). Other peptide-based competitive Casp6 inhibitors include aza-peptide epoxides and Michael acceptors; however, due to the similarity of the active site amongst all caspases, these compounds also inhibit Casp1, Casp2, Casp3, Casp7, Casp8, Casp9 and Casp10 (James et al. 2004, Ekici et al. 2006).

1.5.2. Competitive Small Molecule Inhibitors

Non-peptide small molecule competitive Casp6 inhibitors have also been reported; however, since caspases have overlapping substrate specificities, these inhibitors are non-selective for Casp6 (McStay et al. 2008). Recently, methylene blue, which is in phase three clinical trials for AD, has been reported to inhibit Casp6 (Pakavathkumar et al. 2015). Within the active site containing the Cys/His catalytic dyad, histidine abstracts the proton on the reduced sulfur atom of cysteine leaving it negatively charged. The lone pair of electrons initiates catalysis through a nucleophilic attack on the carbonyl carbon of the substrate. Methylene blue prevents this process through oxidation of the catalytic cysteine residue (Pakavathkumar et al. 2015). However, methylene blue is non-selective since it inhibits Casp1 and Casp3 to the same degree as Casp6 (Pakavathkumar et al. 2015). Using a substrate-based fragment approach with Casp3 and Casp6, Levya et. al. developed three pan-caspase inhibitors that blocked proteolysis of Htt at the Casp3 and Casp6 cleavage sites, suppressed $Hdh^{111Q/111Q}$-mediated toxicity, and rescued rat striatal and cortical neurons from cell death (Leyva et al. 2010). Sulfonamide Isatin Michael acceptors inhibit Casp6 in the nanomolar range and are more selective for Casp6 than Casp3 and Casp7 (Chu et al. 2009).

1.5.3. Allosteric Peptide Inhibitors

An alternative approach to bypass the problem of selectivity is to target allosteric sites. Stanger et al. developed a non-competitive peptide inhibitor that was identified through phage display on the zymogen form of Casp6 (Stanger et al. 2012). Binding of this peptide mediates the tetramerization of two dimers and locks them in a more rigid conformation that exhibits less activity. This peptide was shown to be selective for Casp6 in neuronal cells. Upon treatment with STS, the peptide inhibited lamin A cleavage in a dose-dependent manner, but had no effect on levels of Casp3 substrates spectrin and poly (ADP-ribose) polymerase (PARP). However, this peptide had poor cell penetration properties and was administered to neurons through electroporation. Therefore, it may not have much use in a therapeutic setting.

1.5.4. Allosteric Small Molecule Inhibitors

Small molecule inhibitors that bind zymogen Casp6 at an allosteric site at the dimer interface have been reported (Murray et al. 2014). To identify the compounds, biophysical methods and X-ray crystallography was used to probe the zymogen form of Casp6 for potential allosteric sites followed by a fragment screen using surface plasmon resonance. The compounds identified stabilize the inactive conformation by contacting key residues in the L2 loop. Since rearrangement of the L2 loop is required for formation of the active site, these compounds prevent Casp6 activation. The compounds identified represent the first nanomolar affinity small molecules that bind to the dimer interface site in any caspase family member. However, these compounds prevent activation by stabilizing the zymogen conformation and have a minimal effect on active Casp6. Inhibiting active Casp6 would be more effective for the treatment of AD because active Casp6 accumulates before the onset of AD symptoms (Albrecht et al. 2007).

1.5.5. Uncompetitive Small Molecule Inhibitors

Recently, an uncompetitive inhibitor for Casp6 was reported (Heise et al. 2012). Uncompetitive inhibitors bind only to the enzyme-substrate complex. This compound preferentially binds to the Casp6-VEID complex, therefore making it selective for Casp6. In addition, the compound inhibits Casp6 with a potency of 11 nM. This compound was the first to show uncompetitive inhibition amongst the caspase family. This mode of

inhibition may be of significant importance for drug discovery since these inhibitors recognize a specific enzyme-substrate complex and therefore, do not inhibit the cleavage of other substrates. However, this inhibitor would not be viable for the treatment of AD since Casp6 cleaves a variety of substrates that initiate multiple parallel pathways leading to neurodegeneration. This compound only inhibits cleavage of the fluorophore conjugated VEID peptide used in this study. With the exception of lamin A, Casp6 generally does not cleave its naturally occurring substrates through a VEID motif (Takahashi et al. 1996, Julien et al. 2016).

II. PREFACE

The following chapter contains part of a manuscript being prepared for submission to the Journal of Biological Chemistry. It details the work I conducted on the Sigma-Aldrich commercial library of compounds identified through the *in silico* screen against the postulated Casp6 allosteric pocket.

Title:

Identification of Allosteric Inhibitors against Caspase-6 Activity for the Treatment of Age-dependent Cognitive Decline and Alzheimer's Disease

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Contribution of Authors and Acknowledgements

I conducted the work presented here, however it would not have been possible without the contributions of the other authors listed. *Dr. Andrea LeBlanc* (Department of Neurology and Neurosurgery, McGill University) and *Maria Putorti* (Department of Anatomy and Cell Biology, McGill University) identified the rare variants of human Casp6 which enabled *Alex Beautrait* and *Anne Marinier* (Institute for Research in Immunology and Cancer, University of Montreal) to identify the allosteric pocket and subsequently run the *in silico* screen. *Agne Tubeleviciute-Aydin* and *Gyanesh Sharma* (Department of Neurology and Neurosurgery, McGill University) analyzed the Chembridge Corporation commercial library of compounds as well as those identified through IRIC's medicinal chemistry platform (data not shown). I would also like to acknowledge *Guy Salvesen* for the Casp6 DNA construct used for purification.

2.1 INTRODUCTION

Caspases belong to a highly conserved family of cysteine proteases that have essential roles in apoptosis, inflammation, differentiation, neuronal development and axon pruning (Shalini et al. 2015). All caspases are synthesized as zymogens that must undergo proteolytic processing to become fully activated. During apoptosis, caspase activation occurs in a hierarchical manner such that initiator caspase activation precedes and facilitates effector caspase activation. Although apoptotic caspases are traditionally classified as initiators (Casp2, Casp8, Casp9 and Casp10) and effectors (Casp3, Casp6, Casp7), Casp6 has challenged this classification system since it behaves much differently from the other apoptotic effectors. Rather than being activated by initiator caspases, Casp6 exhibits a unique intramolecular cleavage mechanism that is driven by high enzyme concentrations (Wang et al. 2010). In addition, the substrate specificity of Casp6 is distinct from Casp3 and Casp7 (Talanian et al. 1997). Casp6 has the proteome-wide sequence specificity of VEVD and Casp7 has the proteome-wide sequence specificity of DEVD (Hill et al. 2016). Furthermore, IAPs that interact with apoptotic effectors Casp3 and Casp7 do not interact with Casp6 (Deveraux et al. 1997, Roy et al. 1997). Finally, Casp6 overexpression in HEK293 cells results in excess Casp6 activity in the absence of cell death (Klaiman et al. 2009). These findings were confirmed in another study that used an optimized orthogonal protease to activate each effector caspase in HEK293 cells (Gray et al. 2010). Unlike Casp3 and Casp7 activation, Casp6 activation alone was not sufficient to induce apoptosis. Taken together, these findings suggest that Casp6 has more complex roles outside of apoptosis.

A role for Casp6 in neurodegeneration in the absence of apoptosis has been well established. Nerve growth factor deprivation in mouse spinal neurons induces axonal pruning through the APP-DR6-Casp6 pathway (Nikolaev et al. 2009). DR6 is highly expressed in the adult human hippocampus and temporal cortex; therefore, the APP-DR6-Casp6 pathway may regulate neural connectivity in the adult brain (Iyer et al. 2013). Furthermore, Casp6 activation may also regulate neural connectivity through p75 neurotrophin receptor-mediated axonal degeneration (Park et al. 2010). In primary human neurons, overexpression of WT APP or mutant APP induces Aβ independent, but Casp6

dependent, axonal degeneration (Sivananthan et al. 2010). Although it has been suggested that Casp6-dependent axonal pruning is mediated through upstream Casp9 and Casp3 activity (Cusack et al. 2013), axon pruning is delayed in both Casp6 KO mice and Casp3 KO mice, suggesting that Casp6 may be activated under different contexts (Simon et al. 2012).

Excess neuronal Casp6 activation has been associated with several features of AD neuropathology. Post-mortem studies have shown that AD brains contain significantly higher levels of active Casp6 and TauΔCasp6 localized to NFTs, NTs and NPs in all stages of SAD and in severe cases of FAD (Guo et al. 2004, Albrecht et al. 2007, Albrecht et al. 2009). In addition, Casp6 cleaves numerous substrates that may lead to neuronal dysfunction such as APP (Gervais et al. 1999, LeBlanc et al. 1999, Pellegrini et al. 1999, Weidemann et al. 1999), tau (Guo et al. 2004), α-tubulin (Klaiman et al. 2008), VCP (Halawani et al. 2010); a facilitator of ubiquitinated protein degradation, and actin-regulating postsynaptic density proteins spinophilin, α-actinin and drebrin (Klaiman et al. 2008). Furthermore, Casp6 has also been implicated in age-dependent cognitive impairment since levels of active Casp6 in the entorhinal cortex, the first region to be affected by AD, inversely correlates with global cognitive scores in individuals within normal cognitive range (Braak et al. 1997, Ramcharitar et al. 2013). Knock-in mice that express a self-activated form of human Casp6 in the CA1 region of the hippocampus have also supported a role for Casp6 in age-dependent cognitive impairment (LeBlanc et al. 2014). These mice develop age-dependent spatial and episodic impairment in the absence of NFTs and amyloid plaques. Together these studies suggest that Casp6 represents a potential early therapeutic target in age-dependent cognitive impairment and AD.

Several studies indicate Casp6 to be a viable drug target. The first Casp6 null mice developed normally without any noticeable apoptotic defects (Zheng et al. 1999). Interestingly, B cell differentiation into plasma cells and antigen-stimulated production of antibody is accelerated in these mice; thus, Casp6 inhibition in AD patients may increase their immunity (Watanabe et al. 2008). Another Casp6 KO mouse model exhibited

hypokinetic behavior and learning deficits (Uribe et al. 2012). However, these mice expressed Casp6 without the catalytic domain; therefore, it is unclear if the changes in behavior were due to the absence of Casp6 activity or if the encoded protein had a toxic effect. In humans, pro-Casp6 is not significantly expressed in the brain, suggesting that it does not have an essential role in the brain under physiological conditions (Godefroy et al. 2013). These findings also demonstrate that the high levels of Casp6 seen in AD brains are indeed pathogenic (Godefroy et al. 2013). Moreover, Casp6 is expressed in peripheral tissues and declines with age, but Casp6 expression remains relatively high in the colon (Godefroy et al. 2013). These findings suggest that inhibiting Casp6 could potentially cause colon cancer; however, mice overexpressing human Casp6 and mice null for Casp6 do not show a role for Casp6 in an inflammation-mediated model of colon carcinogenesis (Foveau et al. 2014). Although there are multiple studies indicating an essential role for Casp6 in cancer treatments (Lee et al. 2006, Chen et al. 2009, Suboj et al. 2012, Shahzidi et al. 2013, Martin et al. 2016), no studies have demonstrated a role for Casp6 in preventing cancer. Casp6 may have a dispensable physiological role; therefore, Casp6 inhibition is likely a feasible treatment for AD.

Casp6 is endogenously regulated through several binding partners and post-translational modification. Natural Casp6 inhibitors include Zn^{2+}, which inhibits through an allosteric mechanism, and baculoviral p35, which inhibits through a covalent mechanism (Zhou et al. 1998, Velazquez-Delgado et al. 2012). Sox11 is the only known intrinsic regulator of Casp6, as it reduces Casp6 processing and activity (Waldron-Roby et al. 2015). However, these regulators cannot be used therapeutically since they also target other caspases (Perry et al. 1997, Zhou et al. 1998, Huber et al. 2012, Waldron-Roby et al. 2015). The only selective endogenous Casp6 inhibitor is Mch2β, which is an alternatively spliced isoform of Casp6 that does not possess any catalytic activity (Lee et al. 2010). Mch2β prevents Casp6 activation but has no effect on active Casp6 (Lee et al. 2010). Targeting active Casp6 rather than preventing its activation would be a more effective approach for treating AD since active Casp6 accumulates before the onset of AD symptoms (Albrecht et al. 2007). Moreover, ARK5 regulates Casp6 activity through Ser257 phosphorylation (Suzuki et al. 2004). Casp6 phosphorylation prevents self-cleavage and downregulates

activated Casp6 (Cao et al. 2012, Velazquez-Delgado et al. 2012). However, enhancing ARK5 activity would also not be viable since its expression is associated with tumor formation and invasion (Suzuki et al. 2004).

A number of synthetic competitive Casp6 inhibitors have been developed. Recently, a competitive peptide-based Casp6 inhibitor was designed based on the Htt Casp6 cleavage site (Aharony et al. 2015). This peptide protects cells from mHtt-induced toxicity, and partially restores post-symptomatic behavioral and cognitive deficits in a Huntington mouse model (Aharony et al. 2015). However, this inhibitor may not be selective for Casp6 since the Htt cleavage site can be cleaved by other proteases (Wong et al. 2015). Recently, methylene blue was demonstrated to inhibit Casp6 through oxidation of the catalytic cysteine; however, it also inhibited Casp1 and Casp3 (Pakavathkumar et al. 2015). Additional competitive small molecule Casp6 inhibitors have also been developed, yet these are also non-selective since the active site is structurally similar amongst all caspases and Casp6 has overlapping substrate specificities with other caspases (James et al. 2004, Ekici et al. 2006, McStay et al. 2008).

An approach to develop selective inhibitors is to target allosteric sites unique to Casp6. A synthetic non-competitive peptide inhibitor identified through phage display on the zymogen form of Casp6 selectively inhibited Casp6 activity in neuronal cells (Stanger et al. 2012). But like most peptide inhibitors, this peptide had poor cell penetration properties. In addition, small molecule allosteric Casp6 inhibitors that bind at the dimer interface have also been reported to stabilize the inactive conformation to prevent activation (Murray et al. 2014). However, most of these molecules had poor binding affinity for active Casp6. Furthermore, uncompetitive Casp6 inhibitors have been developed and were demonstrated to be selective, but the ability of these compounds to bind to the enzyme is dependent upon certain substrate sequences (Heise et al. 2012). To our knowledge, no small molecule non-competitive allosteric inhibitors that downregulate activated Casp6 have been reported.

Here, we describe a novel human genetic approach used to identify a putative allosteric site on Casp6. An *in silico* screen of 77,000 diverse small molecules against this pocket identified fifty-four potential Casp6 inhibitors. Among these, compound **10** and its analogues were the most potent and selective Casp6 inhibitors. Kinetic analyses show that compound **10** acts through a non-competitive allosteric mechanism. In human colon carcinoma (HCT116) cells, these compounds are non-toxic and inhibit Casp6 activity. However, in HEK293T cells, they induce cytotoxicity. Extensive compound **10** analogue screens have shown compound **10P** to be our most potent inhibitor to date. Our data serves as a starting point for the development of a small molecule allosteric Casp6 inhibitor and also illustrates that screening of rare variants holds promise as a novel method for the identification of allosteric sites on other therapeutic targets in drug discovery.

2.2 MATERIALS AND METHODS

Caspase Expression Constructs – pET23b-Casp3-His (Addgene plasmid 11821), pET23b-Casp6-His (Addgene plasmid 11823), pET23b-Casp7-His (Addgene plasmid 11825), pET15b-Casp8ΔDED (Addgene plasmid 11827) and pET23b-Casp9 (Addgene plasmid 11829) were a kind gift from Dr. Guy Salvesen (The Burnham Institute, La Jolla, CA, USA). Our lab cloned human Casp6p20p10 in the mammalian pCep4β vector (Klaiman et al. 2009).

Recombinant Protein Purification - Casp6 was expressed in the BL21(DE3)pLysS *E. coli* strain (Stratagene, La Jolla, CA, USA). Overnight starter cultures were diluted 50x in 1 liter of 2X TY media with 100 µg ampicillin, and grown at 37°C until reaching an OD of 600 nm. Cultures were subsequently induced with 50 µM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Bioshop Canada Inc., Burlington, Ontario, CA) and further grown overnight (~18 hours) at 30°C. Cells were pelleted by centrifugation 3,900 x g for 10 minutes and lysed in a buffer containing 50 mM Tris-HCl, pH 8.0 and 100 mM NaCl supplemented with 1 mg/ml lysozyme. Following sonication, cellular debris was removed through centrifugation at 18,000 x g and 4°C for 30 minutes. The supernatant was loaded onto a 5-mL Ni^{2+} affinity column (GE Healthcare Life Sciences, Baie D'Urfe,

QC, CA). The column was washed with a buffer containing 50 mM Tris-HCl, pH 8.0 and 500 mM NaCl. Casp6 was eluted in a buffer containing 50 mM Tris-HCl, pH 8.0, 100 mM NaCl and 200 mM imidazole. The eluted fraction was diluted 10-fold in buffer A (20 mM Tris, pH 8.5, 2 mM dithiothreitol (DTT)) to reduce salt concentration. The protein sample was loaded onto a 5 mL Macro-Prep High Q column (Bio-Rad, Mississauga, ON, CA) pre-washed with 1 X column volume of buffer B (20 mM Tris pH 8.5, 2mM DTT, 1 M NaCl) and subsequently with 4 X column volume of buffer A**.** The column was eluted with a linear NaCl gradient ranging between 80 - 260 nM. The eluted Casp6 fractions were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Peak fractions containing Casp6 were pooled and concentrated by dialysis against polyethylene glycol (PEG) (SnakeSkin 7K MWCO Dialysis Tubing) at 4°C for several hours. The concentrated protein was stored at -80°C.

Measures of Recombinant Caspase-6 Activity - A fluorescence-based activity assay (Fig. 5) was used to measure *in vitro* Casp6 activity in a clear bottom black 96-well plate at 50 µL/well in triplicate at 37°C. RCasp6 (25 nM active site) was mixed with VEID-AFC (10 µM) in Stennicke's Buffer (SB) (20 mM piperazine-N, N-bis 2-ethanesulfonic acid (PIPES) (Bioshop) pH 7.2, 30 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM DTT, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1propanesulfonate (CHAPS), 10% sucrose). The cleavage rate of VEID-AFC was measured in a Synergy H4 plate reader at an excitation wavelength of 380 nm and emission wavelength of 505 nm every sixty seconds for 80 minutes. Cleavage rates were calculated from the linear phase of the assay. Fluorescence units were converted to the amount of moles of AFC released based on a standard curve of 0 – 25.0 µM free AFC.
A two-step luminescence-based activity assay (Fig. 6) (Caspase-Glo ® 6, Promega, Madison, WI, USA) was used to confirm the potency of the initial hits and was performed according to the manufacturer's recommendations. Activity was quantified through peak relative luminescence units after a thirty-minute incubation period at 37°C.

Titration of Active Sites in Purified Recombinant Caspase-6 – A 2/3 serial dilution of Q-VD-OPh (Millipore Canada, Etobicoke, ON, CA), an irreversible active site pan

caspase inhibitor (Caserta et al. 2003), was prepared in Stennicke's Buffer (SB) within a clear bottom black 96-well plate. Casp6 concentration was estimated by spectrophotometry (NanoDrop 2000) and diluted in SB. Casp6 was mixed with Q-VD-OPh dilutions for a final concentration of 200 nM Casp6 with 100 µL/well in triplicate. The plate was incubated for 15 minutes at 37 °C. After the incubation period, 80 µL of each Casp6 / Q-VD-OPh solution was transferred into another clear bottom black 96-well plate containing 20 µL of 500 µM Ac-Val-Glu-Ile-Asp-7-Amino-4-trifluoromethylcoumarin (Ac-VEID-AFC: Enzo Life Sciences, NY, USA) diluted in SB in each well. Enzyme activity was measured in a Synergy H4 plate reader at an excitation wavelength of 380 nm and emission wavelength 505 nm every thirty seconds for 30 minutes at 37°C. Cleavage rates were calculated as described above. Rate of activity was plotted against Q-VD-OPh concentration (Fig. 7A) and active site concentration was estimated by the x-intercept of the line extrapolating the tangent of the curve at low Q-VD-OPh concentration (Fig. 7B). Since the x-value (Q-VD-OPh concentration) when y=0 (100% inhibition) was averaged to be 187 nM (n=3), and the protein concentration in the well was 200 nM, the enzyme preparation was 93% active. Enzyme concentrations were adjusted accordingly for subsequent activity assays

Measurement of Caspase-6 Inhibition - The potency of hit compounds and analogues was quantified through single dose response screening. Compounds were added to RCasp6 (25 nM active site) in the above reaction conditions at a final concentration of 50 µM or 100 µM in 1% dimethyl sulfoxide (DMSO). The potency of inhibitors was measured by normalizing the rate of activity to a DMSO control condition. IC_{50} values of the most potent compounds were determined by plotting relative activity (% DMSO control) to inhibitor concentrations ranging from 1 nM to 1 mM. The data was fitted to a dose response curve equation containing a -1 slope with top and bottom constraints of 1 and 0 respectively, using Prism 6.0 software.

Selectivity Against Other Caspases - Recombinant Casp3, Casp7, Casp8 and Casp9 were purified using standard purification techniques as described in (Garcia-Calvo et al. 1998) and active site titrated (data not shown). Casp1, Casp2, Casp4, Casp5, and Casp10

were obtained commercially (Biovision, Milpitas, CA, USA; Enzo Life Sciences) and concentrations were quantified using spectrophotometry (NanoDrop 2000). Fluorogenic assays were performed using preferred substrates (10 μM) and the following enzyme concentrations: Ac-YVAD with 100 nM Casp1, Ac-VDVAD with 100 nM Casp2, Ac-DEVD-AFC with 5nM Casp3 and 25 nM Casp7, Ac-WEHD-AFC with 100 nM of Casp4 and Casp5, Ac-LETD-AFC with 100 nM Casp8, Ac-LEHD-AFC with 100 nM Casp9 and Ac-IETD-AFC with 100 nM Casp10. Caspase activity and inhibition were calculated as described above.

Kinetic Analyses - Compounds were added to 25 nM (active site) of RCasp6 and enzyme activity was measured with eight different Ac-VEID-AFC substrate concentrations (1.0 μM – 200 μM). The apparent K_m and V_{max} values for the substrate in the absence or presence of compounds were determined through non-linear regression analysis of Michaelis Menten plots (v vs. [S]). A double reciprocal plot (Lineweaver Burk plot) was also used to estimate K_m and V_{max} values through x-axis and y-axis intercepts, respectively. Additionally, IC_{50} curves were generated (as described above) using varying concentrations of Ac-VEID-AFC substrate.

Measures of Casp6 Cytotoxicity - HCT116 and HEK293T cells were cultured in McCoy's 5A (Gibco) and Opti-Minimal essential media (MEM) (Gibco) respectively, supplemented with 10% fetal bovine serum (FBS). Cells were plated in 96-well plates at 10,000 cells per well in 100 μL volume 24 hours before addition of DMSO or inhibitor. Cells were treated with DMSO or inhibitor for two hours and subsequently with 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (0.5 μg/μL) for four hours at 37°C. Absorbance was measured at 560 nm and 670 nm. Cell viability was measured relative to a 1% DMSO control condition.

Transfection of Cell Lines - HCT116 cells were plated in 6 well plates at 250,000 cells per well in 2 mL volumes 24 hours before transfection. Cells were transfected with 8μg polyethylenimine (PEI) and 1 μg pCep4βCasp6p20p10. HEK293T cells were plated in 12 well plates at 150,000 cells per well in 1 mL volumes 24 hours before transfection. Cells

were transfected with 4 µL of Lipofectamine^{2000} (Invitrogen, CA, USA) reagent and 1.6 µg pCep4βCasp6p20p10. Both cell lines were grown for an additional 24 hours before drug treatment.

Measures of Casp6 Activity in Cell Lysate - After DMSO or drug treatment, proteins were harvested in cell lysis buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.1% CHAPS, 100 µM EDTA). Protein concentration was quantified with the Bradford method. Specific VEIDase activity was quantified by cleavage rate of Ac-VEID-AFC (measured as described above) in SB per µg of protein.

2.3. RESULTS

Compound 10 potently and selectively inhibits recombinant Casp6: Structural analysis of the identified Casp6 variants showed that the position of some of these amino acids line a pocket of low sequence conservation. Through the use of molecular docking calculations, an *in silico* screen of 77,000 diverse compounds was performed on the putative allosteric site. These compounds originated from curated commercial libraries (Chembridge Corporation and Sigma-Aldrich) as well as from the IRIC's medicinal chemistry platform in-house collection. To assess the potency of the hits identified by the *in silico* screen, a fluorescence-based activity assay was used to measure enzyme reaction rate. Amongst the Sigma-Aldrich commercial chemical library compounds (Table 1), 100 µM of compound **10** inhibited Casp6 by ~95% (Fig. 8A). Data for compound **3** was not determined due to intrinsic fluorescence. The rate of activity could not be determined due to interference of the fluorescent signal. Data for compound **14** is also not shown because it was not available. With the other compounds only showing moderate inhibition, compound **10** was further pursued as our hit compound. Casp6 inhibition by compound **10** was confirmed through a two-step luminescence-based activity assay (Fig. 8B). Interestingly, compound **5** inhibits luciferase in this assay whereas compound **10** had no effect on luciferase (Fig. 9). Compound **10** exhibited an IC_{50} of 23.1 µM (Fig. 8C). Compared to the other nine mammalian caspases, compound **10** shows a trend towards selective Casp6 inhibition (Fig. 8D). Compound **10** showed about two-fold selectivity at

20 μM concentrations. The IC_{50} values for compound **10** against Casp3, Casp7 and Casp8 also show a trend for selective Casp6 inhibition (Fig. 10; Table 2).

Compound 10G is more potent than compound 10, but less selective: To potentially identify a more potent Casp6 inhibitor, thirteen direct analogues of compound **10** were ordered from Chembridge Corporation (Table 3) and screened at a single dose of 50 μM. All of the analogues tested were more potent than compound **10** with compound **10G** being the most potent, which inhibited Casp6 by ~93% (Fig. 11A). Data for compound **10A** and compound **10J** are absent due to intrinsic fluorescence. Compound **10G** exhibited an IC_{50} of 2.75 μM (Fig. 11B). Compound **10G** was also tested against the other nine caspases and showed potent inhibition of Casp2, Casp4 and Casp6 (Fig. 11C). Compound **10E**, the second most potent Casp6 inhibitor, was also tested in subsequent assays. Compound **10G** was confirmed to be the most potent compound since compound **10E** exhibited an IC_{50} of 5.64 μM (Fig. 10; Table 2). In addition, the IC_{50} values for compound **10E** and compound **10G** against Casp3, Casp6, Casp7 and Casp8 suggest that they are more selective for Casp6 (Fig. 10; Table 2).

Compound 10P was identified as a non-PAIN Casp6 Inhibitor: Structural analysis of compound **10** and compound **10G** revealed that they were pan assay interference compounds (PAINS) (Baell et al. 2010). These are compounds that appear as frequent hitters in many biochemical high throughput screens. The alkylidene barbiturate component of compound **10** (Fig 12A, panel i) and compound **10G** (Fig. 12A, panel ii) was reported to be highly reactive. Therefore, these compounds may not be inhibiting Casp6 in a drug-like way. To identify a non-PAIN Casp6 inhibitor, five compound **10** / **10G** analogues without the akylidene barbiturate component were identified (Table 3). Amongst these analogues, compound **10P** (Fig. 12A, panel iii) showed the most potent inhibition of Casp6 (Fig. 12B). Compound **10P** has a similar potency to compound **10** with an IC_{50} of 31.4 μM (Fig. 12C).

Compound 10G and compound 10P inhibit Casp6 through a non-competitive mechanism: Kinetic analyses were performed to assess the mechanism of inhibition of

these compounds. A Michaelis-Menten analysis of compound **10G** and compound **10P** showed decreases in V_{max} in a dose-dependent manner with minimal changes in K_m (Fig. 13A). This pattern of kinetic parameters was also observed in the double reciprocal plot (Fig. 13B). In addition, increasing VEID-AFC substrate concentration does not significantly affect the IC_{50} of compound **10G** and compound **10P** (Fig. 13C), suggesting that inhibitor binding is unaffected by substrate binding and therefore acts through a non-competitive allosteric mechanism.

Compound 10 and its analogues inhibit Casp6 in HCT116 Cells: To assess the toxicity of these compounds on HCT116 cells, an MTT assay was performed after two-hour treatment with 100 μM of inhibitor. Compound **10** and its analogues compound **10E** and compound **10G**, do not exhibit significant changes in cell viability relative to a DMSO control, whereas STS treatment induces cytotoxicity (Fig. 14A). The ability of these compounds to inhibit Casp6 within cells was tested by treating HCT116 cells overexpressing Casp6 for two hours with 100 μM of each compound. Compound **10**, **10E** and **10G** did not significantly inhibit VEIDase activity but showed a trend towards some inhibition of Casp6 (Fig. 14B).

Compound 10 and its analogues do not inhibit Casp6 in HEK293T cells at sub-toxic doses: These compounds were also tested on HEK293T cells, a cell line known to have high transfection efficiency with Casp6. The toxicity of these compounds was initially tested at a single dose of 100 μM compound using the MTT assay (Fig. 15A). Compound **10** and its analogues induced cytotoxicity in HEK293T cells after two hours of treatment. To find the sub-toxic doses of compound **10**, **10G** and **10P**, cells were treated with doses of each inhibitor ranging from 1 μM to 100 μM (Fig. 15B). The sub-toxic doses were 5 μM for compound **10**, 1 μM for compound **10G** and 10 μM for compound **10P**. To determine if treatment time had an effect on cell viability, cells were treated at sub-toxic doses for times ranging from thirty minutes to twenty-four hours (Fig. 15C). Treatment for as long as twenty-four hours did not induce cytotoxicity. To test Casp6 inhibition *in cellulo*, HEK293T cells were treated with these compounds at sub-toxic doses for two,

four or eight hours (Fig. 15D). The compounds did not have any effect on VEIDase activity under these conditions.

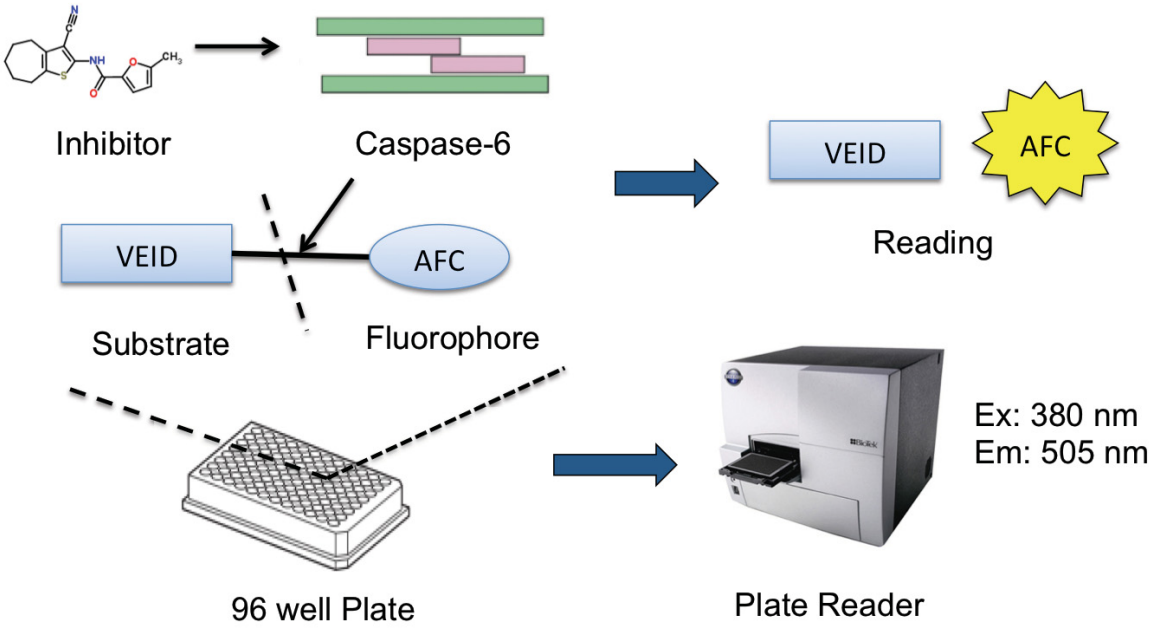


Figure 5 | Schematic of the *In Vitro* Fluorescence-Based Activity Assay. Purified Casp6 (25 nM) was incubated with Ac-VEID-AFC and inhibitor at 37°C in a Synergy H4 plate reader. The rate of activity was measured by the amount of cleaved AFC every minute for eighty minutes. See materials and methods for details.

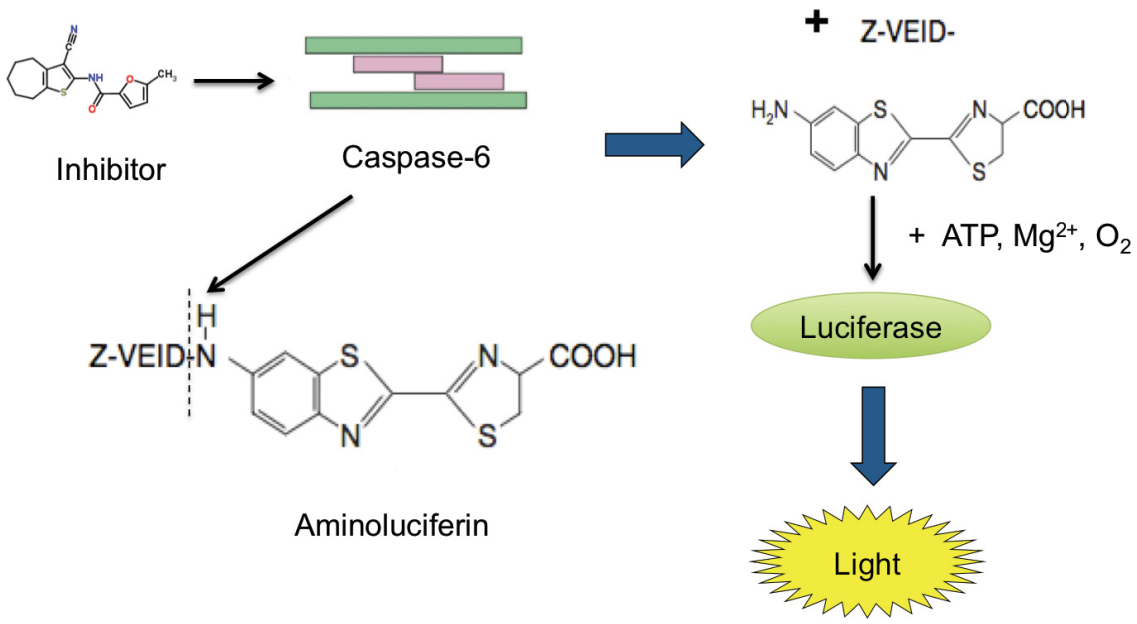


Figure 6 | Schematic of the Two-Step *In Vitro* Luminescence-Based Activity Assay. Purified Casp6 (25 nM) was incubated with Z-VEID-aminoluciferin and inhibitor at 37°C in a Synergy H4 plate reader. Readout is dependent on the amount of cleaved aminoluciferin reacting with luciferase together with the co-factors ATP, Mg^{2+} and O_2.

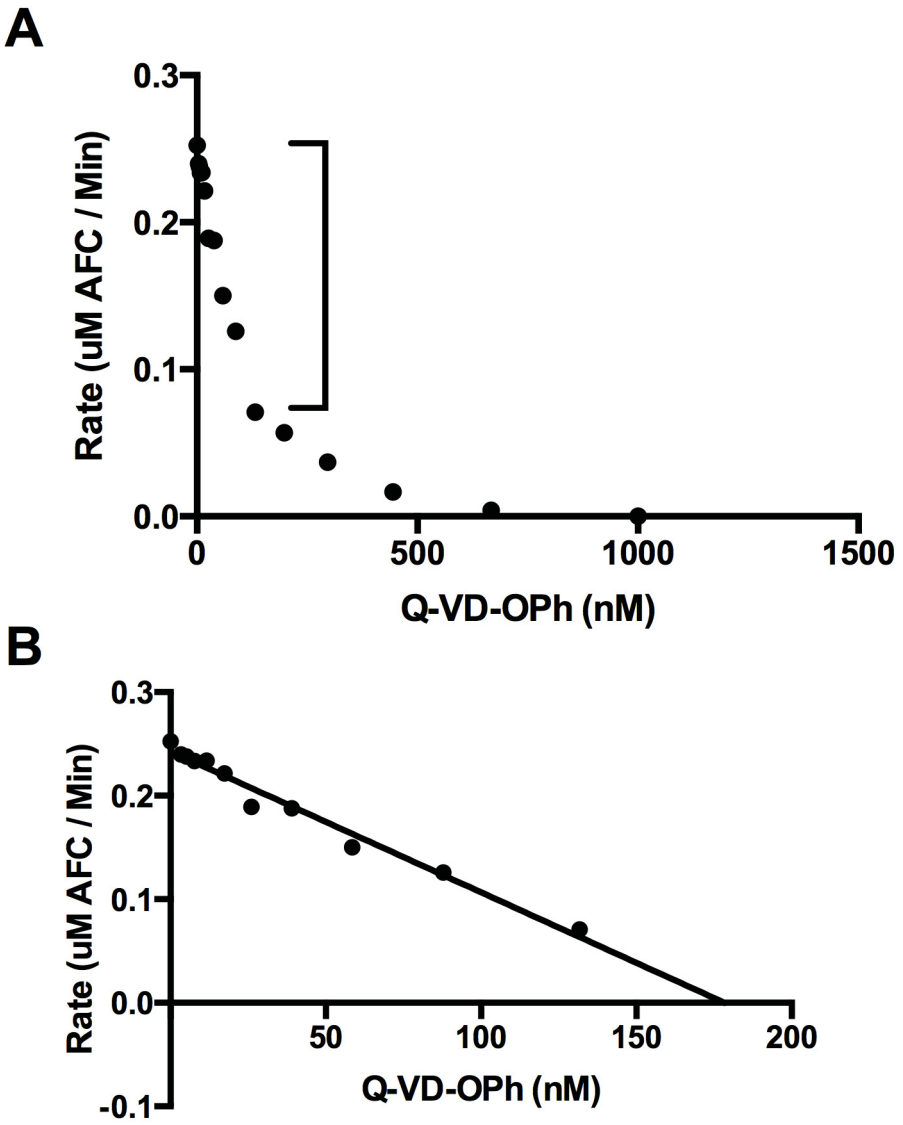


Figure 7 | Casp6 was titrated with Q-VD-OPh to estimate its active site concentration. (A) Reaction rate of 200 nM Casp6 incubated with different concentrations of Q-VD-OPh (0 – 1 μM) using Ac-VEID-AFC (100 μM) as a substrate. The bracket indicates the values used to obtain the active Casp6 concentration. **(B)** Linear regression of the values used to estimate active site titration. Graphs represent one of three independent experiments.

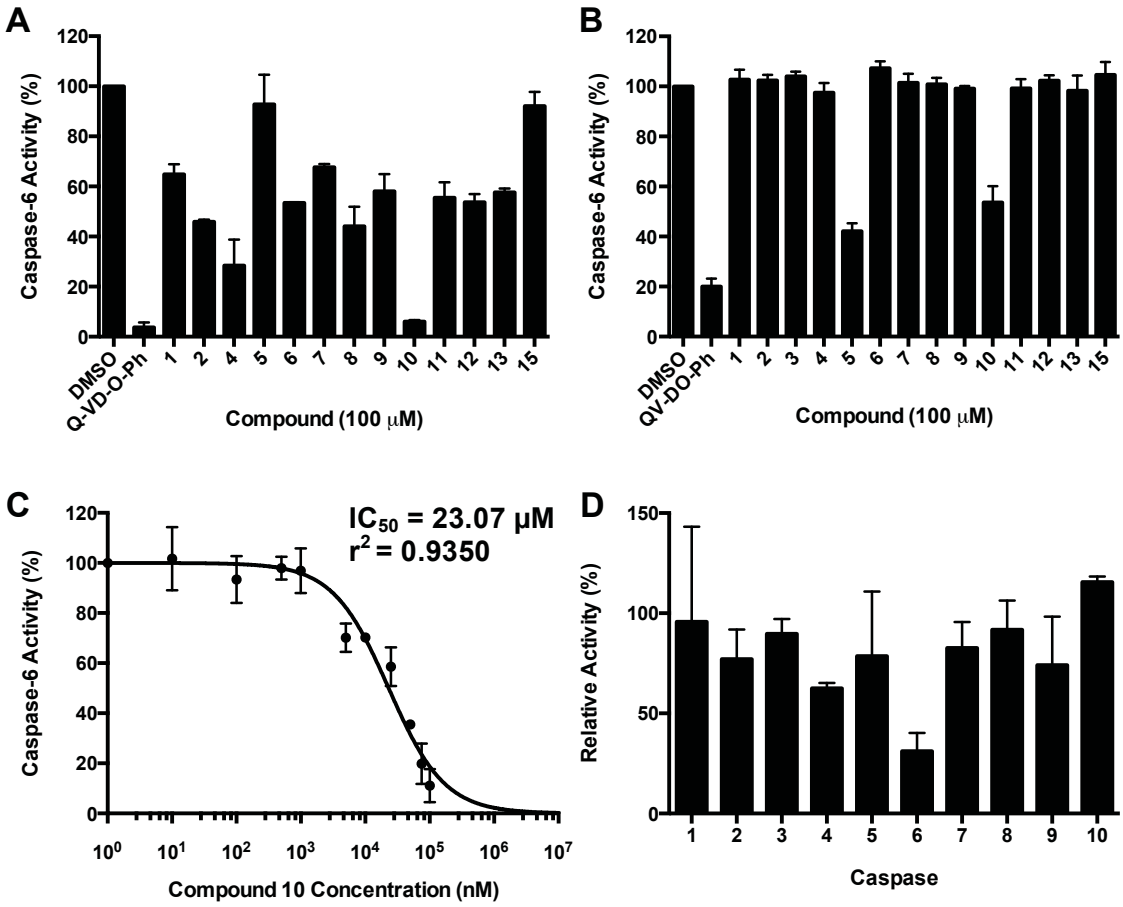


Figure 8 | Compound 10 inhibits active Casp6 potently and selectively. Relative activity of RCasp6 treated with DMSO (1%), Q-VD-OPh (5 μM) and small molecule inhibitors (100 μM) originating from the Sigma-Aldrich Library using a **(A)** fluorescence- and **(B)** luminescence-based activity assay. **(C)** Dose response curve of RCasp6 activity with varying concentrations of compound **10**. Activity is shown relative to a DMSO control. **(D)** Relative activity of recombinant Casp-1 to -10 treated with compound **10** (50 μM). For each caspase, activity is shown relative to a DMSO control. Data represent the average of three independent experiments, and error bars represent standard deviation.

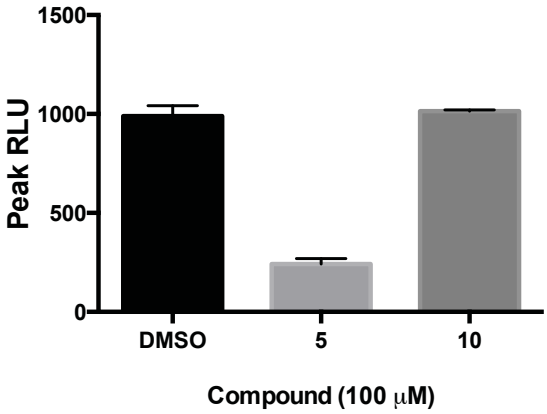


Figure 9 | Luciferase is inhibited by compound 5 but not by compound 10. Bars show peak relative luminescence units after D-luciferin (10 nM) addition to luciferase after incubation with 1% DMSO (black bar), 100 μM compound **5** (light grey bar) and 100 μM compound **10** (dark grey bar). Readings were done in triplicate. Data represents one trial (n=1).

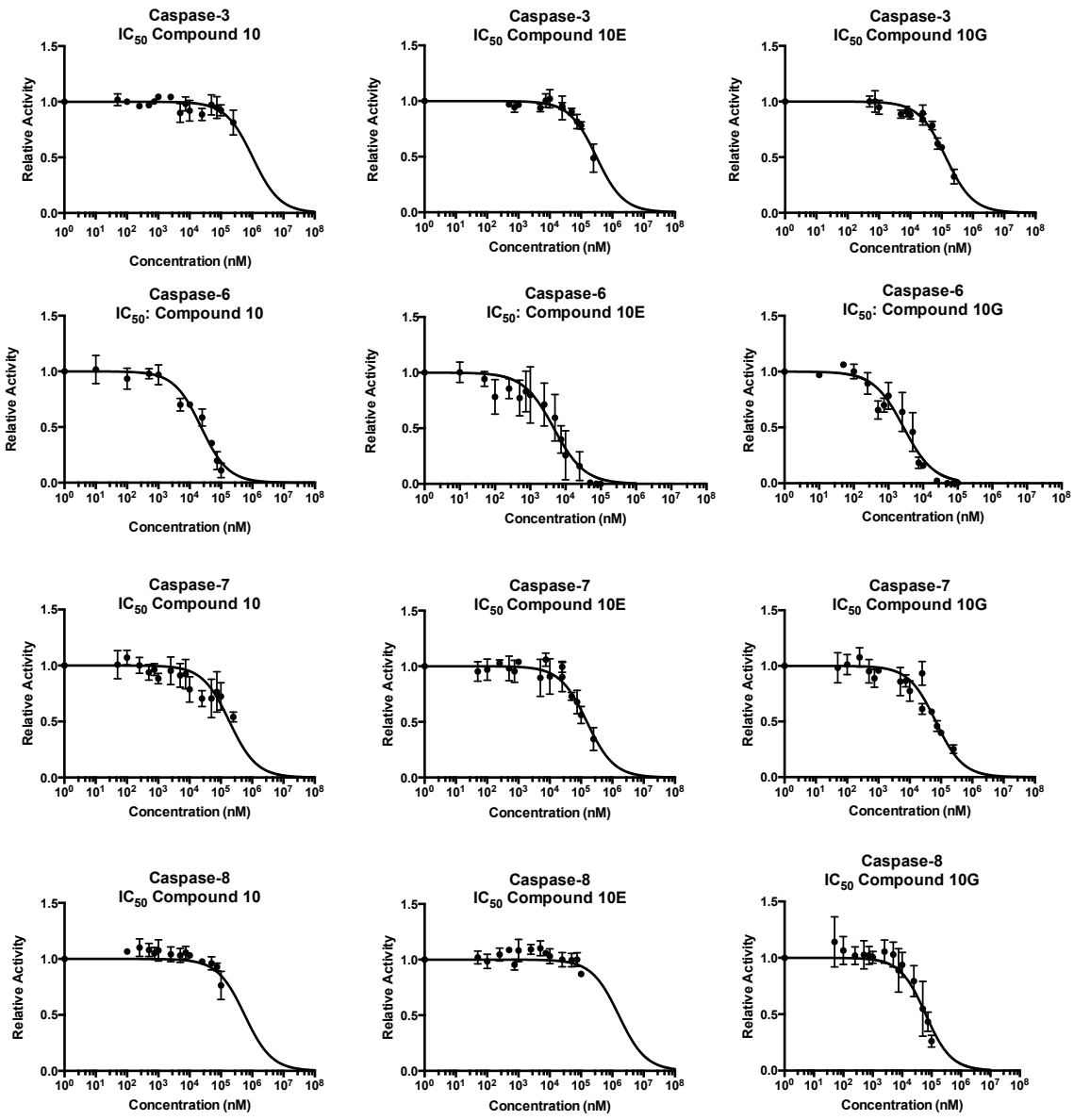


Figure 10 | The selectivity of compounds 10, 10E and 10G were confirmed through their IC_{50} values against other recombinant caspases. Dose response curves of Casp3, Casp6, Casp7, and Casp8 activity with varying concentrations of compound **10, 10E** and **10G**. Activity is shown relative to a 1 % DMSO control. Each curve represents the average of three independent experiments, and error bars represent standard deviation.

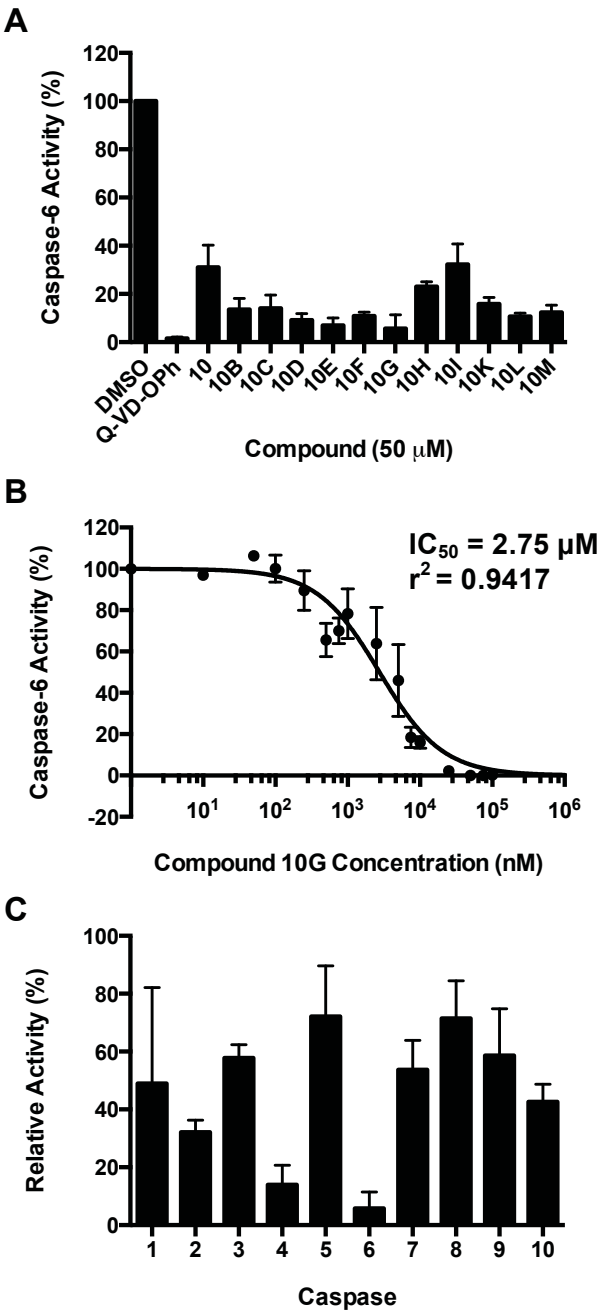


Figure 11 | An analogue of compound 10, compound 10G, inhibits active Casp6 more potently but less selectively. (A) Relative activity of RCasp6 treated with DMSO (1%), Q-VD-OPh (5 μM) and compound **10** analogues (50 μM) using the fluorescence-based activity assay. **(B)** Dose response curve of Casp6 activity with varying concentrations of compound **10G**. Activity is shown relative to a 1% DMSO control. **(C)** Relative activity of recombinant Casp-1 to -10 treated with compound **10G** (50 μM). For each caspase, activity is shown relative to a 1% DMSO control. Data represent the average of three independent experiments, and error bars represent standard deviation.

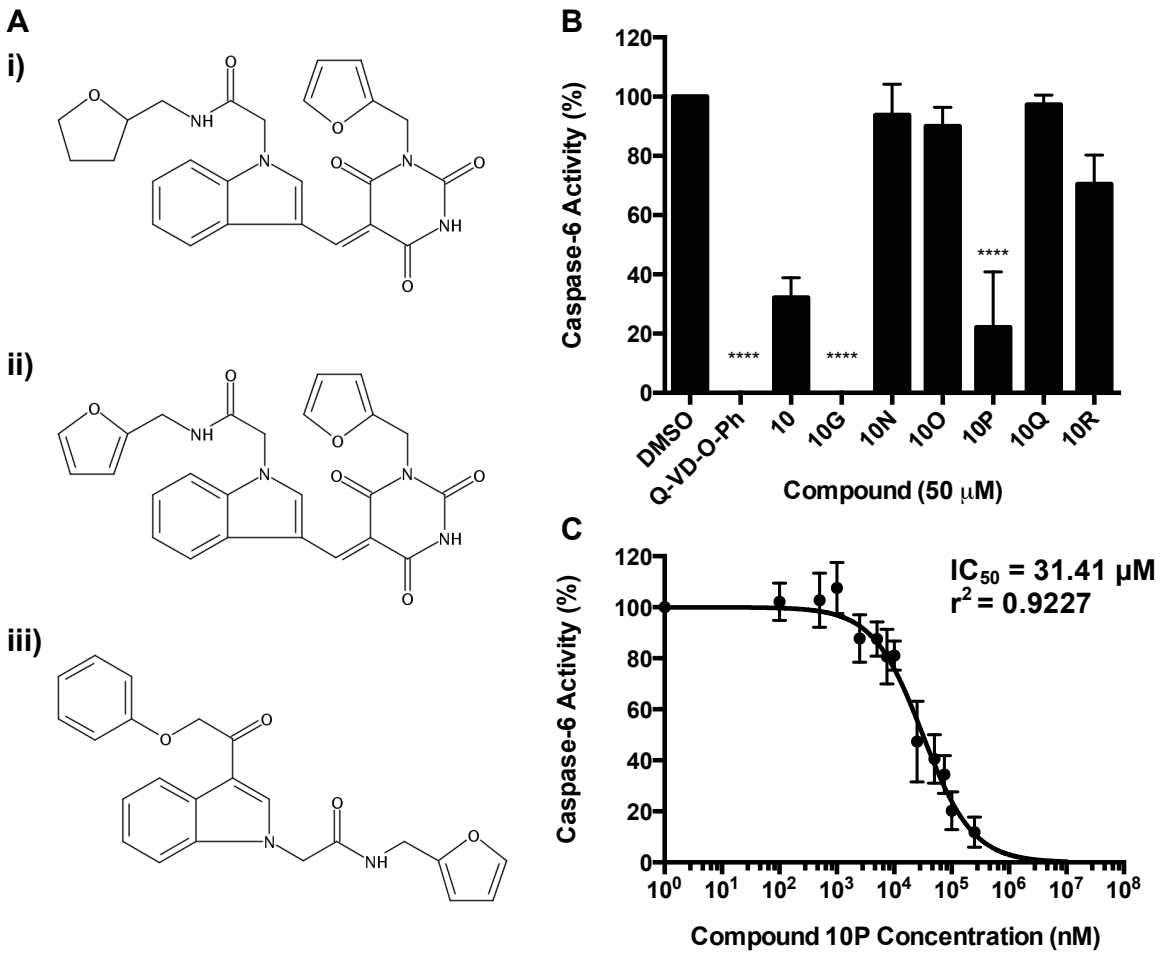


Figure 12 | A non-PAIN compound 10G analogue, compound 10P, inhibits active Casp6. (A) Structures of i) compound **10**, ii) compound **10G** and iii) compound **10P (B)** Relative activity of RCasp6 treated with DMSO (1%), Q-VD-OPh (5 μM) and small molecule inhibitors (50 μM) using the fluorescence-based activity assay. **(C)** Dose response curve of Casp6 activity with varying concentrations of compound **10P**. Activity is shown relative to a 1 % DMSO control. Data represent the average of three independent experiments, and error bars represent standard deviation. Statistical difference between DMSO and addition of compounds was determined by one-way ANOVA $p < 0.0001$.

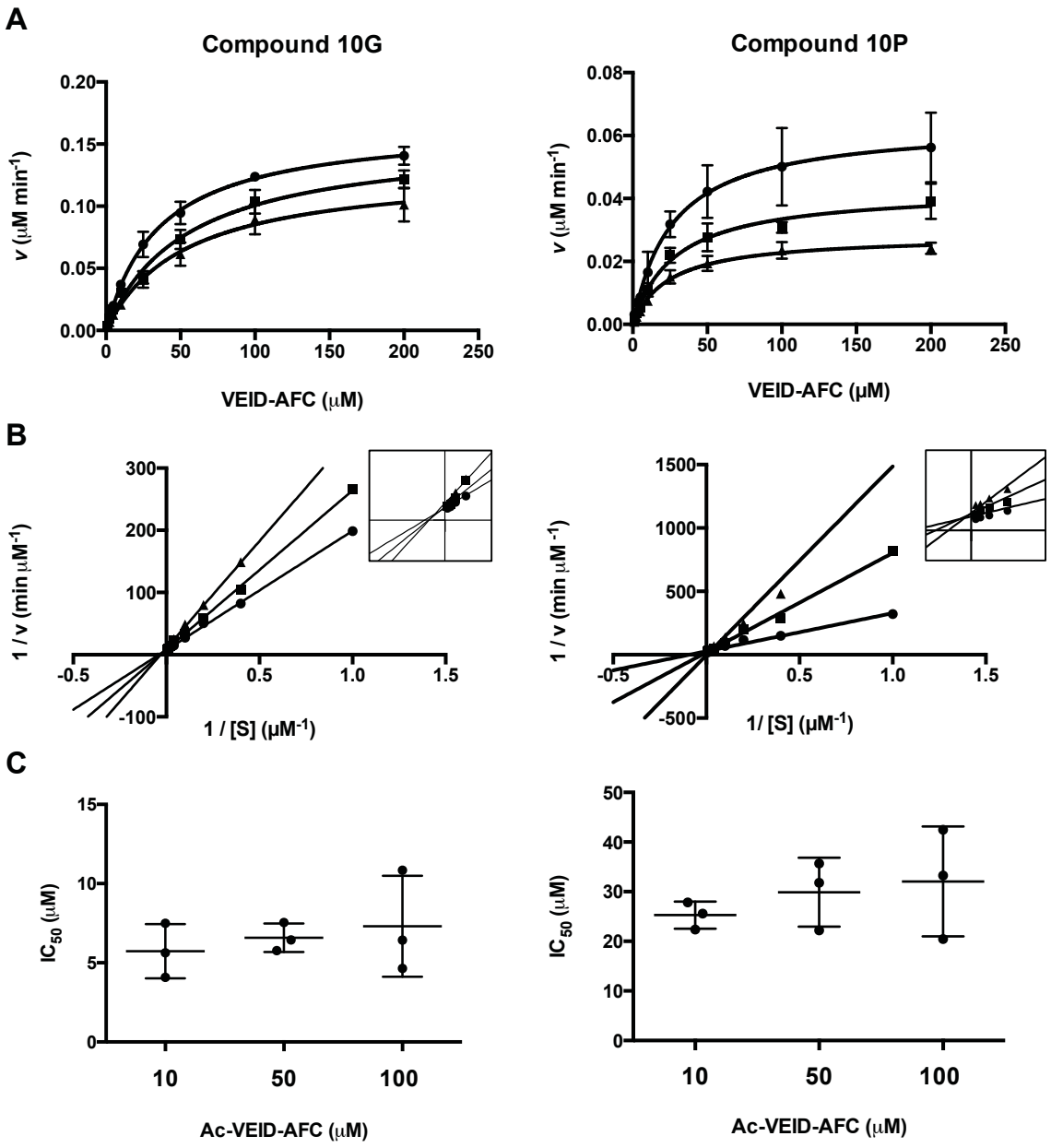


Figure 13 | Compounds 10G and 10P inhibit active Casp6 through a non-competitive allosteric mechanism. **(A)** Michaelis-Menten kinetics plots of Casp6 enzymatic activity in the presence of compound **10G** (2.5 μM, filled squares; 5.0 μM, filled triangles) and compound **10P** (50 μM, filled squares; 100 μM, filled triangles). Filled circles represent a 1% DMSO control. Data represents the average of three independent experiments, and error bars represent standard deviation. **(B)** Lineweaver-Burk double reciprocal transformation of the concentration response curves in A. **(C)** IC_{50} of compounds **10G** and **10P** with increasing concentrations of Ac-VEID-AFC substrate. Data represents three independent experiments for each concentration. There was no statistical significance between each concentration when using a one-way ANOVA $p < 0.0001$.

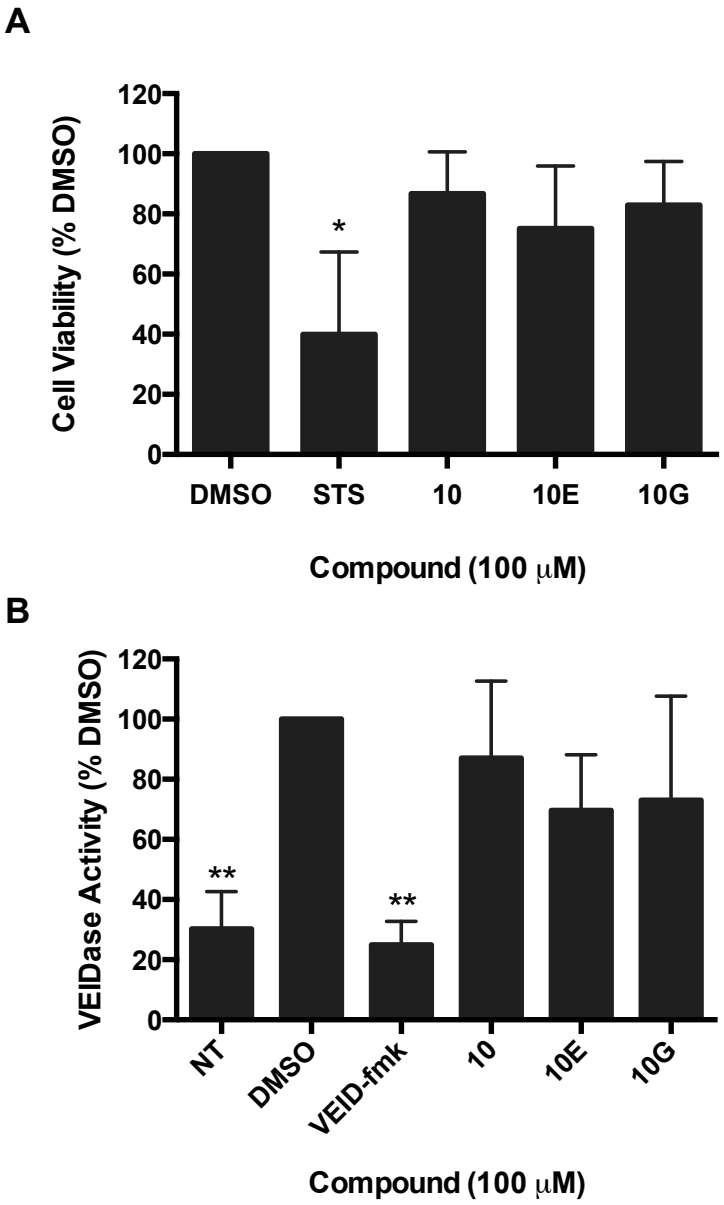


Figure 14 | Compound 10 and its analogues show a trend towards Casp6 inhibition in HCT116 cells without inducing cytotoxicity. (A) Viability of HCT116 cells after two hour treatment with STS (1 μM) or inhibitor (100 μM). Cell viability is expressed relative to a 1% DMSO control. **(B)** VEIDase activity of non-transfected (NT) or Casp6 transfected HCT116 cells after two hour treatment with VEID-fmk (5 μM) or inhibitors (100 μM). VEIDase activity is expressed relative to a 1% DMSO control. Data represents the average of three independent experiments and error bars represent standard deviation. Statistical difference between DMSO and addition of compounds was determined by one-way ANOVA $p < 0.0001$.

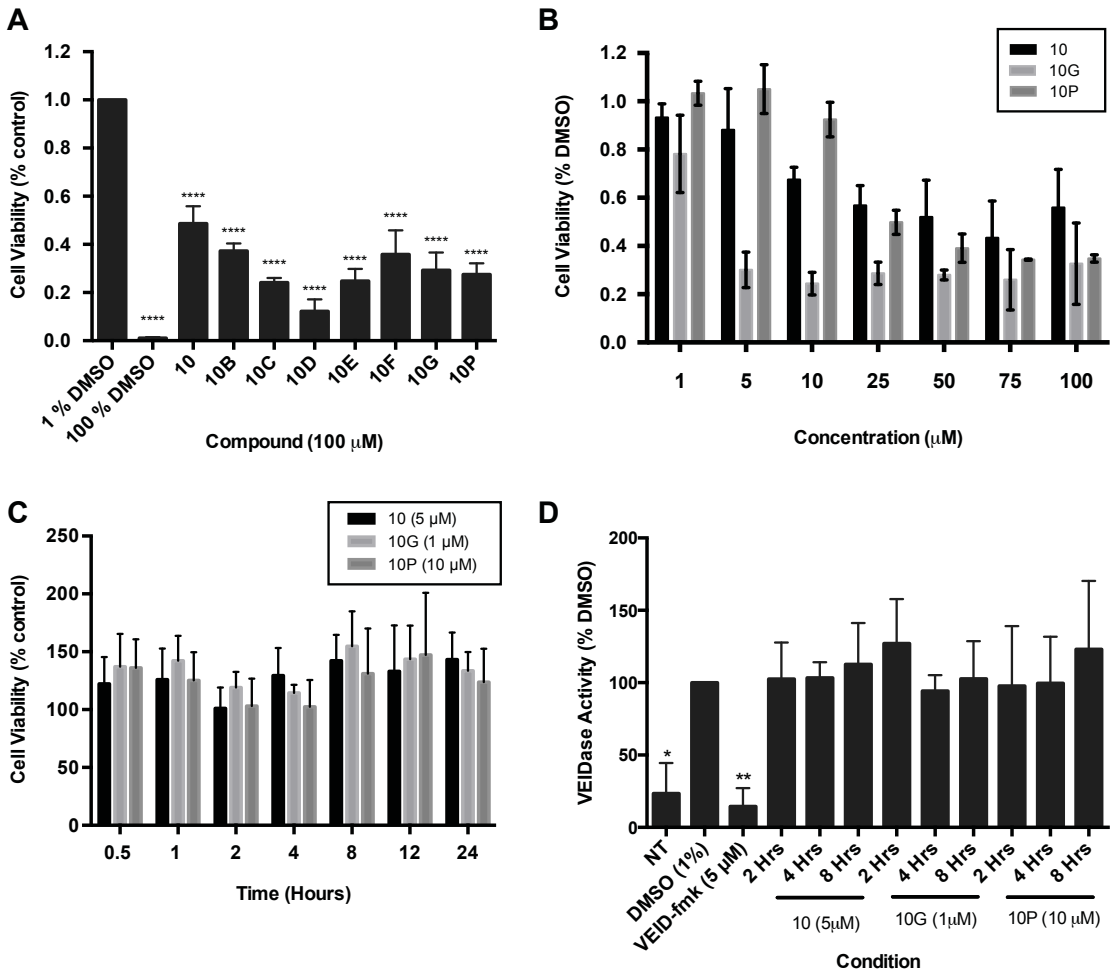
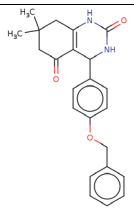
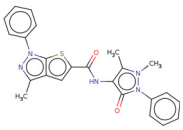
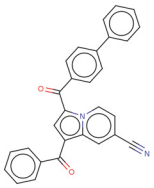
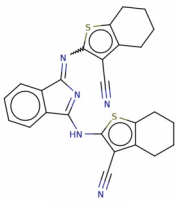
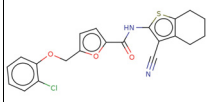
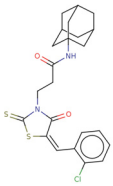
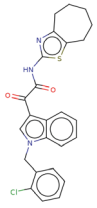
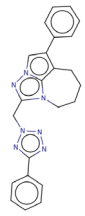
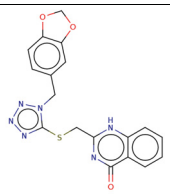
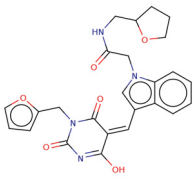
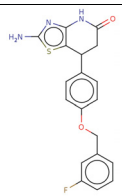
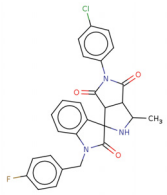
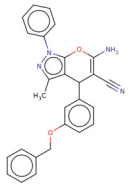
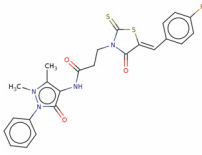
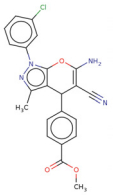


Figure 15 | Compound 10 and its analogues do not inhibit Casp6 activity in HEK293T cells at sub-toxic doses. (A) Cell viability after two-hour treatment with DMSO or inhibitor (100 μM) as determined by the MTT assay. Cell viability is expressed relative to a 1% DMSO control. **(B)** Cell viability after two-treatment with compound **10**, **10G** and **10P** (1 – 100 μM) (compound **10**, black bars; compound **10G**, light grey bars; compound **10P**, dark grey bars) **(C)** Viability of cells treated for varying times (0.5 – 24 hours) with compound **10** (5 μM), compound **10G** (1 μM) and compound **10P** (10 μM) (compound **10**, black bars; compound **10G**, light grey bars; compound **10P**, dark grey bars). **(D)** VEIDase activity of lysate from non-transfected (NT) and Casp6 transfected cells treated with compound **10** (5 μM), compound **10G** (1 μM) and compound **10P** (10 μM) for 2 – 8 hours. Data for each panel represents three independent experiments. Statistical difference between DMSO and addition of compounds was determined by one-way ANOVA $p < 0.0001$.

Table 1. Structures and inhibition data of hit compounds from Sigma Library

Compound	Structure	MW	logP	RB[a]	HBD[b]	HBA[c]	% Inhibition[d]
1		376.45	3.19	4	2	5	35.12
2		443.52	5.09	4	1	7	54.09
3		426.47	6.86	5	0	4	N.D.
4		467.61	6.66	3	1	5	71.57
5		412.89	5.8	5	1	5	7.21
6		461.04	5.53	5	1	4	46.55
7		463.98	5.93	5	1	5	32.35

Compound	Structure	MW	logP	RB[a]	HBD[b]	HBA[c]	% Inhibition[d]
8		395.46	4.36	4	0	7	55.86
9		394.41	2.32	5	1	9	41.92
10		476.48	2	7	2	10	93.93
11		369.41	3.14	4	3	5	44.51
12		489.93	3.45	3	1	6	46.33
13		434.49	5.46	5	2	6	42.38
14		496.58	4.16	6	1	7	N.D.

Compound	Structure	MW	logP	RB[a]	HBD[b]	HBA[c]	% Inhibition[d]
15		420.85	4.48	4	2	7	7.93

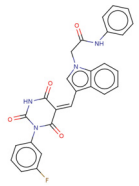
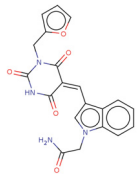
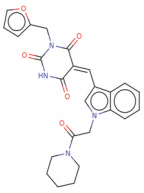
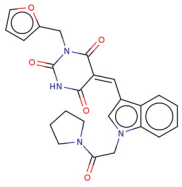
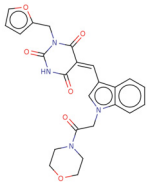
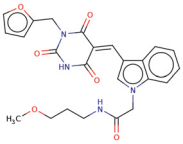
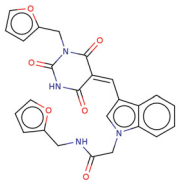
A – Rotatable Bonds
B – Hydrogen Bond Donors
C – Hydrogen Bond Acceptors
D – Mean Inhibition (%) of 25 nM (active site) RCasp6 treated with 100 μM of inhibitor relative to a DMSO control (n =3)
N.D. – Not determined due to intrinsic fluorescence

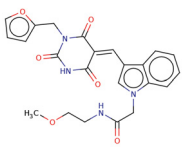
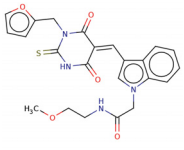
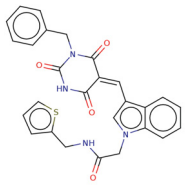
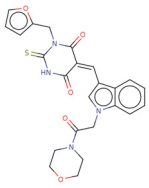
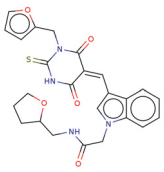
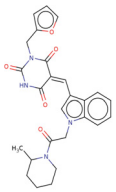
Table 2. IC_{50} values of compound 10, 10E and 10G against recombinant caspases

Protease	IC_{50} (μM)		
	10	**10E**	**10G**
Caspase-3	1043	308	136
Caspase-6	23.1	4.66	2.75
Caspase-7	188	154	68.0
Caspase-8	570	1603	62.6

(n = 3 for each value)

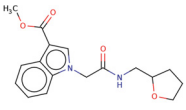
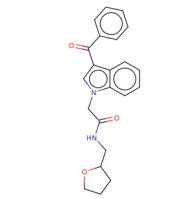
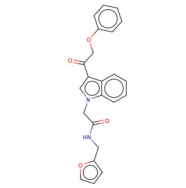
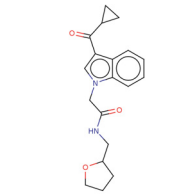
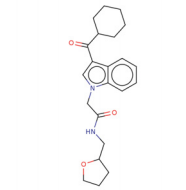
Table 3. Structures and Inhibition data of compound 10 analogues

Compound	Structure	MW	logP	RB[a]	HBD[b]	HBA[c]	% Inhibition[d]
10A		482.46	3.88	5	2	8	N.D.
10B		392.36	0.86	4	3	9	86.42
10C		460.48	2.29	5	1	9	85.91
10D		446.46	1.93	5	1	9	90.83
10E		462.45	1.07	5	1	10	93.07
10F		464.47	1.47	9	2	10	89.12
10G		472.45	1.86	7	2	10	94.30

Compound	Structure	MW	logP	RB[a]	HBD[b]	HBA[c]	% Inhibition[d]
10H		450.44	1.12	8	2	10	76.98
10I		466.51	1.72	8	2	9	67.76
10J		498.55	3.37	7	2	8	N.D.
10K		478.52	1.67	5	1	9	84.16
10L		492.55	2.27	7	2	9	89.30
10M		474.51	2.72	5	1	9	87.67

A – Rotatable Bonds
B – Hydrogen Bond Donors
C – Hydrogen Bond Acceptors
D – Mean Inhibition (%) of 25 nM (active site) RCasp6 treated with 50 μM of inhibitor relative to a DMSO control (n =3)
N.D. – Not determined due to intrinsic fluorescence

Table 4. Structures and Inhibition Data of Non-PAIN compound 10 analogues

Compound	Structure	MW	logP	RB[a]	HBD[b]	HBA[c]	% Inhibition[d]
10N		316.35	1.71	6	1	6	6.20
10O		362.42	3.2	6	1	5	9.96
10P		388.42	3.42	8	1	6	77.81
10Q		326.39	1.99	6	1	5	2.68
10R		368.47	3.62	6	1	5	29.47

A – Rotatable Bonds
B – Hydrogen Bond Donors
C – Hydrogen Bond Acceptors
D – Mean Inhibition (%) of 25 nM (active site) RCasp6 treated with 50 μM of inhibitor relative to a DMSO control (n =3)

2.4. DISCUSSION

Caspases are therapeutic targets of keen interest because of their critical roles in apoptosis, inflammation and neurodegenerative diseases. Targeting the active site of caspases has proven to be an arduous approach as no active site inhibitors have yet succeeded in navigating human clinical trials. The development of selective active site inhibitors is challenging because of the high sequence conservation of the active site amongst the caspase family (Stennicke et al. 1999). In addition, peptide substrate inhibitors have been developed based on the substrate preferences of each caspase; however, the fluoromethylketone moiety often attached to these inhibitors to irreversibly inhibit caspase activity are toxic *in vivo* (Eichhold et al. 1997). To identify selective Casp6 inhibitors, we ran an *in silico* screen against an allosteric site that exhibits low sequence conservation. In this study, we demonstrated that (i) Casp6 activity is modulated by the small molecules identified through an *in silico* screen, (ii) the most potent inhibitors show selectivity for Casp6, (iii) the inhibitors act through a non-competitive mode of inhibition and (iv) these inhibitors show a trend toward Casp6 inhibition in HCT116 cells, whereas they induce cytotoxicity in HEK293T cells. Collectively, these observations validate our novel genetic approach for the identification of allosteric inhibitors that act at a previously uncharacterized site.

In regard to the method used to identify these inhibitors, to our knowledge, no previous studies have taken advantage of rare Casp6 variants to identify an allosteric pocket. Rare variant forms of enzymes often have different binding properties than their WT counterparts. A well-known example of this phenomenon occurs in sickle cell disease in which a single amino acid substitution causes a conformational change within hemoglobin that impairs its ability to carry oxygen (Rees et al. 2010). Previous work done in our lab showed that variants of human Casp6 have reduced catalytic activity due to decreased binding affinity for the substrate. These were used in molecular modeling to identify a non-conserved allosteric pocket formed by these amino acids, and an *in silico* screen was subsequently run against this pocket to identify potential inhibitors (collaboration with Anne Marinier, Alex Beautrait, IRIC). *In silico* screening has previously been used as a tool for the identification of an active site Casp3 inhibitor

(Ganesan et al. 2011) and for the identification of an allosteric Casp8 activator that mediates its dimerization (Bucur et al. 2015). Herein, we are the first to report the use of *in silico* screening to identify allosteric inhibitors against the active conformation of Casp6. By contrast, previously reported allosteric Casp6 inhibitors have been identified through the zymogen conformation of Casp6. An allosteric peptide that inhibits Casp6 activity through the tetramerization of two Casp6 dimers was identified using phage display against the zymogen form of Casp6 (Stanger et al. 2012). Interestingly, the peptide could not be identified from phage panning the active conformation of Casp6 (Stanger et al. 2012). In addition, a fragment merging strategy was employed against zymogen Casp6 to identify nanomolar affinity small molecules that stabilize the zymogen conformation (Murray et al. 2014). The fragment library was also screened against the active conformation but did not yield as many hits (Murray et al. 2014). Therefore, *in silico* screening against zymogen Casp6 could be of interest for the potential identification of additional hits that regulate Casp6 activation.

Most of the compounds identified through the *in silico* screen showed inhibition of Casp6 through the fluorescence-based activity assay. However, only one compound could be validated with the luminescence-based activity assay. This difference could be explained by the readout of each assay. The fluorescence assay measures the rate activity whereas the luminescence assay is an endpoint assay that measures the amount of substrate cleaved. The inhibitors that were hits in the fluorescence assay but not in the luminescence assay may have slowed down the rate of reaction, but may not have had an effect on the total amount of substrate cleaved by the end of the assay. Since compound **10** was our only hit in both assays, we pursued it further and identified a direct analogue that was ten times more potent, compound **10G**. From this novel approach, we identified an inhibitor within the single-digit micromolar range. However, a major limitation of this study is that the *in silico* screen did not filter out PAINS. Compound **10** and compound **10G** are classified as a PAINS because their alkylidene barbiturate functional group comes up as a hit for inhibiting multiple drug targets such as glycosyl transferase MurG (Hu et al. 2004), glycogen synthase kinase 3β (Kim et al. 2008), HIV-1 integrase (Rajamaki et al. 2009), tyrosine kinase p56 (Huang et al. 2004) and retinoid x receptor α

(Park et al. 2008). The alkylidene barbiturate group is highly reactive and acts as a redox cycler since it is easily reduced and oxidized by thiol (Tanaka et al. 1987, Tanaka et al. 1988, Meissner et al. 1994). In addition, compounds containing an alkylidene barbiturate group can act as TNFα antagonists when exposed to light (Voss et al. 2003). Furthermore, the alkylidene barbiturate containing dye mercocyanine 540 causes protein damage when exposed to light (Pervaiz et al. 1992). Therefore, compound **10** and compound **10G** are not stable and may be inhibiting Casp6 in a non-drug like manner.

Despite being PAINS, compound **10** and compound **10G** shows some selectivity for Casp6 thereby supporting our hypothesis that the inhibitors are acting at the postulated allosteric pocket. Relative to the other caspases, compound **10** inhibited Casp6 two- to three-fold more potently than all the other caspases tested. In addition, compound **10** and compound **10G** showed minimal inhibition of the other apoptotic effector caspases. Similarly, the allosteric peptide developed by Stanger et al. showed inhibition of intracellular Casp6 activity but not Casp3 activity; however, the effect of this peptide on other mammalian caspases was not investigated (Stanger et al. 2012). Interestingly, compound **10G** potently inhibited Casp4 (Fig. 11). An alignment of Casp4 with Casp6 showed that the amino acids that line the allosteric pocket in Casp6 are similar to the amino acids in Casp4, suggesting that Casp4 could have a pocket of similar tertiary architecture. Casp4 may have a dispensable physiological role but its function is poorly defined. Casp4 is the human paralogue of mouse Casp11. In mice, Casp11 is essential for Casp1 activation (Wang et al. 1998); therefore, in humans, Casp4 inhibition could potentially inhibit Casp1 activation. Interestingly, Casp1 has recently been implicated in the activation of Casp6 in AD CNS neurons through the NLRP1-Casp1-Casp6 pathway (Kaushal et al. 2015). In addition, Casp4 may also have a role in AD neuropathology. In AD brains, Casp4 expression is increased two-fold in the early stages of neuritic pathology and this elevation remains throughout AD progression (Buxbaum et al. 2009). Moreover, Casp4 expression also correlates with progressive cognitive decline (Buxbaum et al. 2009). Casp4 also has a role in Aβ-induced apoptosis and ER-stress induced cell death (Hitomi et al. 2004). In response to ER stress, Casp4 expression and activation is increased (Hitomi et al. 2004), and in both sporadic and familial AD, ER stress can

induce neuronal cell death (Katayama et al. 2004). These findings suggest that neuronal stress induces Casp4 activation to mediate neuronal cell death in AD. Therefore, inhibition of both Casp4 and Casp6 may be more advantageous for the treatment of AD. A limitation for the selectivity assays is that some of the caspases, such as Casp4, were not titrated since they were purchased from companies. The concentrations of enzymes were measured using spectrophotometry. It is possible that the non-titrated commercially available enzymes lost some of their activity over time and that compound **10G** may not be a potent inhibitor of these caspases.

The kinetic analyses indicate that these inhibitors act through a non-competitive allosteric mechanism, thereby lending further support for their action at the allosteric site. Our conclusion derived from the simplified Michaelis Menten kinetics analysis was supported by the observation that increasing the concentration of the substrate VEID-AFC had no effect on the IC_{50} of compound **10G** and compound **10P** (Fig. 13). In addition, our docking models show that binding of compound **10P** to the allosteric site is feasible. However, a limitation to this study is the absence of a crystal structure. The molecular details of compound **10G**- and compound **10P**-mediated Casp6 inhibition are not known at this time. We speculate that the inhibitors are inducing and stabilizing a conformation that is similar to at least one of the variant conformations.

The compounds were tested in immortal cell lines to determine their toxicity and to determine their capacity to cross the lipid membrane and inhibit Casp6 activity. In the HCT116 cell line, these compounds were non-toxic and there was a trend toward Casp6 inhibition; however this was non-significant. The apparent loss in potency of these compounds could be due to their reactivity. Compound **10** and its direct analogues all contain an unstable PAIN structure (Baell et al. 2010). These compounds could possibly be reacting with components in the cellular media to make it less lipid soluble. In addition, the compounds may also be reacting with intracellular components that modify the ability of the compound to bind to or inhibit Casp6. These compounds were later tested in the HEK293T cell line due to their increased transfection efficiency. Surprisingly, these compounds induced cytotoxicity within these cells with the same dose

tested in the HCT116 cells. The difference in toxicity between the HCT116 and HEK293T cell lines is puzzling. It is possible that these compounds may be reacting with a component present in MEM used to culture HEK293T cells but not with anything in McCoys 5A media used to culture HCT116 cells. Alternatively, the compounds may be reacting with an intracellular component within HEK293T cells that is essential for their viability while the same component in HCT116 cells is not expressed or plays a less essential role for cell viability. When tested at sub-toxic doses, these compounds had no effect on Casp6 activity. These results are not surprising since the sub-toxic doses are well below their IC_{50} values and also because it is likely that not all of the compound is passing through the lipid bilayer. Although the *in cellulo* analyses did not yield positive results, the data suggests that these compounds induce cytotoxicity in certain cell types; therefore, these compounds may not induce cytotoxicity within neurons. The use of primary mouse or human neurons would have been a better model for this project. Ideally, our hit compound would be able to reverse APP-induced neurodegeneration in primary human neurons without inducing cytotoxicity. Surprisingly, most of the previously identified allosteric Casp6 inhibitors have not been tested in cells. The allosteric peptide identified by Stanger et al. showed selective Casp6 inhibition in neuronal cells, but they neglected to show the toxicity profile of the peptide (Stanger et al. 2012). In addition, the peptide has poor cell penetration properties and was administered through electroporation. Therefore, it is unlikely that this peptide could be used in a therapeutic setting.

To date, compound **10P** is our most potent non-PAIN compound; however, the potency of compound **10P** is not considered ideal as a drug candidate. A considerable amount of work remains to be done, as medicinal chemistry will be needed to optimize the compound in several ways. First, the binding affinity and inhibitory properties of the compound will need to be modified to achieve an IC_{50} in the nanomolar range. Second, the binding affinity of the compound will need to be modified to make it more selective for Casp6 and less selective for other caspases. Third, the toxicity of the compound will need to be reduced. Fourth, the lipid solubility will need to be increased so that it can easily surpass the blood brain barrier.

Casp6 inhibition is a relatively novel approach to prevent AD progression and age-dependent cognitive impairment. In regard to selectivity, allosteric inhibitors represent a more favorable avenue than active site inhibitors. We have demonstrated that small molecule allosteric inhibitors can indeed be used to selectivity inhibit Casp6. Our findings serve as a starting point for the development of a Casp6 inhibitor that could potentially be used for preclinical evaluation someday. More importantly, our study provides a template for a novel approach to identify potential regulatory sites on other drug targets. We conclude that taking advantage of mutagenesis data together with structural analyses may hold promise as an approach for the development of novel therapeutics.

III. CONCLUSION

The results from this thesis serve as preliminary data for the development of an allosteric small molecule Casp6 inhibitor. The compounds are far from the stage of preclinical evaluation and future experiments will need to take advantage of medicinal chemistry to make them more specific for Casp6, increase their potencies to nanomolar concentrations, ensure that they are non-toxic and that they cross the blood brain barrier.

More importantly, the results from this thesis also validate a novel approach for the identification of allosteric sites on drug targets. The work described herein provides a framework for the identification of allosteric inhibitors against other potential drug targets and also cautions investigators about the PAINs of drug discovery.

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