# Prospects for Caspase Inhibitors

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**Abstract:** Programmed cell death, or apoptosis, is executed by a series of Cysteine Aspartyl Proteases (Caspases) that form a proteolytic cascade. Each caspase functions either to activate downstream caspases by proteolytic cleavage and/or to proteolytically cleave cellular substrates. Increased levels of apoptosis and caspase activity are frequently observed at sites of cellular damage in both acute (e.g. myocardial infarction, stroke, sepsis) and chronic (e.g. Alzheimer's, Parkinson's and Huntington's Disease) indications. Thus, inhibition of caspase activity with the aim of reducing cell death, and hence tissue damage, is predicted to be therapeutically beneficial. Herein we outline different approaches that have been taken to identify small-molecule caspase inhibitors that include both traditional (e.g. HTS, structure-based design and substrate analog approaches) and novel screening technologies (e.g. Tethering[SM]). In addition, the characterization of inhibitors emerging from these programs will also be presented. Many of these compounds demonstrate efficacy in a wide range of animal models; however, only two examples of caspase inhibitors have progressed to clinical testing. Here we will discuss issues (both compound and mechanism related) associated with developing a caspase program in the pharmaceutical industry.

**Keywords :** Apoptosis, caspases, inhibitor, extended tethering, inflammation, stroke, sepsis.

## 1. INTRODUCTION

Interleukin-1β-converting enzyme (ICE, Caspase-1) was the first human caspase to be identified and isolated [1,2], but since then a total of 12 human caspases have been reported (currently in the NCBI protein database). Caspases represent one of the most specific protease families yet described, since they have an almost absolute requirement for an Aspartic acid residue in the P1 position of their substrate, and require at least three additional amino acids located in the P2-P4 positions [3,4]. Caspases are divided into three groups based upon their preferred substrate preferences [5]. Group I caspases contain caspase-1, -4 and -5, and these have a preference for a hydrophobic amino acid in the P4 position. Group II caspases contain caspase-2, -3 and -7, which have a strong preference for an Aspartic acid residue in the P4 position. In contrast, group III caspases, which contains caspase-6, -8, -9 and -10, show greater tolerance for amino acids in the P4 position.

Caspase proteolytic activity is now known to play an important role in a number of biological processes. Caspases were initially identified in the nematode *Caenorhabditis elegans* as being absolutely required for programmed cell death (apoptosis) during development [6]. In the wrong context however, excessive levels of apoptosis can be detrimental. For example, enhanced levels of cell death occurs in a number of diseases such as stroke, myocardial infarction, sepsis, spinal cord injury, traumatic brain injury, Alzheimer's disease and Parkinson's disease. Caspase family members are also involved in pro-inflammatory responses, and are required for the processing and secretion of pro-inflammatory cytokines [1,2,7-9]. Thus, inhibition of caspase activity, and therefore either apoptosis or an inflammatory response, is predicted to represent novel approaches to the treatment of a wide range of diseases.

## 2. CASPASE ACTIVATION PATHWAYS

Each caspase is initially expressed as a single chain zymogen containing both a large and small subunit (Fig. (**1A**)) which is catalytically inactive (with the exception of caspase-9) [10]. Activation of the pro-form occurs by proteolytic cleavage, frequently by other caspases, to generate a free large and small subunit; the active enzyme species is a hetero-tetramer composed of two large and two small subunits with two identical active sites, each of which is made up of residues contributed by both the large and small subunits (Fig. (**1A**)) [11].

Apoptosis can be stimulated either directly by protein ligands such as Fas or Trail (the extrinsic pathway) or indirectly by chemical stress or DNA damage (the intrinsic pathway) (Fig. (**1B**)) [11]. In the former case, binding of a ligand to the cell surface directly recruits the inactive form of caspase-8 to the inner cell membrane. Even though the inactive form of caspase-8 has poor catalytic activity, the localized high-concentration of caspase-8 at the cell membrane is believed to be sufficient for auto-proteolytic activation. Active caspase-8 then cleaves pro-caspase-3 resulting in its activation, which then proteolytically degrades a wide range of cellular proteins. The intrinsic pathway acts via the release of cytochrome *c* from the mitochondria, which then forms a complex with Apaf-1 and caspase-9 in an ATP dependent manner. This protein complex, known as the apoptosome, can then cleave and activate downstream caspases. It was recently suggested that caspase-2 activity is essential for cytochrome *c* release from the mitochondria, suggesting that caspase-2 may act as an

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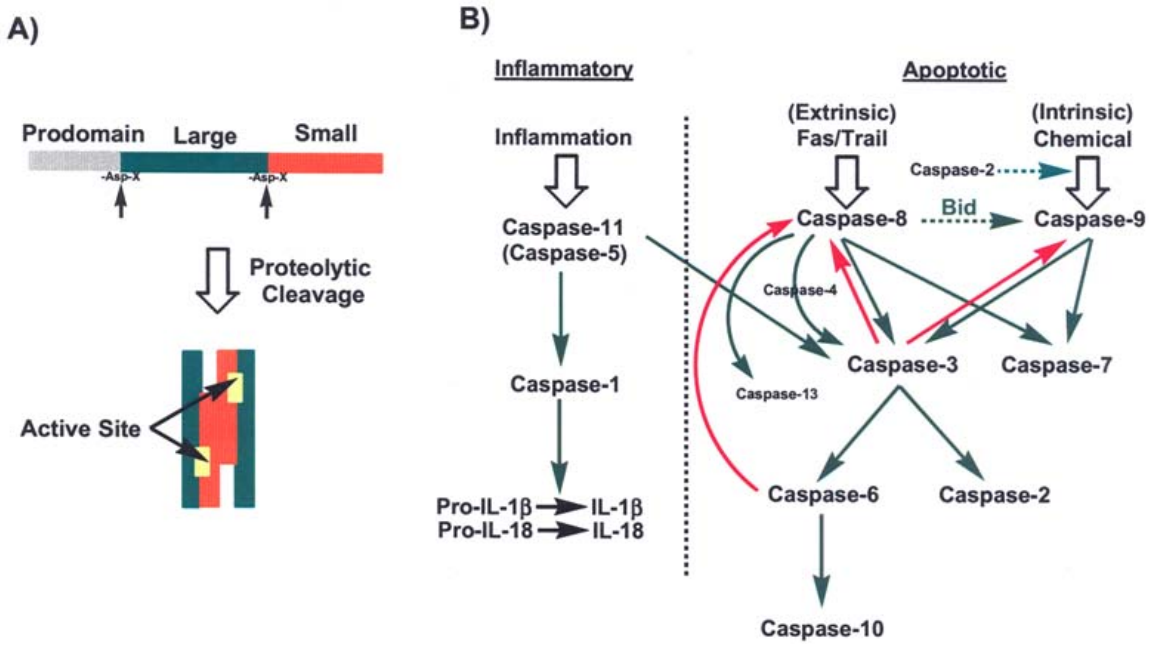


**Fig. (1).** Caspase activation and signaling pathways. **A.** Each caspase is synthesized as a single zymogen which is activated by at least two proteolytic cleavages that occur between the prodomain and the large subunit and between the large and the small subunits. The active form is a hetero-tetramer composed of two large and two small subunits. **B.** The inflammatory and apoptotic signaling cascade.

initiator caspase [12]. Nevertheless, irrespective of the initial stimulus, both the extrinsic and intrinsic pathways feed directly into the main executioner caspases (e.g. caspase-3, -7).

There are also a series of positive feedback cycles that propagate and amplify the apoptotic response. For example, caspase-3 can cleave and activate caspases-2, -6, -8 and –10 and has an important role in the amplification loop involving caspase-9 [13]. Also, when apoptosis is initiated by caspase-9, caspase-6 is necessary to activate caspase-8 and –10 [13]. Furthermore, one of the proteolytic targets of caspase-8 is the protein Bid, which upon cleavage enhances the release of cytochrome *c* from the mitochondria, and thus stimulates the caspase-9 pathway [14,15].

The inflammatory caspases (e.g. caspase-1, -5, -11) are predominantly involved in enhancing secretion of the IL-1β and IL-18 pro-inflammatory cytokines. In mice, an inflammatory challenge such as Lipopolysaccharide (LPS) induces a transcriptional upregulation of the caspase-11 gene [16]. Caspase-11 is implicated in the activation of caspase-1 since it can directly interact with caspase-1 and is also required for caspase-1 activation [17]. However, in humans there is no direct homolog of caspase-11 but data suggests that the functional homolog may be caspase-5 [18]. Similar to murine caspase-11, human caspase-5 is found in a large protein complex containing caspase-1 and two additional adaptor proteins which has been termed the inflammasome [19]. Recruitment of caspase-5 to this protein complex enhances activation of caspase-1. Once activated, both human and murine caspase-1 process pro-IL-1β (cleaved between Asp116 and Ala117)[1,2] and pro-IL-18 (cleaved between Asp36 and Tyr37) [7-9] to generate the mature forms which are then secreted from cells. Interestingly, murine caspase-11 directly interacts with and activates caspase-3 [20], suggesting a direct link between the inflammatory and apoptotic caspase pathways.

## 3. INDICATIONS

Enhanced levels of caspase activity leading to excessive apoptosis or inflammatory responses have been implicated in a large number of indications, suggesting that caspase inhibition could be a viable strategy for therapeutic intervention. As will be discussed below in greater detail, acute indications are attractive to the pharmaceutical industry due to potential toxic side-effect issues associated with inhibiting apoptosis over extended periods of time. Here we present a brief overview of the major diseases in which caspases may be involved.

### 3.1 Cardiac Disease

The most recent data available from the American Heart Association estimates that ~1.1 million Americans will suffer a new or recurrent myocardial infarction (MI) in 2002. In 1999, 85% of individuals who were treated for MI were given beta-blockers; however, even with this treatment 28% of men and 38% of women die within one year of a recognized MI. Thus, there is a very clear unmet medical need for improved pharmaceutical intervention to reduce the death rate from MI.

MI occurs when there is a reduction in blood flow to the heart, resulting in myocyte (heart muscle) cell death. One of the immediate aims is to reopen a blocked artery and restore blood flow to the heart tissue which minimizes the extent of heart muscle damage and preserves the pumping function of the heart. However, even though reperfusion treatment is important for recovery following an MI, reperfusion can enhance myocyte apoptotic cell death [21]. Thus, current treatment protocols, despite being critical for enhancing overall recovery, may also contribute to tissue damage. Administration of a caspase inhibitor in conjunction with reperfusion would potentially increase the effectiveness of this treatment strategy by decreasing myocyte apoptosis.

It was initially observed that myocytes displayed signs of apoptosis in heart samples following MI [22]. Consistent with this finding it was shown that release of cytochrome *c* into the cytosol was significantly increased, and this increase also correlated with enhanced levels of activated caspase-3 in explanted hearts from transplant patients [23] or in cultured myocytes exposed to ischemia [24], suggesting activation of the intrinsic apoptotic pathway (caspase-9). Further evidence implicating a role for caspases in cell death following MI comes from the observation that in rabbit hearts, caspases-2, –3, and –7 were activated following ischemia/reperfusion [25] and proteolytic cleavage of Bid was coincident with increased release of cytochrome *c* from the nucleus [26]. In addition, over-expression of caspase-3 in murine cardiomyocytes increases infarct size following ischemia reperfusion injury relative to control animals and also reduced heart function [27]. Thus, the evidence strongly suggests that apoptosis may contribute to cell/tissue damage following MI.

Peptide inhibitors have further suggested that caspases have a central role in the pathogenesis of MI. An irreversible broad spectrum caspase inhibitor z-VAD-FMK reduced infarct size following experimentally induced MI in a rat model [28], and in rabbits administration of Ac-YVAD-CMK reduced infarct size by 31% [25]. Yet another study has shown that infusion of a variety of caspase inhibitors (z-VAD-FMK, Ac-DEVD-CMK, z-IETD-FMK, and z-LEHD-FMK) during early reperfusion also reduced infarct size [29]. Thus, there is a growing volume of evidence strongly linking caspase expression with myocyte cell death. These data suggest that inhibition of apoptosis may be of great benefit in reducing both infarct size, cell death and tissue damage.

### 3.2 Rheumatoid Arthritis

Arthritis is characterized by the local inflammation of joints and the destruction of cartilage and bone [30] and is a widespread chronic health problem. The two most common forms of arthritis are osteoarthritis (OA) and rheumatoid arthritis (RA). OA affects ~16 million older Americans and is a degenerative joint disease in which there is cartilage erosion around the weight-bearing joints of the hip and knee. RA affects ~2.5 million Americans and is a chronic, systemic and disabling autoimmune disease characterized by inflammation of the connective tissue. In RA the synovial fluid is found to contain high levels of activated mononuclear cells (~20-30% of these are T-cells) with the predominant cytokines being IL-1β and TNF-α, each of which are capable of eliciting further inflammatory responses and cartilage breakdown by binding to their cell surface receptors and stimulating multiple signal transduction pathways [30].

Caspase-1 processes both pro-IL-1β and pro-IL-18 to generate the mature forms which are secreted into the synovial fluid and are involved in the initiation of inflammatory signaling pathways. Synthetic inhibitors have been used to demonstrate the central role that caspase-1 plays in rheumatoid arthritis. For example, the tripeptide irreversible inhibitor Ac-VAD-$CH_2$O-DCB reduces the severity of type II collagen induced arthritis in a mouse model [31] and reduces circulating IL-1β cytokine levels in zymosan stimulated mice [32]. A reversible inhibitor of caspase-1 (Ac-YVAD-CHO) also reduced IL-1β cytokine levels in mice after LPS stimulation [33] and reduced the level of mature IL-1β secreted by macrophages removed by biopsy from patients with inflammatory bowl disease but not from control macrophages [34]. Thus, caspase-1 inhibitors reduce circulating levels of the pro-inflammatory cytokine IL-1β and improve symptoms associated with typical inflammatory disorders in mice.

One potential drawback of using a caspase inhibitor for RA is that treatment requires long-term dosing. Thus, a successful small molecule inhibitor would have to display extreme specificity for caspase-1 and also have acceptable toxicity.

### 3.3 Stroke

A stroke occurs when there is a clot in a blood vessel leading to the brain, resulting in neuronal death due to ischemia. There are two main types of stroke: hemorrhagic (a sudden loss of blood, e.g. vessel rupture), which accounts for ~15% of cases, and ischemic (a temporary or permanent block of blood supply to the brain), which accounts for the remaining 85%. Recent statistics from the American Heart Association indicate that stroke is the third leading cause of death in the US with ~600,000 people suffering a new or recurrent stroke. It is estimated that the total direct and indirect costs associated with stroke in the US in 2002 will be ~$49 billion. Again, as with MI, there is clearly an unmet medical need.

Induction of ischemic injury can be either focal (localized) or global, and in both cases the damage can be either transient or permanent. Animal models mimicking stroke have been used to study the effectiveness of caspase inhibitors as potential therapeutic agents. For instance, in a murine model for focal ischemia, increased levels of caspase-3-like activity and DNA laddering were documented to occur

after six hours of reperfusion [35]. Importantly, the inhibitor z-DEVD-FMK increased neuroprotection (as measured by infarct volume and the number of viable cells) even when administered up to nine hours after ischemic injury. In contrast, in another study the z-DEVD-FMK inhibitor was only effective when administered before ischemic injury but z-VAD-FMK reduced infarct size when given up to six hours following ischemic injury [36]. In a model for global ischemic injury in rats the z-DEVD-FMK inhibitor had no discernable positive effect on neuronal function following global ischemia, but in one of three experiments the inhibitor offered neuroprotection in a focal ischemic model when administered 30 minutes before insult followed by three additional treatments [37]. In another study, malonate injection was used to stimulate stroke in rats (similar to focal ischemic injury) and the general inhibitor z-VAD-FMK reduced both the lesion volume and the percentage of cells characterized to be undergoing apoptosis [38]. Importantly, in this case the inhibitor could be administered up to nine hours following malonate injection. Furthermore, MK-801 (an N-methyl-D-aspartate receptor antagonist) when used in combination with z-VAD-FMK at sub-threshold levels reduced lesion volume, suggesting that combination therapy may be as beneficial, or even better than stand-alone treatments. Even though caspase-1 is not directly implicated in the apoptotic pathway, transgenic mice expressing a dominant negative form of caspase-1 (catalytically inactive by mutating the active site cysteine residue) have reduced infarct size and fewer behavioral changes after a permanent ischemic injury [39,40]. This positive outcome may be due to reduced levels of the pro-inflammatory IL-1β and IL-18 cytokines.

Overall there is a lack of consistency in results from animal stroke models, with differences possibly arising from variations in the protocols and/or methods of injury as well as differences in inhibitor selectivity. Even though the data is intriguing, additional evaluation is needed to determine whether or not inhibition of apoptosis can enhance functional neuronal recovery following ischemic injury.

### 3.4 Traumatic Brain Injury

Traumatic Brain Injury (TBI) occurs when an insult to the brain results in an impairment of cognitive ability or physical function. The Brain Injury Association of America estimates that 5.3 million Americans live with disabilities resulting from brain injury and that one million people are treated every year for TBI. Increased levels of caspase-3 and –8 mRNA and activated protein were found in brain samples of rats subjected to TBI [41]. A separate study also found increased levels of caspase-1 and –3 enzyme like activities following injury, and administration of the z-DEVD-FMK inhibitor reduced the hallmarks of apoptosis and improved neurological recovery in rats [42]. Another study documented an increase in caspase-3 enzyme like activity, and the inhibitor z-DEVD-FMK significantly reduced contusion size; however, unlike the previous study there was no effect on motor function (as measured by balance beam, beam walking, or the Morris water maze) [43]. Thus, even though caspase inhibitors can reduce levels of tissue damage, the resulting effect on functional outcome needs to be further clarified.

### 3.5 Spinal Cord Injury

According to the latest statistics from the National Spinal Cord Injury Association in the US, there are 183,000 to 230,000 people with spinal cord injury which are most commonly caused by automobile accidents, falls or violence. Increased levels of both activated caspase-9 (intrinsic pathway) and –3 were found in spinal cords of rats after infliction of injury and an increase in caspase enzyme activity was detected as early as one hour following treatment [44]. A related study also found increased levels of activated caspase-8 (the extrinsic pathway) in spinal cord tissue as early as 1.5 hours following a transient trauma and also found enhanced levels of Fas ligand after 24 hours (which is known to stimulate the extrinsic pathway) [45]. Increased levels of caspase-3 were also detected, but this occurred at a later time than caspase-8 supporting the model of sequential activation (i.e. caspase-8 activates caspase-3). Thus, it may be necessary to inhibit both the extrinsic and the intrinsic pathways to effectively reduce apoptosis following spinal cord injury. Caspase-1 may also play an important role in tissue death following spinal cord injury, since caspase-1-like activity increased following injury and the z-VAD-FMK inhibitor reduced lesion size and resulted in a significant improvement in motor function [46]. Consistent with a role for caspase-1, expression of a dominant negative form of caspase-1 also had a similar effect on functional outcome [46]. It is not fully understood how caspase-1 exerts this effect, but it may be due to the role that caspase-1 plays in the expression of the pro-inflammatory cytokines IL-1β and IL-18.

### 3.6 Sepsis

A recent study has estimated that there are ~750,000 cases of sepsis (due to severe infection) per year in the US, with a mortality rate of ~29% [47]. Increased levels of apoptotic cells and activated caspase-3 are found in septic patients compared to a control population [48]. Apoptosis predominantly occurred in lymphocytes and intestinal epithelial cells, with a significant reduction of both B-cells and CD4 T-cells in spleens [49]. Mouse sepsis models have a similar pathology since high levels of apoptosis in lymphocytes are also detected following sepsis induction by cecal ligation and puncture (CLP) [50]. Since lymphocytes are important for an immune response, reduced lymphocyte cell numbers due to apoptosis could be a critical factor in the inability to mount a sufficiently strong immune response to overcome sepsis. Thus, inhibiting apoptosis, and therefore lymphocyte cell loss, may increase patient survival rates. Consistent with this, the broad spectrum caspase inhibitor z-VAD-FMK prevented lymphocyte apoptosis and dramatically increased survival rates in mice following CLP [51]. In addition, both a broad spectrum and a caspase-3 specific inhibitor (undisclosed structures) were equally effective at increasing survival rates [52]. Furthermore, neither inhibitor could enhance survival rates in Rag-$1^{-/-}$ mice (lack mature B and T cells), as would be expected if the inhibitors are exerting their effect predominantly through decreasing apoptosis of lymphocytes. Since sepsis also results in heart damage, it was not surprising that z-VAD-FMK reduced apoptosis in the heart and reduced cardiac dysfunction (as measured *ex vivo*) when sepsis was induced

by endotoxin shock [53]. Thus, these data strongly suggest that sepsis may be one of the more promising therapeutic areas for a caspase inhibitor.

### 3.7 Liver Failure

It is well documented that a wide variety of liver diseases are associated with increased levels of apoptosis. Higher levels of apoptotic cells are detected in liver samples from a range of disease states such as alcoholic liver disease, hepatitis B and C, chronic cholecystitis and primary sclerosing cholangitis [54]. Many animal models mimic liver disease by the administration of Fas ligand which activates apoptosis via the caspase-8 (extrinsic) pathway. Liver cells are particularly susceptible to Fas since the Fas receptor is upregulated in a variety of liver diseases [55]. Peptide based inhibitors (z-VAD-FMK, z-DEVD-FMK, or Ac-YVAD-CMK) [56-60] or transgenic mice [61] have been extensively used to demonstrate that inhibiting apoptosis can reduce cell death, improve physiological parameters and enhance survival after liver injury. There have also been reports using small molecule caspase inhibitors (discussed below) to reduce the hallmarks of liver injury induced either by Fas [62] or by Gal/ET (which induces caspase-8 *via* the TNF-α pathway) [63]. Recently, the first Phase I clinical trial with a compound targeting an apoptotic caspase was completed for acute alcoholic hepatitis (IDN-6556, Idun Pharmaceuticals, 2002, http://www.idun.com/text/news/news1.htm), and will be discussed in more detail below.

### 3.8 Neurodegeneration

#### *3.8.1 Alzheimers Disease*

The prevailing hypothesis explaining Alzheimer's Disease is the slow accumulation of the amyloid-β (Aβ) peptide (a proteolytic fragment of the β-amyloid precursor protein (βAPP)) within plaques in the brains of diseased patients. Interestingly, the Aβ peptide alone induces apoptotic cell death in tissue culture cells, which can be inhibited by z-VAD-FMK [64]. However, caspases may have a more direct role in Alzheimer's Disease since they directly cleave βAPP to generate small peptides that induce apoptosis. Caspases-3, -6, -7, -8 and -9 all cleave βAPP at the same position to generate a C-terminal fragment (designated C31) which is found in senile plaques in Alzheimer's Disease patients and in hippocampal neurons during acute brain injury [65]. Interestingly, the C31 fragment itself is a potent inducer of apoptosis [66]. Further emphasizing the link between caspases and C31, both activated caspase-9 and the C31 peptide were found in the brains of Alzheimer's Disease patients but not in control brains [66], supporting the notion that apoptosis may be involved in disease progression. Additional evidence implicating caspases in the cleavage of βAPP comes from the characterization of the Swedish familial mutation of APP, which results in early onset of Alzheimer's Disease and elevated production of the Aβ peptide. This mutation creates a consensus caspase cleavage site (which can be efficiently cleaved by caspase-6 *in vitro*) at a position corresponding to the β-secretase cleavage site [65]. Therefore, caspase cleavage may be directly responsible for enhanced levels of Aβ in these patients. Murine caspase-12 may also be important in apoptosis induced by Aβ since cortical neurons in mice lacking functional caspase-12 are resistant to Aβ induced apoptosis [67]. However, recent results have suggested that the human homolog (as determined by sequence homology) may be not be functional due to the presence of deleterious mutations [68]. Therefore, it remains to be determined how relevant caspase-12 may be in the development of Alzheimer's Disease in humans.

Whether caspases contribute globally or more specifically to the pathogenesis of Alzheimer's Disease is still not completely elucidated. For instance, it was suggested that caspase-3 may have a more specific role only in the loss of neurons involved in learning and memory [69]. Nevertheless, current evidence strongly suggests that caspases are associated with the progression of Alzheimer's Disease. More evidence is necessary, however, before any firm conclusions can be reached as to whether caspases contribute to disease progression, and if they do, whether inhibition of apoptosis is a viable means of therapeutic intervention.

#### *3.8.2 Parkinsons Disease*

Parkinson's Disease is an age related deterioration of parts of the nervous system, and is characterized by the loss of dopaminergic neurons and the presence of Lewry bodies in altered neurons. Numerous studies have shown a correlation between activation of caspase-3 in animal models of Parkinson's Disease and progression of the disease. For example, a correlation was observed between the levels of activated caspase-3 positive neurons and the degree of neuronal loss in brain samples from patients compared to control samples [70]. However, stronger evidence linking caspases with Parkinson's Disease needs to be obtained.

#### *3.8.3 Huntington's Disease*

The huntingtin protein contains an N-terminal CAG/polyglutamine repeat and mutations associated with Huntington's Disease result in an expansion of this polyglutamine repeat, which is associated with enhanced levels of neuronal loss and dysfunction. It was initially shown that caspase-3 can cleave the huntingtin protein and that cleavage occurs at the N-terminal region releasing the polyglutamine tract [71]. Interestingly, the rate of caspase cleavage was observed to increase as the length of the polyglutamine repeat increased and brain samples from patients were shown to contain cleaved forms of the huntingtin protein consistent with caspase-3 proteolysis [72]. As suggested by a recent report, it is possible that caspase-3 may be activated via the caspase-8 pathway in Huntington's Disease [73]. In this case it was found that the protein Hip-1 is normally sequestered in a complex with wild-type huntingtin protein, but not with huntingtin protein containing an expansion of the polyglutamine repeat. This free Hip-1 protein is then able to interact with caspase-8 resulting in its activation, which presumably then activates caspase-3. Caspase-1 is also implicated in progression of Huntington's Disease since transgenic mice overexpressing a dominant negative form of caspase-1 had a later onset of symptoms, an increase in the length of disease progression, and an extended lifespan [74]. It was suggested that these effects may be partly due to reduced levels of mature IL-1β, which itself is implicated in neuronal cell death.

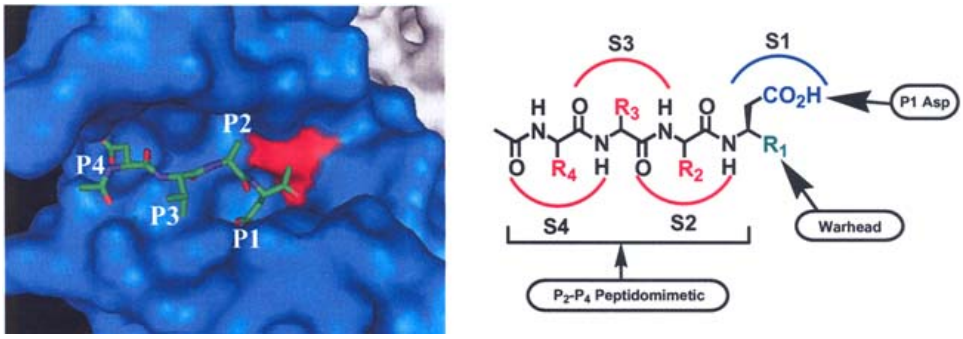


**Fig. (2).** X-ray crystal structure of caspase-3 bound with the tetrapeptide inhibitor (Ac-DVAD-FMK) [105]. Shown in red is the active site cysteine residue. Shown along side is a schematic of a typical caspase inhibitor. Caspase inhibitors are usually composed of a warhead, a $P_1$ Aspartic residue and a $P_2$-$P_4$ peptidomimetic. The corresponding pockets of the active site where each of these groups bind is shown, $S_1$-$S_4$.

Again, the evidence is suggestive that caspases may be involved is disease pathogenesis, but more definitive studies are needed.

## 4. DRUG DESIGN

Small tetrapeptide-based inhibitors can selectively inhibit caspase family members [75], and the structures of many caspases have been solved (caspase-1 [76,77], –3 [1,2,78], –7 [79], –8 [80,81], and –9[82]) in most cases complexed with an active site inhibitor (for an example see Fig. (**2**)). Substrate selectivity is derived from the $P_1$ position, which requires an Asp residue, the $P_2$ and $P_4$ positions, where most of the binding specificity occurs, and to a lesser extent the $P_3$ position [83]. The $P_1$ aspartic acid residue is engaged in several hydrogen bonds within the $S_1$ pocket. With just two exceptions, all active inhibitors reported to date contain an Asp in the $P_1$ position. The $S_4$ subsite is the position where specificity can be most readily obtained. For example, caspase-3 prefers a negatively charged amino acid in this position (such as Asp), whereas caspase-1 prefers a more hydrophobic residue (such as Trp) [83]. Many inhibitor design strategies have taken advantage of these differences in binding requirements to identify either caspase-specific or pan inhibitors.

A typical caspase inhibitor can be divided into three structural components: the warhead, the $P_1$ aspartic acid and the $P_2$-$P_4$ peptidomimetic (Fig. (**2**)).

### 4.1 Warhead

The warhead consists of an electrophile that reacts with the nucleophilic cysteine residue of the active site. Depending upon the nature of the groups flanking the warhead, an inhibitor can be either reversible or irreversible. Examples of some commonly used warheads are shown in

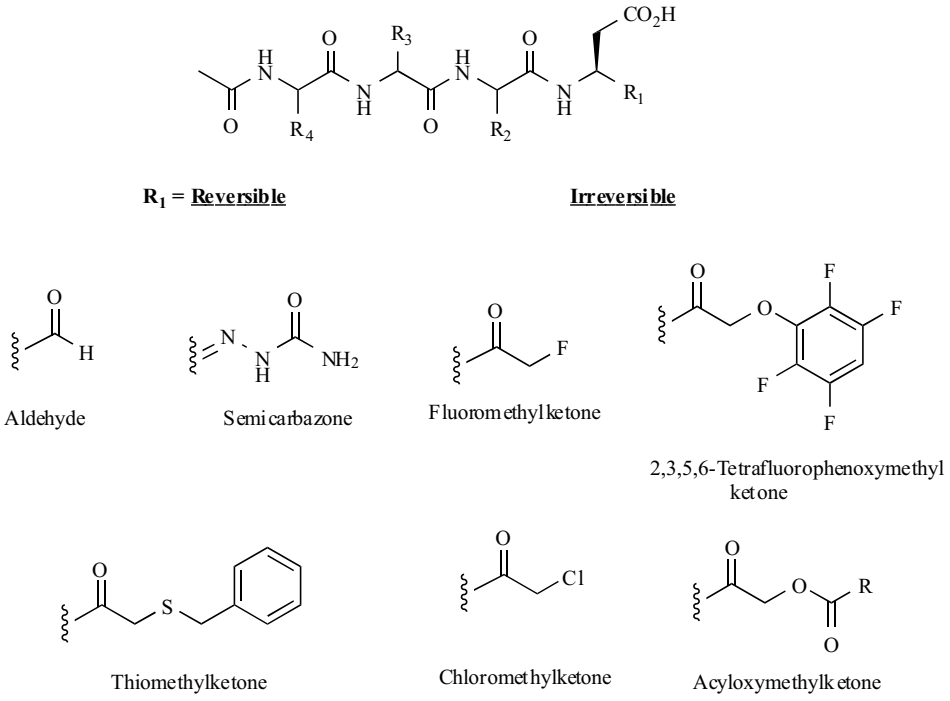


**Fig. (3).** Examples of both reversible and irreversible binding warheads.

Fig. (**3**). From a safety perspective, reversible inhibitors are generally preferred since they will not irreversibly bind to non-target proteins, which has been observed with some irreversible inhibitors. For example, in addition to caspases, the irreversible inhibitor z-VAD-FMK inhibits non-related cysteine proteases such as purified cathepsins B and H [84] and the irreversible inhibitor Ac-YVAD-CMK was shown to irreversibly bind cathepsin B in cells [85]. Despite this potential lack of specificity, a significant advantage associated with irreversible inhibitors is greater potency in cellular assays of apoptosis (discussed below).

### 4.2 $P_1$ Asp

Since caspases have a strong preference for an aspartic residue in the $P_1$ binding position [3,4], most efforts have incorporated this into lead molecules. However, there are two examples of compounds lacking the $P_1$ aspartic acid sub-structure but still retain potent caspase inhibitory activity. In one case, Compound **1** (Fig. (**4A**)) was initially identified by high-throughput screening and subsequently optimized into a potent and selective low nanomolar inhibitor of caspases-3 and -7 (Compound **3**) [86,87]. The warhead is a cyclic ketoamide and the X-ray crystal structure of this compound bound to caspase-3 revealed a tetrahedral intermediate formed between the active site thiolate and the ketone carbonyl group. Despite the lack of significant binding in the $S_1$ subsite, this series possessed potent caspase inhibitory activity, selectivity versus other cysteine proteases and could inhibit apoptosis in cell-based assays.

In a second example, an acyl-sulfonamide (a classic carboxylic acid replacement) was substituted for the aspartic acid residue (Compound **5**, Fig. (**4B**)) [88]. In this case the X-ray crystal structure revealed that the methanesulfon-aminocarbonyl group in the $P_1$ position is buried in the $S_1$ pocket and engages in hydrogen bonding interactions with Arg179 and Arg341 (residues that typically hydrogen-bond with the $P_1$ Asp residue). The additional steric bulk of the methanesulfonyl group is accommodated by rearrangement of the surrounding amino acids. This compound displayed potent cellular activity since it inhibited secretion of mature IL-1β with an $IC_{50}$ of 0.23 μM. It remains to be seen whether other caspases can accommodate this group.

Other than these two examples, there is little evidence that removing or modifying the $P_1$ aspartic acid side chain without a significant decrease in activity can be accomplished. Thus, based on the fact that the great majority of inhibitors published to date possess an aspartic acid unit, one must conclude that the $S_1$ subsite possesses very little tolerance for binding groups other than aspartic acid.

### 4.3 $P_2$-$P_4$ Peptidomimetic

This is the area of caspase inhibitor design that has generated the greatest novelty in structure. In most cases, inhibitor design relies on a substrate analog approach in which a tetrapeptide substrate sequence is the starting point. After a suitable warhead is incorporated, non-peptide substitutions for the $P_2$-$P_4$ regions are identified that improve the pharmacokinetic properties of the molecule. Since the caspase family is rich in structural information, structure-based design plays a major role in the optimization process. These efforts have resulted in the identification of several novel peptidomimetic motifs (e.g. Compounds **6**-**14**). For example, compound **11** was shown to rescue mice from lethal experimental hepatitis and decreased circulating levels of plasma markers associated with fulminant hepatic failure [89]. A series of dipeptides has also been reported by Cytovia (e.g. Compound **14**) to have neuroprotective effects in a rat model of transient focal ischemia [63,90]. However, these inhibitors are irreversible broad spectrum inhibitors which raises questions regarding their *in vivo* specificity and potential toxicity.

Recently, two caspase inhibitors have entered clinical trials. Vertex Pharmaceuticals announced the completion of Phase IIa clinical trials with Pralnacasan (VX-740; Compound **6**, Fig. (**5**)), a caspase-1 specific inhibitor, for the treatment of Rheumatoid Arthritis (RA) (2002 Vertex

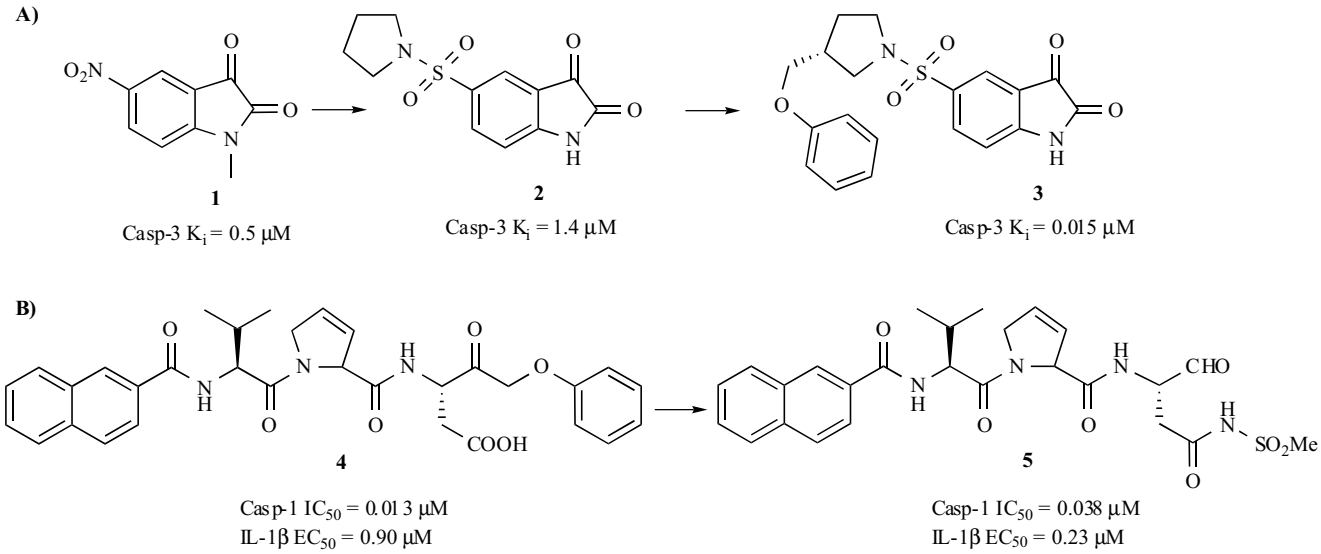


**Fig. (4).** $P_1$ Aspartic acid replacements. **A.** A low micromolar caspase-3 inhibitor (compound **1**) was rapidly optimized to generate compound **3** [86,87]. **B.** A second compound showing the original Asp in the $P_1$ position (compound **4**) which was replaced with an acyl-sulfonamide to generate compound **5**, which also inhibited caspase-1 both *in vitro* and in cells [88].

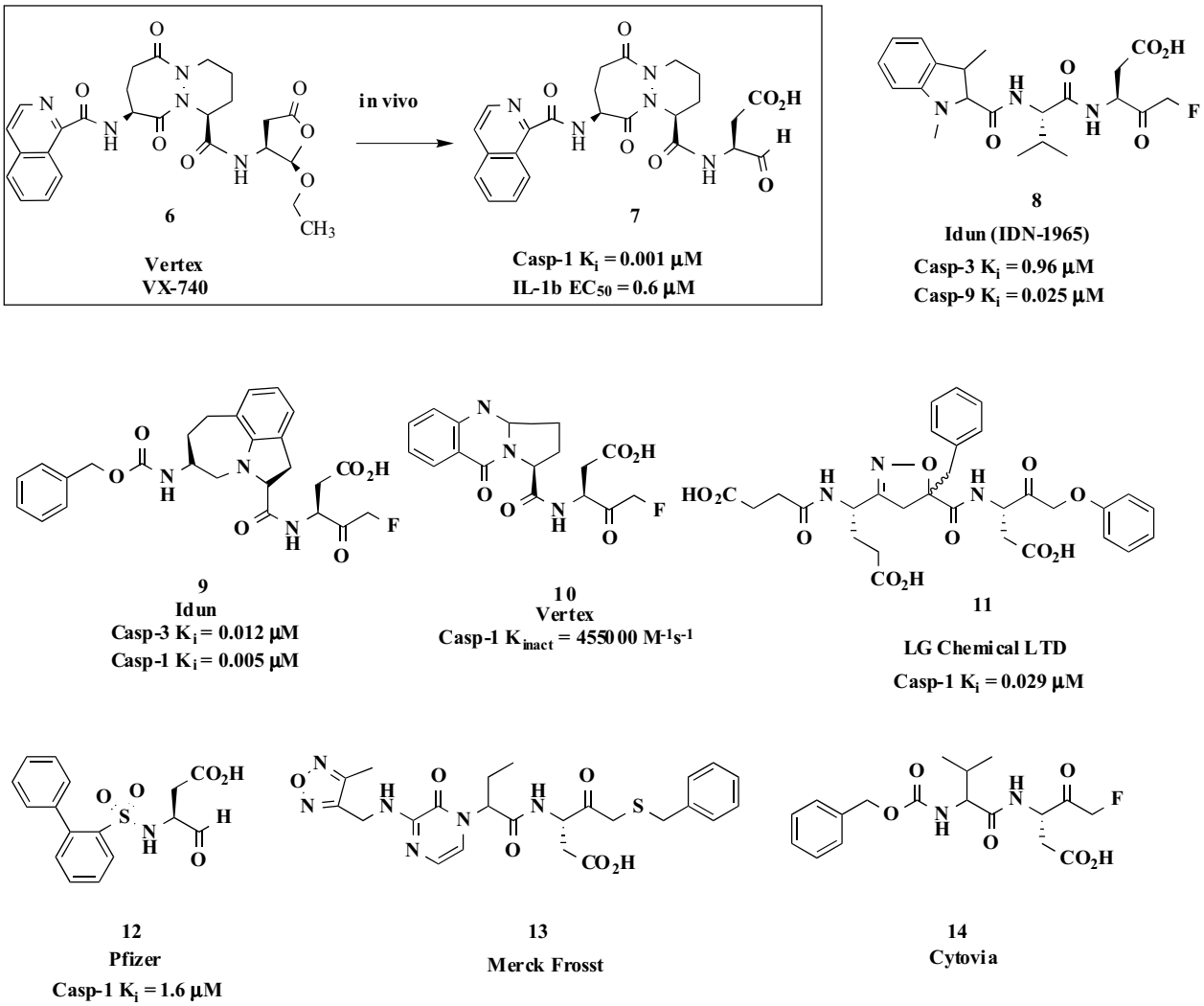


**Fig. (5).** Example of caspase inhibitors. VX-740 (compound **6**) [91] is delivered as an acetal prodrug which is rapidly hydrolyzed to compound **7** *in vivo*. Examples of other reported caspase inhibitors are also shown, Compound **8** [94], **9** [93], **10** [106], **11** [89], **12** [107], **13** [108] and **14** [90].

Press Release, http://www.vpharm.com/frame09.html). This compound is administered orally as an acetal pro-drug which undergoes *in vivo* hydrolysis to the active species possessing an aldehyde warhead (compound **7**). The aldehyde version of the compound (compound **7**) has a reported $K_i$ of ~ 1 nM for caspase-1 and inhibits IL-1β secretion from human peripheral blood mononuclear cells (PBMCs) with an $EC_{50}$ of ~0.6 μM [91]. Pralnacasan was found to significantly reduce joint symptoms and inflammation (as measured by $ACR_{20}$) in all patients who were not on methotrexate or on methotrexate for greater than six months (p=0.0084). However, it was recently announced that this compound was voluntarily withdrawn from Phase IIb clinical studies due to abnormal liver toxicity observed in long-term animal studies. Despite this, Vertex is continuing additional Phase I trials with this compound. Additionally, Vertex has already disclosed that a second generation caspase-1 inhibitor, VX-765, is currently in Phase I clinical trails. The results from these clinical studies not only demonstrate the effectiveness of inhibiting caspase-1 as a therapeutic for RA, but also illustrate the difficulties associated with long-term dosing o f a caspase inhibitor with a reactive warhead.

The second caspase inhibitor in clinical trials, IDN-6556, successfully completed a Phase I trial for acute alcoholic hepatitis (IDUN Pharmaceuticals, 2002, http://www.idun.com/text/news/news1.htm). The structure of this compound has not been disclosed, but it does possess an irreversible warhead [92]. However, the structures of earlier compounds (which are also irreversible) developed by IDUN have been disclosed (e.g. compounds **8** and **9**) [93,94]. One of these (compound **8**, IDN-1965) was shown to dramatically increase survival in rats following Fas-induced liver damage (normally lethal within ~five hours) even when administered up to three hours after Fas administration [62], and prolonged survival of mice after exposure to endotoxic shock [95].

Recently, a novel approach has been reported that allows the rapid discovery of non-peptidomimetic inhibitors of caspases [96-98]. Tethering$^{SM}$ is based on the covalent capture of binding fragments within the active site of caspase-3 using an extender (a small-fragment that irreversibly labels the active site cysteine residue and contains a free thiol) (Fig. (**6A-B**)). The modified caspase-3/extender protein complex was screened against a library of

fragments modified to contain a free sulfhydral group to identify those fragments that bind nearby and form a disulfide bond with the extender. This method is used to identify ligands that have weak but measurable binding affinities for the active site. By modulating the level of reductant in the reaction it is possible to covalently trap these weak binding ligands and identify them by mass spectrometry. Once identified, the selected fragment is combined with a version of the extender containing a reversible binding warhead and the disulfide bond is replaced with a more pharmaceutically appropriate linkage. Using this approach, one selected fragment was rapidly converted into a potent reversible binding caspase inhibitor with a $K_i$ of 2.8 μM (compound **15**), which was then converted into compound **16** with a $K_i$ of 0.2 μM by replacing the n-pentyl linker with a more rigid aminobenzyl moiety. Additional medicinal chemistry, guided by molecular modeling and X-ray structural information, enhanced the potency of one series into low nM inhibitors (Compounds **17**-**18**) [98]. Given the ease of this approach, it should be readily applicable to other caspase family members.

## 5. CHALLENGES ASSOCIATED WITH DEVELOPING CASPASE INHIBITORS AS DRUGS

Even though caspase drug discovery efforts have been under way for over 10 years, only two compounds (VX-740 and IDN-6556) have entered clinical trials. Some of the

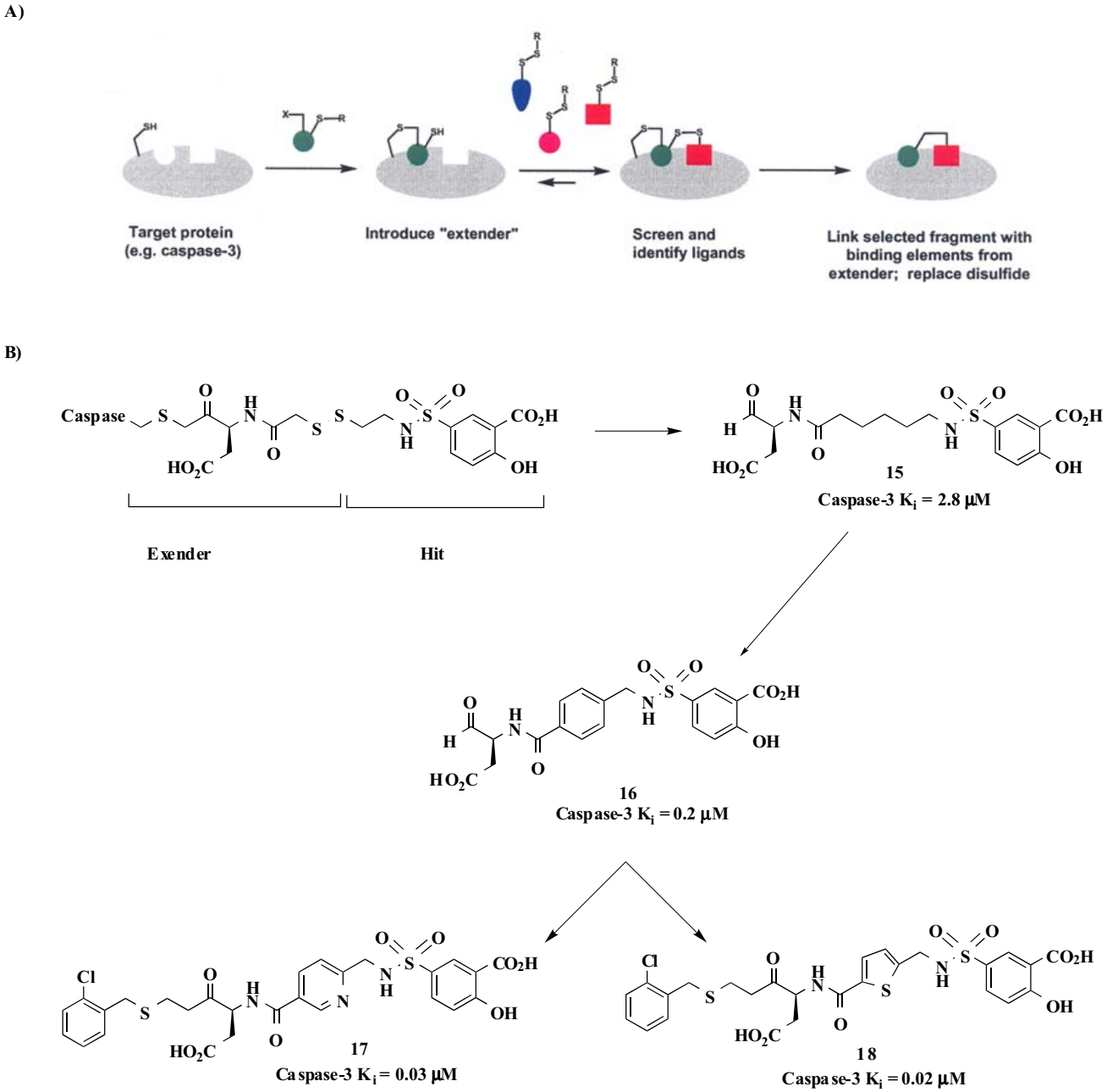


**Fig. (6).** Tethering$^{SM}$ can discover novel caspase inhibitors. **A**. Schematic illustration of Tethering$^{SM}$ using an extender [98]. **B.** Caspase-3 inhibitors derived from Tethering$^{SM}$. Shown is the structure of the extender and the selected hit fragment, which was converted into a reversible inhibitor (compound **15**). Further optimization resulted in compounds **16**-**18** [97].

reasons that have likely contributed to this slow progression towards the clinic are discussed here.

### 5.1 Which Caspase(s) Need to be Targeted?

It is fairly clear that inhibition of caspase-1 is sufficient to yield an anti-inflammatory effect. However, it is less clear which of the apoptotic caspases need to be inhibited in order to observe reduced cell/tissue damage. Since there are now twelve different human caspases, the identification of inhibitors with the appropriate caspase selectivity profile has been an ongoing challenge.

One approach is to inhibit an upstream caspase, such as caspase-8 or -9 (see Fig. (**1B**)), since inhibiting either one may reduce activation of the downstream caspases. However, inhibiting the initiator caspase of the extrinsic or intrinsic pathways may not be sufficient, since in many instances cells rarely die by just one pathway. For example, following MI there is evidence that the mitochondrial pathway is activated (i.e. caspase-9) [23,24], but evidence also suggests that at least one ligand responsible for inducing the caspase-8 pathway (CD95/Fas) is released by damaged heart cells [99]. Additionally, evidence indicates activation of both the caspase-8 and -9 pathways following cerebral ischemia [45,100,101]. Thus, since both pathways may be activated in certain indications (although which comes first is not clear), a caspase inhibitor specific for one pathway but not the other may be insufficient. A second approach is to target the executioner caspases since both the intrinsic and extrinsic pathways converge on these caspases (Fig. (**1B**)). In some indications this may be a viable approach, as for example sepsis where it has been shown that a caspase-3 specific inhibitor performs as well as a pan-caspase inhibitor [52]. A third approach is a combination of the first two approaches, with the aim being to inhibit a broad subset of both initiator and executioner caspases. So far, this appears to be the approach that is most commonly taken. It is likely that inhibitors with different profiles will be tested for efficacy in each indication since different indications may need to be targeted with inhibitors with different selectivity profiles.

### 5.2 When do Caspase Inhibitors Need to be Administered?

Most apoptotic caspase efforts focus on acute indications in which timely treatment after injury is required (e.g. myocardial infarction, stroke or traumatic brain injury). However, the biology of these indications suggest that apoptosis may be a relatively early event following injury, and thus a caspase inhibitor may have to be rapidly administered in order to produce favorable outcomes. In many animal studies, the maximum benefit of a caspase inhibitor occurs when it is administered either before or concurrent with injury [25,28,29,43,46,102], a situation unlikely to occur when targeting numerous acute indications in humans. However, there are examples from animal models in which a caspase inhibitor can be administered following injury and still produce a positive outcome. In one case, z-DEVD-FMK could be administered at six or nine hours (but not at twelve hours) following cerebral ischemic injury and still reduce infarct volume [35]. In a similar example, z-VAD-FMK, in combination with MK-801 (an N-methyl-D-aspartate receptor antagonist), extended the therapeutic window following injury to twelve hours [38]. In cardiac ischemia, either z-DEVD-FMK or z-VAD-FMK could be given six hours after reperfusion (but not eighteen hours) and still reduce infarct size and neurological deficits [36]. However, in a different MI model system, z-VAD-FMK or z-DEVD-FMK reduced myocardial dysfunction only when administered less than two hours following trauma [103]. Thus, choosing the appropriate animal model will be very important for compound evaluation.

Depending upon the indication, early administration may or may not be feasible. For instance, patients with stroke often do not present themselves to the hospital until many hours have progressed since onset of symptoms, especially in cases of mild stroke. Thus, the window of opportunity may have already passed. However, in cases of cardiac ischemia, administration could potentially occur early following the ischemic attack, especially following more severe MI. Thus, the therapeutic window for administration of a caspase inhibitor may vary depending upon the type of injury, the length of the ischemic period and whether it can be administered in combination with other drugs (as combination therapies may represent another option for extending the therapeutic window). Unfortunately, there are still relatively few examples where the timing of inhibitor administration has been extensively examined.

### 5.3 Reversible or Irreversible Warhead?

Reversible inhibitors of caspase-1 are effective in cell-based assays (for their ability to reduce levels of IL-1β, the main proteolytic target of caspase-1) and in animal models of inflammation [32,33,91]. However, the situation is not as clear when trying to inhibit the apoptotic caspases. Even though reversible inhibitors can be extremely potent *in vitro* (with $K_i$'s typically <20 nM), it is often the case that they can have poor cell-based activity (usually >200 μM) (see [94, 98]). One possible explanation for this discrepancy is the potential for positive feedback within the cascade (see Fig. (**1B**)). It may be necessary to inhibit most, if not all, apoptotic caspase activity to ensure cell survival, otherwise the small percentage of remaining active caspase may be sufficient to propagate an apoptotic signal.

One strategy for improving cellular activity is to incorporate irreversible warheads into inhibitors. This increases potency and broadens the caspase inhibition profile *in vitro*. As a result of one or both of these effects, increased cellular activity is also observed [94]. However, there is a significant concern with the development of irreversible inhibitors compared to reversible inhibitors since they have broader inhibition profiles and are less selective (can label non-target proteins) [84,85]. Furthermore, there is the additional possibility that irreversibly labeled protein has the potential of undesirable side-effects, such as an antigenic response to labeled protein. Despite these potential limitations, some irreversible inhibitors are actively being developed. For example AG7088, an inhibitor of the human rhinovirus 3C cysteine protease, contains a reactive vinylester functionality and has recently completed Phase II clinical trials [104]. IDUN Pharmaceuticals has successfully completed a Phase I study with an irreversible caspase inhibitor (IDN-6556, structure undisclosed) [92], and has

initiated a Phase II clinical trial in liver transplantation. Thus, the challenge associated with developing irreversible caspase inhibitors will be the design of molecules possessing high selectivity for their target caspases.

### 5.4 Is there a Mechanism-Related Toxicity Associated with Apoptotic Caspase Inhibition?

An additional concern with developing an apoptotic caspase inhibitor is the potential for initiating or enhancing tumor growth due to inhibition of cell death. As yet, there is little evidence as to how much of an issue this may be, if any. Thus, initial pharmaceutical efforts against the apoptotic caspases have been directed towards acute diseases in which toxicity can be minimized with short treatment protocols.

## 6. CONCLUSIONS

Evidence from a wide range of animal models suggests that inhibiting caspase activity is an important therapeutic intervention point for a large number of diseases. Although small-molecule inhibitors have been slow to progress to the clinic, there are now two unrelated caspase inhibitors that have shown promising results in early clinical trials. The lack of progress in developing compounds directed against the apoptotic caspases reflects our incomplete understanding of the biological pathways and uncertainty about developing irreversible inhibitors. Nevertheless, given these uncertainties, the available data suggests that caspases are viable targets and have great potential as novel therapeutics for a large number of diseases.

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