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Caspases as therapeutic targets

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Abstract

The development of small molecules to modulate caspase activity offers a novel therapeutic strategy in the treatment of apoptosis-related and inflammatory diseases. Caspases are key mediators of apoptosis and inflammation; deregulation of their activation or expression can lead to the development of conditions such as neurodegenerative and autoinflammatory disorders. This review details the different caspase-associated disorders while focusing on caspase-1 inhibition as a potential therapeutic strategy. Problems facing the development of effective and safe caspase therapeutics will also be addressed.

Keywords: apoptosis • inhibitors • inflammation • transplant

Introduction

Cysteine aspartyl-specific proteases (caspases) are a family of proteases that cleave substrates at sites next to aspartic acid residues. These proteases are executioners of the cell and also have a role in inflammation and differentiation. There are a total of 14 mammalian caspases thus far identified, 12 of which are human (namely caspases 1–10, 12 and 14). The caspases are divided into two groups, dependent on their main function. Members, who act in the process of apoptosis, are one group and include caspases 2, 3 and 6-10. The second group are involved in maturation of cytokines and include caspase-1, -4, -5 and -12 [1, 2]. These caspases are present in the cell as inactive zymogens consisting of a prodomain followed by a P20 subunit and P10 subunit, which contain substrate recognition and catalytic domains. Activated caspases cleave a variety of substrates with approximately 400 targets having been identified to date. These include proteins involved in signal transduction (apoptosis regulators, cytokines, serine/threonine kinases), structural proteins (cytoskeletal and nuclear) and proteins involved in regulation of transcription, translation and RNA editing [3, 4]. In contrast to the apoptotic and inflammatory caspases, the precise function of caspase-14 remains undefined. However, a role in keratinocyte differentiation has been determined [5–7].

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Activation of caspases

Initiator caspases of apoptosis contain large prodomains and occur as inactive monomers. The inflammatory caspases are similar in structure to initiator caspases and are also present as inactive monomers. Initiator caspase activation occurs when the caspase is recruited into a multiprotein complex. The activation of different initiators is associated with several complexes, thus caspase-9 is activated in the apoptosome [8], caspase-2 in the PIDDosome [9, 10] and caspase-8 or -10 in the death-inducing signalling complex (DISC) [11, 12]. The method of initiator caspase activation may occur through proximity-induced autoprocessing or dimerization mediated through these complexes [13, 14]. Similarly, it is believed that inflammatory caspases require proximity-induced oligomerization for activation through a complex called the inflammasome [15, 16].

Activation of apoptotic caspases

Apoptotic cell death may occur through the intrinsic pathway or the extrinsic pathway. The intrinsic apoptotic pathway may be stimulated by chemotherapeutics, mitochondrial damage or ionizing radiation. Upon stimulation, the mitochondria releases Cytochrome c which is required for caspase-9 recruitment to the apoptosome [8]. The apoptosome complex contains the apoptotic protease-activating factor-1 (Apaf-1). Apaf-1 contains an N-terminal caspase recruitment (CARD) domain (which is concealed in its inactive form), WD-40 repeats and a CED4 homology domain. Cytochrome c binding promotes a conformational change in Apaf-1 and, upon ATP/dATP binding, Apaf-1 oligomerizes. The oligomeric Apaf-1 structure can be compared to a seven-spoked wheel with the caspase-9 recruitment domain in the centre, this structure allows for the recruitment and activation of caspase-9 [17].

The extrinsic apoptotic pathway is activated in response to ligation of death receptors of the tumour necrosis factor receptor type I family. One member of this family is the Fas receptor, upon ligation the Fas-associated death domain (FADD) adaptor molecule is recruited to Fas [18] and FADD in turn recruits caspase-8 or -10. This complex is known as the DISC and facilitates the activation of caspase-8 or -10 [11, 12, 19].

The third multiprotein activating complex is the PIDDosome required for caspase-2 activation [9, 10]. Under genotoxic stress, caspase-2 is recruited into the PIDDosome, which contains the p53-induced death domain containing protein (PIDD) and the adaptor molecule, RIP-associated ICH-1/CED-3-homologous protein with a death domain (RAIDD). The death domain of PIDD interacts with the death domain of RAIDD, which leads to recruitment of caspase-2 by RAIDD through CARD interaction. Subsequent caspase-2 activation requires autoprocessing of PIDD to generate a 37 kD C-terminal fragment termed PIDD-cc [20]. Activation is believed to occur in a similar manner to activation of other initiators, through dimerization followed by autocleavage [21].

Activated initiator caspases can then activate effector caspases, which leads to the cleavage of structural and regulatory proteins culminating in the features of apoptosis. In contrast to initiator caspases, the effector caspases exist as inactive dimers [22]. Insight into the mechanism of effector caspase activation was provided from analysis of the crystal structure of caspase-7 where cleavage between the small and large subunits was shown to lead to rearrangement of active site loops and activation [23, 24].

Activation of inflammatory caspases

The inflammatory caspases (1, 4, 5 and 12) are involved in cytokine maturation and consequently the inflammatory response. Caspase-1 was the first caspase identified and was discovered in 1989 by Black *et al.* while attempting to determine the protease responsible for the cleavage of interleukin-1β (IL-1β) [25]. Subsequent research, utilizing caspase-1-deficient animal models, demonstrated the requirement of caspase-1 for IL-1β maturation [26, 27] Caspase-1 is also involved in the cleavage and activation of interleukin-18 (IL-18) and interleukin-33 (IL-33) [28–30]. A role of caspase-1 in neuronal apoptosis has been proposed. Caspase-1-deficient mice have demonstrated normal developmental cell death [26, 27], however, these models have also shown resistance to cerebral ischaemic injury and also Rip2 and trophic factor withdrawal-mediated neuronal cell death [31–33]. Whether caspase-1 has a direct apoptotic role in these models is unclear, further research is required to determine the true function of caspase-1 in neuronal apoptosis. In contrast, caspase-5 is involved only in IL-1β cleavage [15]. It is believed that caspase-4 and -5 arose from gene duplication due to their sequence homology [1], although the role of caspase-4 in inflammation needs to be elucidated further.

Activation of inflammatory caspases requires assembly of the inflammasome, a multiprotein complex whose main component is the Nalp proteins, part of the Nod-like receptor (NLR) family. These proteins contain the nucleotide binding domain NACHT and the leucine rich repeat (LRR) domain of 20–29 residues [34]. There are 14 human Nalp proteins so far, Nalp 1, 2, 3, 6, 10 and 12 have been shown to modulate caspase-1 activation [15, 35–39], the mechanism by which these proteins facilitate this activation is not fully understood. Nalp 1, the first Nalp protein to be discovered, contains a pyrin (PYD) domain, NACHT domain, five LRR domains similar to other Nalp proteins but also contains an F-interacting domain (FIIND) and a CARD at its carboxy terminal [34].

The proposed mechanism of caspase-1 activation in the Nalp 1 inflammasome involves the recruitment of an adaptor molecule known as apoptosis-associated speck-like protein (Asc) through the PYD domain of Nalp 1 [16]. Asc then recruits caspase-1 through its CARD domain while Caspase-5 recruitment occurs through the CARD domain of Nalp 1 [15]. The assembled Nalp 1 inflammasome is believed to facilitate the dimerization and activation of caspase-1 and -5 [15]. In contrast, the Nalp 3 inflammasome activates only caspase-1 and contains the adaptor Asc along with cardinal, a second adapter protein [37].

Regulation of inflammasome assembly involves the CARD containing proteins COP and ICEBERG. These consist of a CARD domain, which binds ASC preventing caspase-1 recruitment to the inflammasome [40, 41]. In contrast, cellular pyrin only proteins (POPs) and poxvirus PYD containing proteins bind to the PYD domain of ASC preventing Nalp binding, inflammasome assembly and caspase-1 activation, [42–44]. Additionally, the bcl-2 family members, Bcl-2 and Bcl-X_L, suppress caspase-1 activation through Nalp 1 binding which prevents Asc recruitment [45].

While lipopolysaccharide (LPS), uric acid crystals and ATP are known inducers of caspase-1 activation and IL-1β processing [46–48], to date, only bacterial muramyl dipeptide has been identified as directly inducing inflammasome formation [49]. The mechanism by which these molecules activate the inflammasome is unknown. Evidence suggests the involvement of signal transduction through the LRR domain of Nalps and changes in potassium levels due to cellular stress [49, 50].

Recently, there has been much interest in the role of caspase-12 in inflammation. In most species examined, the caspase-12 gene encodes a full-length caspase protein, in mouse, caspase-12 is involved in endoplasmic reticulum (ER) stress [51, 52]. In the majority of people, the caspase-12 gene, due to a premature stop codon, produces a truncated protein consisting only of a caspase recruitment domain (CARD). Some individuals of African descent possess a gene that encodes the full-length caspase-12 protein (casp-12-L) [53]. Casp-12-L may inhibit caspase-1 activation leading to a reduction in IL-1β, IL-18 and interferon gamma (IFN-γ) levels [54].

Interleukins, their activation by caspases and their role in inflammation

The interleukin-1 family consists of eleven different ligands including IL-1, IL-33 and IL-18, which are regulated by caspases. IL-1α and IL-1β are synthesized as 31 kD precursors, which are cleaved to generate 17 kD mature forms at the site Tyr-Val-His-Asp^{116}/Ala^{117} [25]. IL-1α is cleaved by calpain and is active both in its precursor and mature form [55]. In contrast, IL-1β is cleaved by caspase-1 and is only active after cleavage [25].

IL-1α and IL-1β bind to three different receptors namely IL-IRI, IL-RII and IL-RAcP [56, 57]. IL-1 receptor activation leads to a multitude of actions including the induction of fever and modulation of the inflammatory response by increasing expression of adhesion molecules allowing for leucocyte infiltration. IL-1 also increases expression of cytokines, chemokines, proinflammatory mediators and is involved in Th1 differentiation [58].

Interleukin-18 is synthesized as a 24-kD proform, which is cleaved by caspase-1 at Asp^{36}-Tyr^{37} to its mature 18-kD form [28, 29]. Interleukin-18 binds to two sites on the receptor IL-18Rα and an additional site on IL-18Rβ initiating signal transduction through the receptor toll domains [59]. IL-18 induces IFN-γ, the Fas ligand, increases the level of cell adhesion molecules, induces chemokine production and is involved in T-cell polarization [60].

Finally, IL-33 is synthesized as a 30-kD proform, which is cleaved into the 18 kD active form by caspase-1 [30]. The active IL-33 binds to the ST2 receptor leading to the activation of NF-κB and mitogen activated protein kinases (MAPK) and increased cytokine production in Th2 cells. Treatment of recombinant IL-33 in a mouse model leads to increased levels of eosinophils and serum IgE, additionally pathological changes have been reported namely enlargement of the spleen, stomach and duodenum [30].

Owing to the diverse role of these interleukins in inflammation, modulation of caspase-1 provides an efficient means of controlling interleukin activation and the ensuing onset of the inflammatory response.

Small molecule inhibitors of caspase activity and activation

Modification of caspase activation

Research into the utilization of modulators of caspase activation in the treatment of certain conditions offers a novel therapeutic approach. These modulators may indirectly activate or repress caspase activation by targeting the proteins involved in caspase activation such as Apaf-1 of the apoptosome [61, 62]. Direct modification of caspase activity is also possible through the use of molecules such as peptides, which bind the active site of caspases.

Caspase regulation may also occur through binding of molecules to allosteric sites. Inhibition of caspases-1, -3 and -7 has been reported through binding of thiol-containing inhibitors to cysteine residues of allosteric sites. The conformational changes produced by inhibitor binding are similar to those found in the inactive form of these caspases and culminates in the prevention of peptide binding in the active site [63, 64]. This review will focus on caspase inhibition *via* active site binding as the therapeutic potential and clinical effects of caspase inhibitors are based largely on this type of inhibitor.

Synthetic molecules as inhibitors of caspase activity

Initially, peptide-based small molecular inhibitors of caspases were developed based on the cleavage sites of caspase substrates such as DEVD site of PARP. These molecules took advantage of the requirement of caspases for an aspartic acid at the P1 position of the substrate [2]. Specificity of these caspase inhibitors is influenced by the preference of different caspases for certain amino acids in positions P2 to P5 of the active site.

Position P4 is a key determinant for specificity; inflammatory caspases prefer large aromatic or hydrophobic amino acids at this position with the preferred cleavage site sequence of WEHD.

Table 1 Specificity of small molecule inhibitors of caspase activity

Reversible inhibitors (K_i nM)	Caspase 1	Caspase 4	Caspase 5	Caspase 3	Caspase 7	Caspase 2	Caspase 6	Caspase 8	Caspase 9	Caspase 10	Reference
Ac-WEHD-Cho (caspase 1, 4, 5 inhibitor)	0.056	97	43	1960	>10,000	>10,000	3090	21.1	508	330	[164]
Ac-YVAD-Cho (caspase 1 inhibitor)	0.76	362	163	>10,000	>10,000	>10,000	>10,000	352	970	408	[164]
Ac-DEVD-Cho (caspase 3, 7 inhibitor)	18	132	205	0.23	1.6	1710	31	0.92	60	12	[164]
Boc-IETD-Cho (caspase 8 inhibitor)	<6	400	223	195	3280	9400	5.6	1.05	108	27	[164]
Boc-AEVD-Cho (broad specificity)	<12	375	438	42	425	>10,000	52	1.6	48	320	[164]
CrmA (broad specificity)	0.01	1.1	<0.1	1600	>10,000	>10,000	1300	<0.34	<2.3	17	[164]
VX-765 (caspase 1 inhibitor)	0.8	<0.6	-	21,500	16,000	-	560	100	1030	-	[118]
5-nitroisatin (caspase 3, 7 inhibitor)	12,000	4000	-	500	290	>50,000	1700	>50,000	21,000	-	[72]
M867 (caspase 3, 7 inhibitor)	107	495	805	1.4	8.9	>100,000	3442	4032	-	29,490	[142]
Irreversible inhibitors (2^{nd} order rate constant $m^{-1}s^{-1}$)											
Z-VAD-dcb (pan-caspase)	700,000	450,000	-	13,000	3600	-	-	-	-	-	[165]
Ac-YVAD-CHN2 (caspase 1 inhibitor)	15,200	6300	-	<500	<200	-	-	-	-	-	[165]
Z-VAD-fmk (pan-caspase)	280,000	5500	130,000	16,000	18,000	290	7100	280,000	180,000	-	[164]
M-808	75,733	-	-	37,550	2107	-	-	12,850	-	-	[166]
IC_{50} value	**Caspase 1**	**Caspase 4**	**Caspase 5**	**Caspase 3**	**Caspase 7**	**Caspase 6**	**Caspase 2**	**Caspase 8**	**Caspase 9**	**Caspase 10**	**Reference**
Pralnacasan	0.0013 μM	-	-	2.3 μM	-	-	-	0.12 μM	-	-	[74]

Caspase-2, -3 and -7 prefer an aspartic acid at P4 whereas caspases-6, -8 and -9 prefer large aliphatic side chains and the sequence (L/V)EXD [2]. Caspase-2 prefers an additional substrate interaction, favouring small hydrophobic residues at position P5 [65]. Specificity data for several caspase inhibitors are illustrated in Table 1. These data demonstrate the preference of caspases for certain amino acids. It must be noted, however, that the specificity of inhibitors may alter within biological systems where cross-reactivity with abundant non-target caspases may occur [66].

Initial modifications to peptide-based inhibitors involved the addition of electrophilic groups on the P1 aspartic acid residue such as aldehydes, nitriles or ketones to produce reversible inhibitors. These inhibitors have demonstrated low potency in cell-based systems possibly due to inability to reach necessary concentrations to inhibit sufficient caspase activation [67, 68]. In

contrast, the addition of chloro, fluoro-, acyloxy, or diazomethyl ketones produces more potent irreversible inhibitors [69].

Several adaptations have been made to these reversible and irreversible inhibitors to improve cell penetration and stability allowing for the development of effective therapeutics. A number of P2 and P3 replacements have been developed; mainly pyridone, pyrimidone and pyridazinodiazepine scaffolds [70, 71]. Non-peptide caspase inhibitors are also currently in development. So far, non-peptide molecules that have been described include isatin sulfonamides and onilinoquinazolines [72, 73].

At present, only a small number of caspase inhibitors are undergoing clinical trials. This includes the reversible caspase-1 inhibitors Pralnacasan [74] and VX-765 [www.clinicaltrials.gov ID:NCT00205465] manufactured by Vertex Pharmaceuticals (Cambridge, MA, USA) and the irreversible pan caspase inhibitor IDN6556 manufactured by Idun Pharmaceuticals (San Diego, CA, USA) [75, 76].

Role of caspases in disease

Caspases are key mediators of apoptosis and inflammation, the loss or gain of caspase activity/expression is the basis of a number of diseases caused by deregulation of the apoptotic and inflammatory pathways. While important diseases are associated with a lack of caspase activity (*e.g.* cancer where apoptosis is typically decreased), this review will focus on those diseases and conditions associated with increased caspase activity, as it is in these cases that the role of caspases as therapeutic targets is most clear.

Caspases as targets in conditions associated with increased levels of apoptosis

Increased levels of apoptosis are associated with a number of disorders including neurodegenerative diseases and ischaemia/reperfusion-related conditions namely stroke, myocardial infarction and transplant failure.

Neurodegenerative disease

Caspases have been implicated in a number of neurodegenerative diseases including Alzheimer's, Parkinson's and Huntington's disease.

Huntington's disease (HD) is believed to be caused by the polyglutamine expansion of the huntingtin protein (Htt). The symptoms of this disease include uncontrollable movement and deterioration of cognitive ability leading to dementia. The mechanism by which mutated Htt leads to the manifestation of HD symptoms is unknown. Research suggests that accumulation of mutated Htt fragments in cells may be toxic and lead to neurodegeneration [77–79]. The diminished association of mutant Htt with Htt interacting proteins may also play a role in disease progression [80].

Caspase-cleaved Htt has been detected in both HD and normal brains [81], with caspase cleavage sites defined at amino acids 513 and 552 for caspase-3, amino acid 586 for caspase-6 and the N-terminal region of Htt for caspase-1 [82, 83]. Cleavage of Htt at a caspase-6 site appears to be required for neuronal degeneration in a mouse model of HD. Other caspases that have also been implicated in development of HD include caspase-1, -2, and -8 [80, 84–86].

Alzheimer's disease (AD) is characterized by the degeneration of synapses and neuronal death leading to cognitive deterioration. The brains of Alzheimer's patients have aggregates of β-amyloid known as plaques, and also tangles which are intracellular aggregates that contain high levels of modified Tau [87, 88]. However, the relationship between these features and disease development and progression is uncertain. Activated caspases have been detected in AD brains [89–91]. Cleavage of proteins associated with AD by caspases has also been reported such as Tau processing and cleavage of the β-amyloid precursor protein (APP) to produce fragments which include β-amyloid [92, 93]. Research to date provides evidence of an association between caspases and AD, however, does not implicate caspases in disease development or progression.

Parkinson's disease (PD) is characterized by loss of motor skills and speech, the symptoms of this disease are associated with the loss of dopaminergic neurons of the substantia nigra. The level of caspases has been investigated in the brains of PD patients with increased levels of caspase-1, -3 and -8 having been reported [94, 95]. Several animal models of PD induced by the neurotoxins 6-hydroxydopamine (6-OHDA),1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and its metabolite 1-methyl-4-phenylpyridinium, have also reported increased levels of caspase activation upon treatment [94, 96, 97]. However, a causal role for caspases in the loss of dopaminergic neurons is not certain and inhibition of caspases may not prevent neuronal cell death in Parkinson's disease [94].

The role of caspases in neurodegenerative diseases is still unclear; identification of caspase activity in these diseases is not evidence of a causal role of caspases or the implication of a beneficial effect upon caspase inhibition. Research into disease progression performed with animal models offers an insight into these conditions, however animal models must be chosen carefully. For example, the use of certain neurotoxins that mimic PD may not give a true representation of the disease pathology in human beings. The use of caspase inhibitors as therapeutic agents will prove difficult until the mechanisms behind disease progression are elucidated.

Ischaemia

Ischaemia is the restriction of blood supply to tissue, the subsequent return of blood flow is known as reperfusion. Ischaemia leads to increased pH, ATP depletion and reactive oxygen species

(ROS) production which initiate signalling cascades leading to cell death and inflammation. Ischaemia-induced cell death may occur by apoptosis, necrosis or autophagy [98], and the factors that determine the extent to which each of these pathways contribute to cell death during ischaemia is largely unknown.

Caspase-1, -2, -3, -7 and -9 have been associated with animal models of global and focal ischaemia [31, 99, 100]. Other apoptotic mediators have also been reported to be expressed or released such as cIAP, Smac/Diablo and Cytochrome c [101, 102]. Caspase inhibitors have been utilized to determine the role of increased caspase expression in ischaemia. This strategy has given varied results. For example, the caspase inhibitors Z-DEVD-fmk, YVAD-cmk and Z-VAD-fmk have been reported to protect neurons against ischaemic death in a rodent model of focal ischaemia [103]. In contrast, a separate study showed the treatment of rats with the caspase inhibitors Z-VAD-fmk and Z-DEVD-fmk did not reduce ischaemic injury significantly [104].

One possible reason for these varying results may be due to the different contributions of apoptotic death occurring in these models. Research into the timing of apoptosis during ischaemic/reperfusion injury has been performed and suggests a delayed induction of apoptosis in response to ischaemia after an initial wave of necrotic cell death [105–107]. The therapeutic window available for caspase inhibitor administration is consequently unclear. VRT-018858, the active metabolite of the caspase-1 inhibitor Pralnacasan (HMR3480/VX-740), has been utilized in a rat model of focal cerebral ischaemia. A reduction in lesion volume was ascribed to VRT-018858 administration within 3 hrs of ischaemic injury. However, no significant reduction in ischaemic injury was observed if VRT-018858 treatment was delayed to 6 hrs after ischaemic injury [108].

Despite the difficulty in ascribing a clear role to apoptosis during ischaemia-induced tissue injury, caspase inhibitors, such as VRT-018858, have been evaluated for clinical usefulness.

Ischaemia/reperfusion injury – implications in transplant injury

A major obstacle of organ transplantation is ischaemia/reperfusion (I/R) injury, which occurs in the course of organ preservation and subsequent transplant to the recipient. The use of caspase inhibitors may offer a therapeutic strategy to reduce transplant injury and failure as an increase in apoptotic cells and caspase activity has been reported in transplanted organs including the heart, kidneys, liver, lungs and pancreas [109–113].

The irreversible pan-caspase inhibitor IDN6556 has been tested in a rat model of lung transplantation [114]. The beneficial effects of IDN6556 on transplanted lung function was dependant on length of cold ischaemia storage with a significant increase in lung function when grafts were stored for 18 hrs, transplanted and reperfused for 2 hrs.

IDN6556 is currently in clinical trials for use during liver transplantation. A Phase II trial demonstrated that addition of this caspase inhibitor to organ preservation solution significantly reduced apoptosis and caspase-3 and -7 activity after reperfusion. However, additional administration of this inhibitor to the recipient abrogated this reduction leading to apoptotic levels similar to placebo, together with increased levels of neutrophil infiltration in the liver. IDN6556 administration did not reduce the rate of acute cellular rejection in this trial [75].

The use of caspase inhibitors to reduce I/R injury in transplant organ does show promise due to the short-term nature of treatment. Addition of caspase inhibitors to organ preservation solution also allows for selective treatment of target cells. Further research is required to determine the contribution of apoptotic cell death in I/R transplant injury in order to establish the therapeutic benefits of caspase inhibition.

IDN6556 has also been utilized in the treatment of liver disease such as hepatitis. Clinical trials performed with IDN6556 have demonstrated a reduction in transaminase levels, which are markers for hepatic injury. However, transaminase levels returned to pre-treatment levels following discontinuation of IDN6556 treatment [76, 115] suggesting chronic drug treatment may be required. This could be problematic due to the increased risk of adverse side effects that accompany long-term treatment.

Inflammatory diseases

Deregulation of the inflammatory response can lead to the development of autoimmune and autoinflammatory disorders. Caspases activate a number of key cytokines required for the inflammatory response. Therefore, caspases represent a target for drugs to prevent induction of this response in inflammatory diseases.

Mutations that lead to defects in the components of the inflammasome effect caspase-1 activation and therefore affect the inflammatory response by altering interleukin activation. Mutations in exon 3 of the *nalp3* gene have been linked to the auto-inflammatory disorders Muckle-Wells syndrome, familial cold urticaria and chronic infantile neurological cutaneous and articular syndrome. These mutations have been shown to cause hyperresponsiveness to LPS stimulation in peripheral blood mononuclear cells of familial cold urticaria patients and spontaneous secretion of IL-1β in monocytic cells [116, 117]. The reasons for this increase in activity in response to NACHT domain mutations of *nalp 3* are unknown. In principle, mutations may enhance inflammasome assembly as the NACHT domain is involved in oligomerization or mutations may reduce the binding of inhibitory proteins to Nalp 3 [37].

The caspase-1 inhibitor VX-765 may have the potential to be used in the treatment of familial cold urticaria and other autoinflammatory diseases. The treatment of peripheral blood mononuclear cells (PBMC) and whole blood from familial cold urticaria patients with VX-765 suppresses LPS-induced IL-1β activation [116, 118]. Caspase-1 inhibition may also be beneficial in the treatment of the auto-inflammatory disorder, familial Mediterranean fever which is associated with mutations of the pyrin protein [119, 120]. Mutations in this protein can lead to induction of Asc

oligomerization and inflammasome assembly *in vitro* [121]. Pyrin may also bind to caspase-1 directly [122].

Autoimmune disorders occur due to deregulation of the inflammatory response causing self-directed inflammation mediated through the adaptive immune response. Disorders include rheumatoid arthritis and inflammatory bowel disease. Regulation of interleukins through modulation of caspase activity may provide a method to control these disorders. The caspase-1 inhibitor Pralnacasan has been utilized in the treatment of dextran sulfate sodium (DSS)-induced colitis in mice. DSS-induced colitis is a model for human inflammatory bowel disease. Combined treatment with DSS and Pralnacasan showed reduction in inflammatory cells, IL-18 levels in colons and INF-γ in para-aortal lymph nodes compared with the DSS/placebo group [123]. In a separate study, cytokine expression was investigated in DSS-treated mice. DSS treatment led to an increase in IL-18, TNF-α, IL-12 and INF-γ protein levels with Pralnacasan suppressing the increase of IL-18, IL-1β, TNF-α and IFN-γ-inducible protein-10 (IP-10) [124].

Pralnacasan is a peptidomimetic compound containing a pyridazinodiazepine scaffold at the P2-P3 site, which selectively inhibits caspase-1, demonstrating IC_{50} values of 1.3 nM, 2.3 μM and 0.12 μM for caspase-1, -3 and -8, respectively [74]. Upon administration, the ethoxyl group of the compound is hydrolyzed producing the active compound RU36384/VRT-018858 [125]. Pralnacasan inhibits LPS-induced IL-1β release and *S. aureus cowan*-induced IL-1β and IL-18 release in human PBMC. In a mouse model, Pralnacasan also suppresses IL-1β release induced by LPS injection [126].

In 2001, data on the effect of Pralnacasan in a murine model of rheumatoid arthritis (RA) were published. Oral administration of Pralnacasan in DBA/1 mice with established collagen-induced arthritis reduced paw inflammation and disease progression. A reduction in cartilage damage and bone erosion in Pralnacasan treated mice compared to vehicle treated mice was also reported.

The use of Pralnacasan was also extended to osteoarthritis (OA). Treatment in two murine models of knee OA showed lower histopathological scores and a reduction in hydroxylysylpyridinoline (HP) /lysylpyridinoline (LP) ratio of collagen cross-links in the urine indicating a reduction in cartilage degradation. In this study, OA was induced in Balb/c mice by injection of bacterial collagenases, STR/1N mice were also utilized due to their ability to develop OA spontaneously [125].

Clinical trials of Pralnacasan have focused on its effect in RA. Phase IIa clinical trials for Pralnacasan treatment began in October 2002. In this 12-week randomized trial, 285 adult RA patients were enrolled. An improvement in patients was reported as determined by the ACR20 response rate (20% or higher improvement in RA symptoms) when treated with Pralnacasan and methotrexate. Pralnacasan-treated patients also showed ACR20 improvement independent of treatment with methotrexate. The highest dose of Pralnacasan (1200 mg/day) showed significant reduction in C-reactive protein, erythrocyte sedimentation rate and serum amyloid A levels, which are indicators of inflammation [74]. In November 2003, phase IIb clinical trials were terminated due to the development of liver fibrosis in Pralnacasan-treated dogs during a 9-month toxicology study [press release 10 Nov 03 www.vrtx.com].

The use of caspase-1 inhibitors in the treatment of inflammatory diseases has proved successful in a number of trials. However, adverse side effects, such as liver fibrosis, associated with caspase modulation pose a major obstacle in the development of caspase therapeutics and will be discussed in more detail in later sections.

Role of caspases in sepsis – mediators of apoptosis and inflammation

Sepsis is broadly defined as the systemic inflammatory response to infection. Systemic inflammatory response syndrome (SIRS) is not only caused by infection but a range of processes including ischaemia, multiple trauma, tissue injury and haemorrhagic shock [127]. The mechanism of sepsis is complex and not fully understood. One model proposes that following the SIRS response, the body tries to regain homeostasis by initiating the compensatory anti-inflammatory response syndrome (CARS). A combination of both SIRS and CARS responses may also occur and is termed mixed antagonistic response syndrome (MARS) [128]. The mechanisms by which caspases contribute to sepsis include the release of cytokines and induction of apoptosis, the degree to which these processes contribute to sepsis is largely unknown.

Role of caspase-1 and -12 in sepsis

The mechanism by which caspase-1 mediates its response in sepsis is not fully understood but is believed to involve the activation of interleukins through the inflammasome. IL-18 activation stimulates the inflammatory response by INF-γ production and T-cell polarization and may act as a predictive factor for lethal sepsis [129]. Additionally, caspase-1 activation of IL-1β leads to the up-regulation of pre-B cell colony-enhancing factor in neutrophils inhibiting apoptosis of these cells [130, 131]. Delayed neutrophil apoptosis has been reported to occur during sepsis [132, 133]. Neutrophil numbers are usually tightly regulated *in vivo* by the induction of apoptosis approximately 5 hrs after maturation [134], delayed apoptosis leads to an increase in neutrophil numbers contributing to hyperresponsive immunity and the symptoms of SIRS. A role of caspase-1 independent of cytokines Il-1β and IL-18 in sepsis has also been proposed following a study utilizing a murine model of sepsis. Caspase-1 knockout mice but not IL-1β/IL-18 double knockout mice demonstrated increased survival and protection from splenic B lymphocyte apoptosis [135].

The full-length version of inflammatory caspase-12 (casp12-L) is implicated in sepsis. Casp-12-L confers hyporesponsiveness to LPS as measured by cytokine release and is associated with increased mortality rates in patients with severe sepsis [53]. This

increased mortality in casp-12-L patients is believed to be caused by an inhibitory effect on caspase-1 [54]. Caspase-12 deficient mice have shown increased survival in two sepsis models with an increased ability to clear bacteria following infection [54].

Role of apoptotic caspases in sepsis

The apoptotic caspases also play a part in the development of sepsis. Increased lymphocyte apoptosis may occur in sepsis contributing to the symptoms of CARS. Reduced levels of B-, CD4 T- and dendritic cells have been reported in septic patients and also in animal models of sepsis [133, 136, 137]. Caspase-2, -3, -6 and -9 have been implicated in lymphocyte apoptosis [138] and prevention of lymphocyte apoptosis by caspase inhibitors improves survival in sepsis models [137, 139]. Lymphocyte apoptosis not only contribute to CARS by reducing the number of B- and T-cells, but also the uptake of apoptotic cells can lead to macrophage and dendritic cell non-responsiveness known as anergy [140].

The utilization of caspase therapeutics may prove problematic in the case of sepsis due to lack of knowledge regarding the complex nature of sepsis development and progression and also the diverse roles that caspases may perform in this condition.

Challenges facing the development of caspase therapeutics

In the development of a safe and effective caspase therapeutics, a number of criteria, such as drug specificity and selectivity, must be considered. This section highlights some of the key questions, which must be addressed in the development of caspase therapeutics.

Firstly, an understanding of the contribution of apoptosis to cell death in the condition to be treated is essential, along with knowledge of the caspases implicated and the therapeutic window for treatment. As described in previous sections, apoptosis may not account for all cell death occurring in a certain condition. In ischaemia/reperfusion injury, three types of cell death may take place, namely apoptosis, autophagy and necrosis. Use of caspase inhibitors may only partially block cell death in this situation [141].

Trials designed to determine the effectiveness and potency of caspase therapeutics in the prevention of apoptosis must consider appropriate techniques to detect apoptosis. The hallmarks of apoptosis include chromatin condensation, DNA fragmentation, membrane blebbing and cleavage of a variety of caspase substrates. A study by Methot *et al.* reported a 3.5-fold increase in caspase inhibitor concentration required to inhibit DNA fragmentation by 95% compared to the concentration required to reduce α II spectrin cleavage by 95% in primary thymocytes [142]. This study illustrates the importance of accurate determination of apoptosis in the evaluation of caspase inhibitors.

Following on from this, detection of apoptosis performed with these parameters does not give an indication of cell death occurring through apoptosis independent pathways, leading to the question: will inhibition of caspase activation protect cells from death? Evidence that cells are not protected includes the study of OCI-AML3 cells treated with a variety of chemotherapeutic drugs, which lead to activation of caspase-2, -3, -7, -8 and -9, and also PARP cleavage and DNA fragmentation. The caspase inhibitors IDN-1529 and IDN-1965 blocked apoptosis as determined by the above parameters. Cell death, however, was not prevented in these cells as determined by cell number, release of Cytochrome c, Smac and Omi from the mitochondria and loss of mitochondrial membrane potential (MMP) [143]. In a second study, Bax-induced death in Jurkat cells demonstrated the features of apoptosis; however, caspase inhibition performed with Z-VAD-fmk in these cells led to cell death induced by loss of MMP [144].

The effects of caspase therapeutics can be more clearly assessed in animal models where parameters, such as survival and reduction of symptoms, can be measured alongside off-target effects. For example, in the case of Pralnacasan clinical studies, liver fibrosis developed in an animal model during long-term treatment. Adverse effects may occur due to inhibition of non-target caspases, the targeting of apoptosis-independent processes performed by caspases or indeed caspase independent processes.

Adverse effects of caspase therapeutics may occur during treatment if caspases unrelated to the condition under treatment are inhibited due to lack of specificity. The caspase-1 inhibitor VX-765 is undergoing phase 2 clinical trials by Vertex. VX-765 is converted to the active metabolite VRT-043198 by esterases. The compound inhibits caspase-1 and -4 with a K_i value of 0.8 nM and 0.6 nM, respectively, the compound also demonstrates a K_i value for caspase-8 of 100 nM. Preclinical trials of VX-765 demonstrated Fas-induced apoptosis induction at concentration of 200 μM [118]. The concentrations used in inflammatory disorders are much lower than in this study; however, long-term dosage at lower concentrations may potentially cause adverse side effects. Long-term toxicity studies are required to establish the adverse effects of treatment.

Introduction of the caspase therapeutics to target cells may pose several problems. The caspase therapeutic should be delivered exclusively to target cells where possible as in the case of introduction of caspase inhibitors to organ preservation solution. Where select treatment of target cells is not feasible, the effect of the therapeutic on non-target cells and organs should be monitored.

Finally, caspase modulation may have adverse effects due to the role of caspases in other aspects of the apoptotic and inflammatory response or indeed separate processes such as differentiation and proliferation. The next section discusses the role of caspases in a variety of processes, which must be taken into account when determining the therapeutic potential of caspases.

Role of caspases in other aspects of cell death

There are a number of different cell death pathways including autophagy, necrosis and mitotic catastrophe. The type of cell death chosen as well as the degree of overlap between these pathways is largely unknown in certain conditions, such as I/R injury. In the development of effective and safe caspase therapeutics, a number of questions need to be addressed: does some level of redundancy occur in cell death pathways so that blocking the apoptotic pathway leads to activation of autophagy or necrosis and do these pathways have common elements?

Autophagic cell death is characterized by production of vesicles containing cytoplasm and organelles, which are degraded in the lysosome. This type of cell death is induced upon cellular stress such as starvation, accumulation of misfolded proteins and irradiation. The link between apoptosis, caspases and the process of autophagy is still unclear, inhibition of apoptosis and caspases has been reported to induce autophagy suggesting an inhibitory role of caspases in autophagy. Cells deficient in the Bcl-2 family members Bax and Bak fail to undergo apoptosis following etoposide treatment, however, autophagy occurs instead [145]. Treatment of mouse L929 fibroblastic cells with the pan-caspase inhibitor Z-VAD-fmk directly induces autophagic death, treated cells contained intact mitochondria and endoplasmic reticulum, however, also contained membrane-bound vesicles. Caspase-8 and a calpain-like cysteine protease are believed to inhibit autophagic cell death in these cells [146, 147]. The possibility of inducing autophagic cell death upon caspase inhibition must be considered during the development of beneficial caspase therapeutics.

Role of caspases in non-apoptotic and non-inflammatory processes

While caspases play critical roles in apoptosis and inflammation, it has emerged that some caspases contribute to other important biological processes including differentiation and proliferation. These additional roles are as yet poorly understood but must be considered when assessing the risk of adverse effects following treatment with caspases inhibitors.

Role of caspases in differentiation and cell fusion

The epidermis is composed of keratinocytes, which during turnover from basal to upper epidermal layers differentiate into flattened corneocytes lacking nuclei and other organelles. Differentiating keratinocytes share some features with apoptosis such as DNA fragmentation. Inhibition of caspase activity performed with the inhibitors Z-VAD-fmk and Boc-D-fmk impairs differentiation through the failure of nuclei to degrade [148]. Caspase-14 has been implicated in the process of keratinocyte differentiation. This caspase is expressed mainly in epidermal cells and expression increases upon differentiation including cleavage of the proenzyme to the active form. Moreover, retinoic acid, a modulator of keratinocyte differentiation, suppressed caspase-14 mRNA in an *in vitro* model [5–7].

A similar process of denucleation occurs in lens fiber cells. Caspases have been implicated in this process due to the observation of PARP cleavage and inhibition of denucleation performed with the pan caspase inhibitor Z-VAD-fmk in differentiating cells. However, the caspases implicated are unknown, both caspase-3 and -9 are putative mediators of denucleation [149–151]. Caspases have also been implicated in the differentiation of erythrocytes and dendritic cells [152–155].

Caspases are involved in the process of cell fusion. Caspase-8 has been associated with the syncytical fusion of cytotrophoblasts to form syncytiotrophoblasts in the placenta. Antisense oligonucleotides for caspase-8 and the caspase-8 inhibitors IETD-cho and Z-IETD-cho reduces fusion in these cells [156].

Caspases, in particular caspase-3, are also associated with the formation of myotubes from fusion of myoblasts. The link between caspase-3 and myoblast differentiation may occur *via* mammalian sterile twenty-like kinase cleavage, which is an effector of myoblast differentiation and a caspase-3 substrate. Deletion of caspase-3 in myoblasts or the inhibition of caspase-3 activity *via* Z-DEVD-fmk treatment has been shown to reduce myotube formation [157].

Role of caspases in cell proliferation

Caspases are implicated in T-cell proliferation due to the suppression of growth upon treatment with caspase inhibitors [158, 159]. Caspase-8 may be involved in this process as the caspase-8 inhibitor c-FLIP1 has been shown to modulate T-cell proliferation [160]. Similarly, caspase-8 and -6 act as positive regulators of B-cell proliferation. B-cells deficient in caspase-8 have been shown to fail to proliferate in response to LPS and double-stranded DNA in a mouse model [161]. In a second study, inhibition of caspase-6 and -8 caused suppression of B-cell proliferation. Additionally, inhibition of caspase-6 alone blocked Cyclin D induction and CDK 4 expression levels required for cell cycle progression [162].

In contrast, caspase-3 may act as a negative regulator of B-cell proliferation. A study by Woo *et al.* demonstrated increased B-cell proliferation in caspase-3 null mice compared to wild-type specimens. The cyclin-dependant kinase inhibitor, p21, is a substrate for caspase-3 and may mediate the anti-proliferative effects of caspase-3 [163].

The future of caspase therapeutics

The potential to produce therapeutics through the regulation of caspases is evident, to date a number of caspase inhibitors, such

as Pralnacasan and IDN6556, have demonstrated beneficial therapeutic effects upon administration. However, there are a number of challenges in the development of safe and effective drugs. One major obstacle is the lack of knowledge regarding the role of caspases in certain apoptosis-associated diseases, and an incomplete understanding of the different cell death pathways that contribute to these diseases. Information regarding the therapeutic window for administration is also required. Administration of caspase therapeutics without this information may have deleterious effects to the patient.

Another challenge facing the utilization of caspase therapeutics is the development of adverse effects upon treatment. Off-target effects may occur due to lack of drug specificity leading to the modulation of non-target caspases or indeed unrelated proteins. Additionally, interference with non-apoptotic or non-inflammatory roles of caspases may result in adverse effects and must also be considered. Research relating to the long-term consequences of caspase inhibition, in particular for the treatment of chronic conditions, is required to determine the safety of these drugs and the future of caspase therapeutics.

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