Review

Inhibitors of c-Jun N-terminal kinases—JuNK no more?

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Abstract

The c-Jun N-terminal kinases (JNKs) have been the subject of intense interest since their discovery in the early 1990s. Major research programs have been directed to the screening and/or design of JNK-selective inhibitors and testing their potential as drugs. We begin this review by considering the first commercially-available JNK ATP-competitive inhibitor, SP600125. We focus on recent studies that have evaluated the actions of SP600125 in lung, brain, kidney and liver following exposure to a range of stress insults including ischemia/reperfusion. In many but not all cases, SP600125 administration has proved beneficial. JNK activation can also follow infection, and we next consider recent examples that demonstrate the benefits of SP600125 administration in viral infection. Additional ATP-competitive JNK inhibitors have now been described following high throughput screening of small molecule libraries, but information on their use in biological systems remains limited and thus these inhibitors will require further evaluation. Peptide substrate-competitive ATP-non-competitive inhibitors of JNK have also now been described, and we discuss the recent advances in the use of JNK inhibitory peptides in the treatment of neuronal death, diabetes and viral infection. We conclude by raising a number of questions that should be considered in the quest for JNK-specific inhibitors.
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1. Introduction

The c-Jun N-terminal kinases (JNKs) were initially described in the early 1990s as a family of serine/threonine protein kinases, activated by a range of stress stimuli and able to phosphorylate the N-terminal transactivation domain of the c-Jun transcription factor. This phosphorylation enhances c-Jun-dependent transcriptional events in mammalian cells [1,2]. Further research has revealed three JNK genes (JNK1, JNK2 and JNK3) and their spliceforms as well as the range of external stimuli that lead to JNK activation. A number of independent approaches have since suggested the importance of JNK-dependent signalling events in both normal development and in disease. This has been highlighted by the striking beneficial phenotypes of JNK gene knockout mice in disease models, including neuroprotection against stroke and improved insulin responsiveness in diabetes (reviewed in [3]).

Inhibitors have been used increasingly to explore the biological functions of JNK in mammalian systems without the need for JNK gene knockout. In this review, we explore recent developments in the discovery of JNK inhibitors and their potential in the treatment of human disease. We first focus on small molecule, ATP-competitive JNK inhibitors as summarised in Fig. 1. Our initial discussion centres on SP600125 developed by Signal Pharmaceuticals/Celgene. In addition, we provide a brief overview of an increasing number of other small molecule ATP-competitive JNK inhibitors now described in the published literature. We then discuss the recent advances in the use of ATP-non-competitive JNK inhibitory peptides. These inhibitors are also highlighted in Fig. 1.

Lastly, we consider questions that arise with the development of JNK inhibitors and their possible therapeutic application.

These questions centre on the controls needed to establish specificity of actions of JNK inhibitors, whether JNK isoform-selective inhibitors are possible or desirable, whether other compounds have off-target effects to inhibit JNK, and what concerns accompany the chronic use of JNK-specific inhibitors. Further work will be needed to address these issues, however the demonstrated efficacy of the current generation of JNK inhibitors in improving outcomes in disease models suggests that this further effort will be worthwhile.

2. SP600125, an anthrapyrazolone inhibitor of JNK

2.1. Discovery and initial characterisation of SP600125

In late 2001, the small molecule JNK inhibitor, SP600125 (anthra[1,9]pyrazol-6(2H)-one), was reported following the screening of a proprietary library (Celgene/Signal Pharmaceuticals) for inhibitors of JNK2 activity towards the c-Jun trans-activation domain [4]. The chemical structure of SP600125 is shown in Table 1, alongside the structures of other small molecule inhibitors of JNK discussed in subsequent sections of this review.

The highly planar nature of SP600125 and poor solubility in aqueous solution, both consequences of its anthrapyrazolone core structure, were noted in its initial description [4]. JNK inhibition by SP600125 was further observed to be reversible and ATP-competitive, showing IC_{50} values for JNK inhibition in the range of 40–90 nM (see Table 1) with >300-fold selectivity over the related mitogen-activated protein kinases (MAPKs), ERK1 and p38-2 and between 10-fold and 100-fold selectivity over another 14 protein kinases tested [4]. These results suggested high affinity and specific interactions of SP600125 with residues in the JNK ATP-binding site.

These interactions of SP600125 with JNK have been further explored following the co-crystallisation of SP600125 with JNK3 [5]. The resulting structure (Protein DataBase (PDB): 1PMV) is shown in Fig. 2, where the JNK3 residues not conserved in the related MAPK, p38-2, have been highlighted (specifically I70, V79, V196, L206 and Q155). These residues produce a narrow ATP-binding pocket in JNK that accommodated the planar SP600125 molecule and were predicted to contribute to the specificity of SP600125 towards JNK over the p38 MAPKs [5]. Although this prediction was investigated in mutagenesis studies targeting the JNK ATP-binding site residues, single mutations such as I70V or V196A did not significantly alter SP600125 binding to JNK [6]. Further work is needed to evaluate whether the mutation of residues in combination might produce more striking effects. Direct evidence for the JNK3 residues that interact with SP600125 should drive further structural refinements to increase inhibitor affinities and/or specificities.

2.2. Use of SP600125 to dissect signalling mechanisms and JNK-dependent cell events

In initial testing for biological efficacy of SP600125 in stimulated Jurkat T cell cultures, c-Jun phosphorylation was inhibited with an IC_{50} of 5 to 10 μM [4]. The concentrations required for intracellular effects were therefore significantly higher than the *in vitro* IC_{50} values estimated with the purified JNK proteins (40 to 90 nM, see Table 1). These differences were attributed to the ATP concentrations competing with SP600125 in these different assays; the *in vitro* biochemical assays were performed at ATP concentrations lower than would be normally found *in vivo*. Thus, the intracellular IC_{50} values were higher than those observed *in vitro* [4].

The use of SP600125 to evaluate JNK-dependent events in cells has grown rapidly since 2001. As >850 publications have now reported the use of SP600125 in cells or *in vivo*, we have restricted our discussion here to two broad areas highlighting different areas for possible therapeutic applications of SP600125 and other JNK inhibitors.

We begin by considering the effects of SP600125 to enhance recovery following ischemia/reperfusion damage or other insults in a variety of tissue types. An underlying theme emerges in the actions of SP600125 to prevent cell death. As we will describe, SP600125 can inhibit a number of pro-apoptotic events such as the activation of pro-apoptotic Bcl2 family members, the release of mitochondrial cytochrome *c* into the cell cytosol, or the activation of pro-apoptotic caspase family of proteases. The reader is referred to recent excellent reviews on apoptosis for further details on the hallmarks of this cell death process [7,8]. In some cases, it also appears that SP600125 can modulate immune cell responses, and thus provide beneficial effects. We then consider the possible therapeutic applications of SP600125 in the treatment of infectious disease, most notably in its actions to alter the outcomes of viral disease. Taken
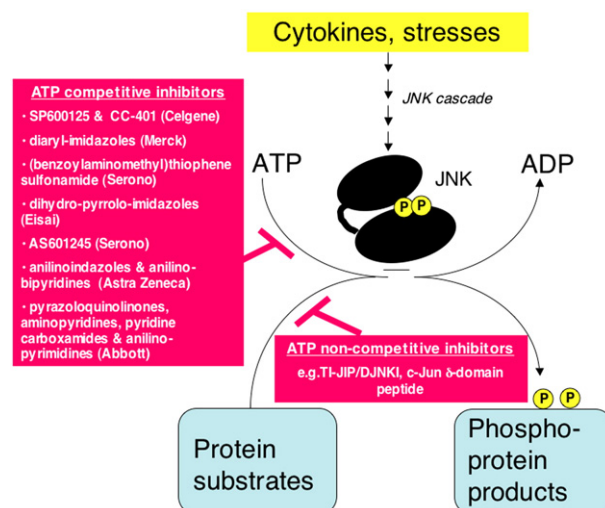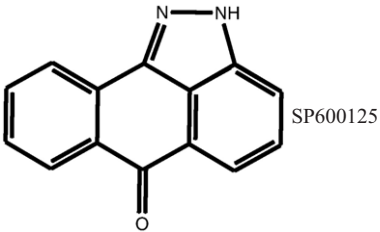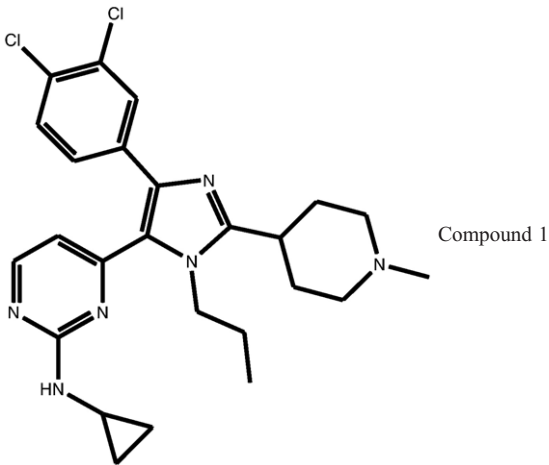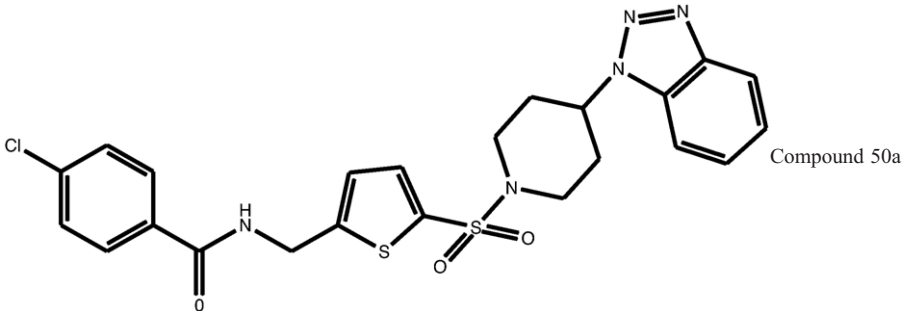
Fig. 1. General overview of the reaction catalysed by active JNK, highlighting the different classes of JNK inhibitors described to date. JNK is activated via a specific protein kinase cascade when eukaryotic cells are exposed to cytokines or stress. Active JNK catalyses a phosphorylation reaction in which the γ-phosphoryl residue from ATP is transferred to a range of protein substrates [92]. This review centres on the JNK inhibitors described in the published literature. These inhibitors can be divided into those that directly interfere with ATP-binding and thus termed "ATP-competitive" or JNK inhibitory peptides that compete with the protein substrates and thus termed "ATP-non-competitive".

Table 1
Small molecule JNK inhibitors identified in high throughput screening of proprietary chemical libraries

Core structure/Class description in initial publications	Inhibitor Structure (note: Compound number refers to that given in the original report)	Identification	IC$_{50}$ (and/or K$_i$ when reported)	PDB entry	Reference
Anthrapyrazolone	SP600125	High throughput screening of Celgene compound collection using JNK2 activity assay, followed by cell-based testing	IC$_{50}$ JNK1=40 nM IC$_{50}$ JNK2 =40 nM IC$_{50}$ JNK3=90 nM	1UKI 1PMV	[5]
Diaryl-imidazoles	Compound 1	Reported by scientists at Merck, but discovery approach not described. Note, this compound also interacts with and inhibits p38 with higher potency (IC$_{50}$ p38= 0.078 nM)	IC$_{50}$ JNK3 = 7 nM	1PMN	[5]
(Benzoylaminomethyl) thiophene sulfonamides	Compound 50a	High throughput screening of Serono compound collection using JNK3 activity assay, followed by structure–activity relationship studies and neuronal cell based assays for the inhibition of NGF-deprivation-induced cell death.	IC$_{50}$ JNK2=650 nM IC$_{50}$ JNK3=150 nM	–	[58]
Dihydro-pyrrolo		Design by Eisai scientists	IC$_{50}$ JNK1=2.5 nM	–	[59]

imidazoles

Compound (S)-5
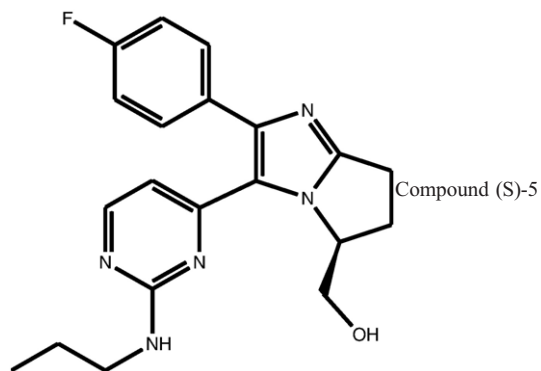
based on structures of known p38 inhibitors with additions to increase JNK3 inhibitory potency and selectivity; testing inhibition of c-Jun phosphorylation and survival following K^+ withdrawal from cerebellar granule neurons. Note, this compound inhibits p38 with lower potency (IC$_{50}$ p38 = 28 nM)
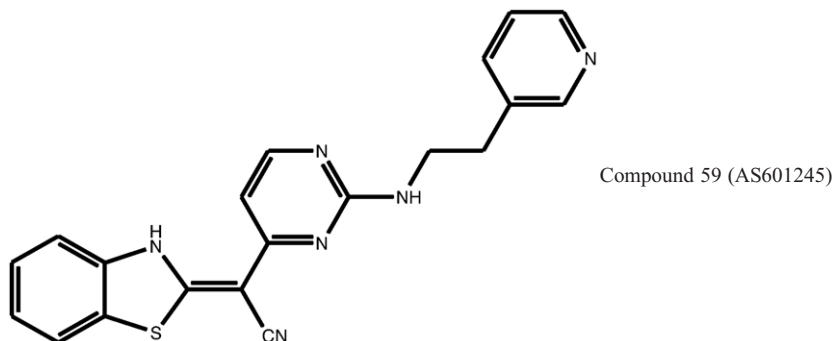
(Benzothiazol-2-yl) acetonitrile	High throughput screening of Serono compound collection using JNK3 activity assay, followed by structure–activity relationship studies, cell-based assays and anti-inflammatory effects *in vivo*.	IC$_{50}$ JNK1 = 150 nM IC$_{50}$ JNK2 = 220 nM IC$_{50}$ JNK3 = 70 nM	– [60]
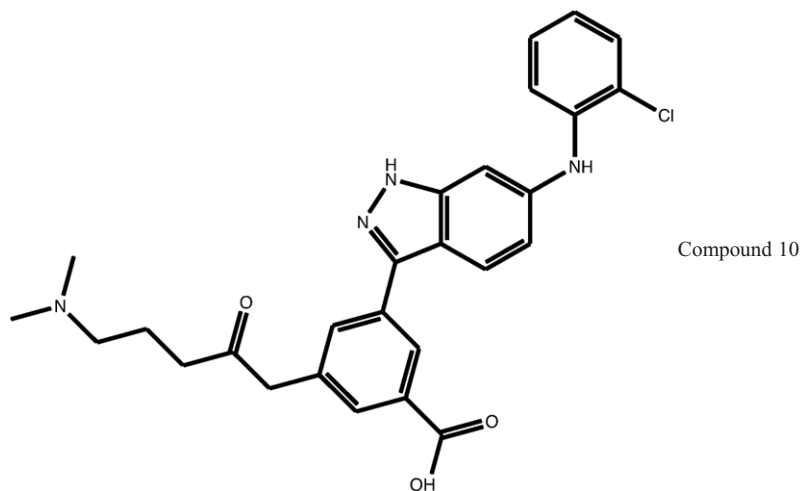
Compound 59 (AS601245)

Anilinoindazoles

Compound 10

High throughput screening of Astra Zeneca compound collection using JNK3 activity assay, followed structure–activity relationship studies.

IC$_{50}$ JNK1 = 101 nM 2B1P [61]
IC$_{50}$ JNK3 = 3 nM

High throughput screening of Abbott compound collection using JNK1 activity assay, followed by testing in cell-based assays for inhibition of tumour

IC$_{50}$ JNK1 = 290 nM 2GO1 (structure with a related compound) [63]

Table 1 (*continued*)

Core structure/Class description in initial publications	Inhibitor Structure (note: Compound number refers to that given in the original report)	Identification	IC$_{50}$ (and/or K$_i$ when reported)	PDB entry	Reference
Pyrazoloquinolinones	Compound 16	necrosis-factor stimulated c-Jun phosphorylation			
Aminopyridines	Compound 35	High throughput screening of Abbott compound collection using JNK1 activity assay, followed by testing in cell-based assays for inhibition of tumour necrosis-factor stimulated c-Jun phosphorylation	IC$_{50}$ JNK1=36 μM (K$_i$ JNK1 =3 nM) IC$_{50}$ JNK2 = 70 μM (K$_i$ JNK2 =13 nM) (K$_i$ JNK3=61 nM)	2GMX (structure with a related compound)	[64]
Pyridine carboxamide	Compound 12	High throughput screening of Abbott compound collection using JNK1 activity assay, followed by testing in cell-based assays for inhibition of tumour necrosis-factor stimulated c-Jun phosphorylation.	IC$_{50}$ JNK1=24 nM) IC$_{50}$ JNK2=74 nM)	2H96 (structure with a related compound)	[65]

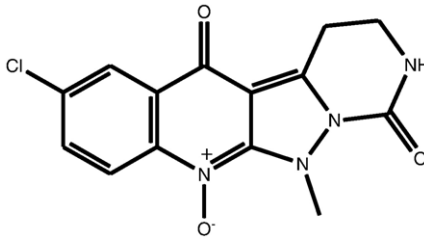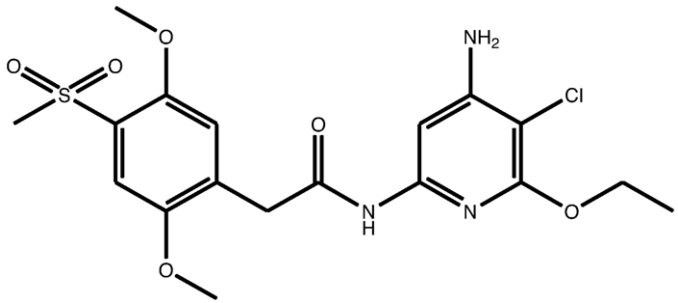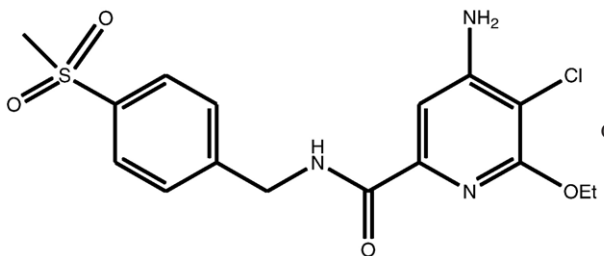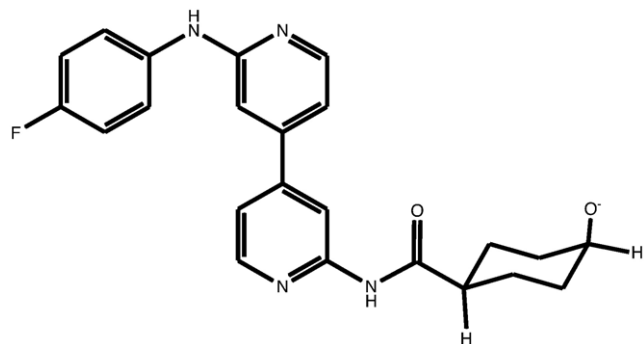|				
Anilino-bipyridines	\n\nCompound 11	High throughput screening of Astra Zeneca compound collection using JNK3 activity assay, followed structure–activity relationship studies. Note, this compound also inhibits p38 with comparable potency (IC_{50} p38 = 40 nM)	IC_{50} JNK1 = 88 nM\n$IC_{50}$ JNK3 = 15 nM	2EXC	[62]
Anilino-pyrimidines	\n\nCompound 2b	High throughput screening of Abbott compound collection using JNK1 activity assay, followed by rational structural design then testing in cell-based assays for inhibition of tumour necrosis-factor stimulated c-Jun phosphorylation.	IC_{50} JNK3 = 9 nM	2NO3	[66]
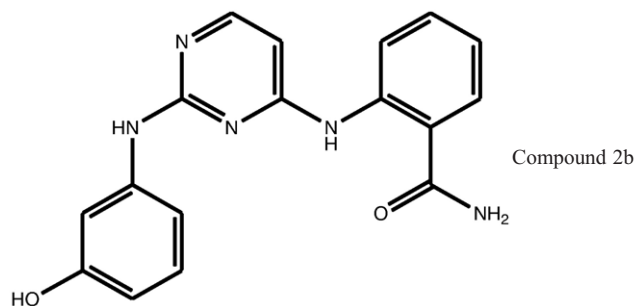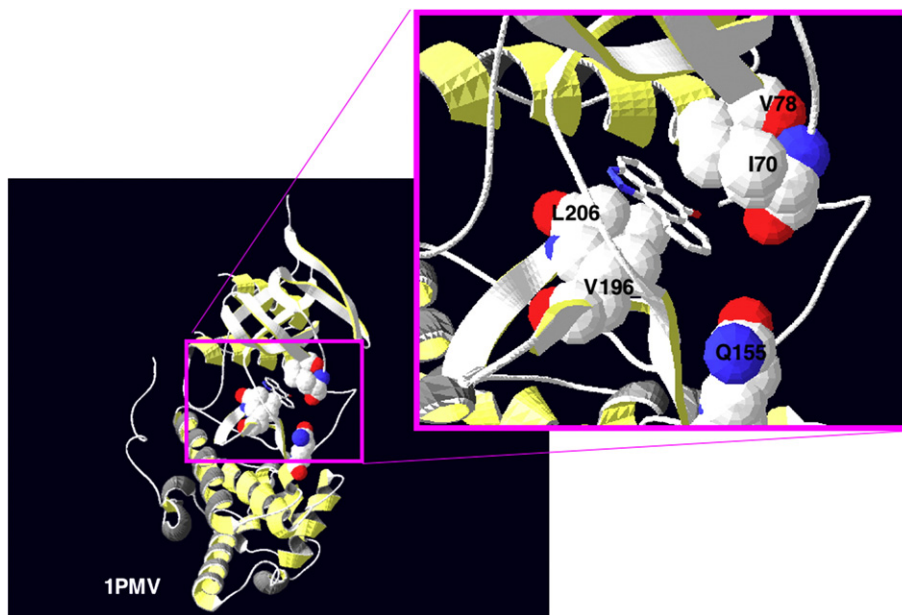
Fig. 2. Structure of the JNK complex with the ATP-competitive inhibitor SP600125. SP600125 has been co-crystallised with JNK3, and the resulting structure has been recorded in the Protein DataBase (PDB) as 1PMV [5]. Here, the residues in JNK3 not conserved in p38-2, namely I70, V79, V196, L206 and Q155, are highlighted. Although these residues were predicted to most likely contribute to the specificity of SP600125 towards JNK1/2/3 over the p38 MAPKs [5], subsequent mutagenesis studies further work is required to evaluate which residues make major contributions to binding [6].

together, these studies suggest that SP600125 administration will be beneficial in a range of therapeutic applications.

2.3. Efficacy of SP600125 in the treatment of ischemia, ischemia/reperfusion damage and other insults

JNK activation follows insults such as ischemia/reperfusion in many tissues including lung, kidney, liver, brain, and heart [9–13]. For the lung, a challenge facing its transplantation remains primary graft failure following ischemia/reperfusion injury during the initial removal and subsequent transplantation surgery. The inclusion of SP600125 in the preservation and reperfusion solutions reduced lung injury as visualised directly by histological examination of lung tissue and the assessment of apoptotic cell numbers [14]. These benefits have been accompanied by improved biochemical markers such as decreased release of total protein, lactate dehydrogenase, and tumour necrosis factor-α into the bronchoalveolar lavage fluid, indicating maintenance of tissue integrity despite the ischemia/reperfusion insult [14].

In other forms of lung insult, SP600125 administration has also been beneficial. The administration of SP600125 1 h after smoke inhalation decreased airway cell apoptosis, decreased mucous plugging, lowered the influx of inflammatory cells, decreased the release of cytokines and improved animal survival [15]. These in vivo data suggest a critical role for JNK in smoke-induced lung injury, highlighting the beneficial effects of SP600125. Similarly, the administration of SP600125 has implicated JNK in the regulation of the expression of the acute phase protein, pentraxin 3, in the lung in response to the pro-inflammatory cytokine, tumour necrosis factor [16]. As higher levels of pentraxin 3 exacerbate lung injury, JNK inhibition is expected to be an attractive therapeutic approach to protect the

lung from the increased tumour necrosis factor levels that accompany many inflammatory and other insults.

Ischemia/reperfusion insult can also accompany renal transplantation and surgery, renal failure and trauma. Direct protective effects of SP600125 during kidney ischemia/reperfusion have been observed and have been attributed to JNK inhibition suppressing apoptotic cell death events in a Fas ligand-initiated extrinsic pathway [17]. The involvement of macrophages in renal tissue injury in vivo has also been suggested, with macrophage accumulation being a prominent feature in most forms of human glomerulonephritis and correlating with renal dysfunction. The exposure of bone marrow-derived macrophages to SP600125 prior to transfer into a sheep model of glomerulonephritis caused a 75% reduction in proteinuria, thus highlighting a critical role for the JNK signaling pathway in macrophage-mediated renal injury [18]. The benefits of JNK inhibition in ischemia/reperfusion may therefore include altered inflammatory cell responses that initiate damage.

Possible benefits of SP600125 for the liver following insult have also been demonstrated. Marked protective effects of SP600125 was observed for acetaminophen-induced toxicity both in vitro and in vivo, through the actions of SP600125 were observed to block apoptotic cell death [19]. This has been extended recently to the study of acute hepatic failure following paracetamol poisoning in which SP600125 administration in vivo markedly reduced mortality and hepatic tumour necrosis factor production [20]. Similarly, the subcutaneous injection of SP600125 prior (6 and 2 h) and after (2 h) insult reduced hepatocyte apoptosis, suppressed lethality, and decreased the elevation of serum markers of liver damage in an experimental model of fulminant hepatic failure [21]. In contrast, SP600125 administration was not protective against carbon tetrachloride

or concanavalin A toxicity [19]. This highlighted that JNK inhibition will not be beneficial for all forms of hepatic injury, and instead suggests that the targeting of other stress-initiated events should be tested as alternative therapeutic approaches.

Similar, or potentially more extreme, problems also face those striving to improve the survival of neurons following insults to the brain. SP600125 treatment has prevented cell death following ischemia or ischemia/reperfusion of the brain [22–25]. As one example, SP600125 decreased neuronal apoptosis induced by global ischemia/reperfusion in the hippocampal CA1 subregion. Specifically, SP600125 suppressed the expression of Fas ligand that initiates the extrinsic death pathway, the translocation of the proapoptotic protein Bax to mitochondria, the release of cytochrome *c* to the cytosol, and the activation of proapoptotic caspases [25]. Similarly, in models of early brain injury after subarachnoid hemorrhage, SP600125 administered intraperitoneally 1 h before and 6 h after haemorrhage demonstrated benefits such as the suppression of caspase activation and concomitant neuronal injury, improved blood–brain barrier preservation, reduced brain swelling, and improved neurological function [26]. SP600125 also prevented apoptosis of dopaminergic neurons in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of Parkinson's Disease [27] as well as neurons in the acute injury accompanying spinal cord trauma [28]. Taken together, these results support the further development of JNK inhibitors as neuroprotective agents and their use in a range of brain insults.

In contrast to the positive findings supporting the benefits of SP600125 administration as described in the preceding paragraphs, detrimental effects of SP600125 have been reported in ischemia/reperfusion injury in other tissues and cell types. For example, when SP600125 was administered both at the onset of partial hepatic ischemia and during the subsequent reperfusion events, a number of markers of liver damage such as serum alanine aminotransferase levels were increased [29]. This was accompanied by deterioration of liver histology and increased neutrophil infiltration that augmented oxidative stress in the reperfused liver tissue [29]. Thus, detrimental effects to the liver appeared to be mediated, at least partially, via circulating immune cells. SP600125 exacerbated these detrimental effects.

There have also been detrimental effects of SP600125 noted for the cells of the heart. SP600125 significantly enhanced the activation of the proapoptotic protease, caspase-3, and increased the numbers of apoptotic cardiac myocytes in culture in response to their energy depletion following exposure to potassium cyanide and 2-deoxy-D-glucose [30]. Similarly, chronic SP600125 treatment *in vivo* in the cardiomyopathic hamster model of heart failure SP600125 increased the number of apoptotic (TUNEL-positive) myocytes and the area of interstitial fibrosis [31]. This was accompanied by enhanced left ventricular chamber dilation and dysfunction indicating the negative effects on cardiac structure and function [31]. Whilst these results suggest a role for JNK in cardiac myocyte survival, they contradict the observations that SP600125 protected cardiac myocytes from cell death following β-adrenergic stimulation [32]. Again, this has emphasized that the cardiac effects

of SP600125 must be evaluated in a range of different insults and pathological conditions.

Additional studies are now needed to explore how SP600125 alters the balance between death and survival in different cell types. At a biochemical level, the cell context-dependent differences, as noted in the preceding paragraphs, may reflect the differences in the expression and/or localisation of JNK substrates within the various cell types. In addition, it is also becoming clearer that defining the impact of JNK signalling on immune cell function will be critical to understanding those diseases in which there is a significant immunological response. The differences observed may also reflect the various administration and insult protocols employed in these studies, or the concentrations of SP600125 achieved *in vivo*. The availability of additional JNK inhibitors should allow these issues to be addressed directly.

2.4. Efficacy of SP600125 in the treatment of viral infection

Increasingly, it has been shown that viral infection can lead to JNK activation. Examples include infection by Epstein–Barr Virus [33], Herpes Simplex Virus [34], Reovirus [35], Kaposi's Sarcoma Virus [36], or Varicella–Zoster virus [37,38]. Whilst the exact mechanisms leading to JNK activation remain to be evaluated in many of these cases, it is of interest that Kaposi's Sarcoma Virus encodes the viral kinase ORF36 that interacts with JNK as well as the upstream JNK pathway kinases MKK4 and MKK7. ORF36 expression can lead to the phosphorylation and activation of MKK4/7 and, thus, to JNK activation [36]. Further interventional studies, primarily in cultured cells *in vitro*, have supported a role for JNK activation in viral infection processes and/or subsequent cellular events. In the following paragraphs, we discuss the results of recent studies evaluating the effects of SP600125 in models of viral infection that suggest that JNK inhibitors may provide new therapeutic interventions.

In a number of situations following exposure to virus or viral proteins, SP600125 treatment has prevented viral-induced cell death. This is consistent with the studies discussed in the preceding sections that highlighted that SP600125 could prevent cell death in many tissues following a range of different stresses. Specifically, SP600125 treatment prevented apoptotic death following the exposure of human monocytic cells to the Human Immunodeficiency Virus (HIV) accessory protein viral protein Vpr [39]. Similar positive effects to protect cells from death have been observed when SP600125 treatment either rescued influenza epitope-specific human cytolytic T lymphocytes from activation-induced cell death [40] or prevented the death of cultured hippocampal cells exposed to Herpes Simplex Type 1 Virus [41].

Conversely, SP600125 inhibited the proliferation of primary erythroleukemic cells isolated from Friend spleen focus-forming virus-infected mice [42]. Furthermore, in cell lines established from these animals, SP600125 caused significant apoptosis as well as an increase in the fraction of cells in the G2/M phases of the cell cycle and undergoing endoreduplication [42]. These latter data suggest that JNK plays an important role in cell proliferation and/or the survival of erythroleukemia cells,

and thus that SP600125 administration could provide a novel approach in the treatment of viral-induced erythroleukemia [42].

In other examples of viral infection, the use of SP600125 has altered viral replication or cellular persistence. For example, rotavirus is a double-stranded RNA virus that affects the gastrointestinal system resulting in vomiting and diarrhoea. The use of SP600125 in combination with p38 MAPK inhibitors has suggested that maximal rotavirus-induced interleukin-8 and c-jun transcription required JNK and p38 activity. Significantly, both p38 and JNK were required for rotavirus replication but not viral structural antigen expression [43]. Similarly, SP600125 used together with inhibitors of phosphatidylinositol 3-kinase inhibited the establishment of persistent SARS-CoV infection in Vero E6 cells [44]. Clearly, there are now many opportunities to evaluate how SP600125 acts in concert with other inhibitors of intracellular signaling pathways to modulate aspects of viral biology. The most appropriate therapeutic strategy may ultimately require combination therapies of signal transduction modulators.

Despite these successes, there have also been some situations when SP600125 treatment has not been beneficial. These have emphasized the need for caution. For example, the use of SP600125 did not significantly change disease progression following infection with Coxsackievirus B3 (CVB3), an enterovirus in the Picornavirus family that is the most common human pathogen associated with myocarditis and idiopathic dilated cardiomyopathy [45]. SP600125 reduced CVB3-induced phosphorylation of activating transcription factor 2 (ATF2), but did not alter CVB3 viral protein synthesis, viral progeny release, cell death, or caspase-3 activation in infected cells. In contrast, p38 MAPK inhibitors altered progeny release [45]. Thus, it remains critical to test the effects of SP600125 on a range of different virus types and cellular effects.

SP600125 treatment may also alter gene expression changes that have significant effects for virus structure and/or life cycle. For Hepatitis C Virus non-structural protein 3 (NS3) protein-expressing cells, exposure to SP600125 abolished a number of transcription factor activities, notably AP1 and ATF2, inhibited c-jun expression, and inhibited NS3-induced cell growth [46]. Similarly, SP600125 blocked Cytomegalovirus IE1-mediated induction of AP-1 and relB promoter activity in NIH 3T3 and cultured smooth muscle cells [47]. Furthermore, nuclear localisation of the viral encoded proteins may be regulated by JNK as seen for the human Papillomavirus E1 DNA helicase [48]. Thus, these newly recognized roles for JNK may open new anti-viral strategies with the use of JNK inhibitors such as SP600125.

2.5. What might the future of SP600125 hold?

Despite the obvious successes of SP600125, and its repeated use in both *in vitro* and *in vivo* systems, some scepticism surrounds its continued use, particularly when its specificity for JNK inhibition is more closely evaluated. Despite the initial claims of the selectivity of SP600125, with little or no inhibition shown for 17 tested protein kinases or 18 inflammatory enzymes [4,49], its subsequent testing has shown inhibition of 13 of 30 tested protein kinases [50]. Notably, serum and glucocorticoid-regulated kinase, p70 ribosomal S6 kinase, AMP-dependent protein kinase, cyclin dependent kinase 3, casein kinase 1δ and dual-specificity tyrosine–regulated kinase 1A were all inhibited by 10 μM SP600125 to a greater extent than the inhibition observed for JNK [50]. Additional data showing SP600125 binding to a range of kinases (39 of the 119 tested protein kinases) in phage interaction screening assays [51], suggests there may be many additional kinase targets of SP600125.

Despite these concerns raised on the specificity of SP600125, its value as a therapeutic agent will be confirmed with its continued usefulness *in vivo* with minimal toxicity or few undesirable side-effects. Some caution should be exercised when the core anthrapyrazole structure of SP600125 is considered. Anthrapyrazoles have been used as anticancer agents due to their toxic effects associated with reactive oxygen species (ROS) production, topoisomerase inhibition and DNA-interactions [52,53]. Thus, SP600125 administration *in vivo* may be associated with similar toxicity that would be undesirable when an aim is to prevent cell death. This will be of greater concern when the effects of long-term dosing are evaluated. Thus, the continued development of SP600125 as a new therapeutic or therapeutic lead will require further evaluation if it shows toxic effects via JNK-independent actions.

A second generation ATP-competitive anthrapyrazolone JNK inhibitor, CC-401, has also been developed by Celgene based on the chemistry of SP600125. Despite limited publicly available details of the compound and its use, Celgene has stated that CC-401 completed a Phase I trial in healthy volunteers. Celgene is also evaluating CC-401 in a phase II clinical trial for acute myelogenous leukemia. Given the anticancer activity of some anthrapyrazoles, further evidence to support the actions of CC-401 via JNK inhibition will be required.

CC-401 has shown efficacy in an experimental model of immune-induced renal injury [54]. Specifically, CC-401 treatment of a rat anti-glomerular basement membrane disease model reduced proteinuria in the first 24 h. The rapid transient neutrophil influx was not affected, but the continued treatment with CC-401 suppressed glomerular and tubulointerstitial damage usually seen at 14 days. As CC-401 had no effect upon glomerular macrophage infiltration at day 14, it was proposed that this protection was due to modulation of macrophage activation. Thus, JNK signalling would appear to promote renal injury in acute and progressive rat anti-glomerular basement membrane disease, so that JNK inhibitors may be a novel therapeutic approach for the treatment of human glomerulonephritis. Similarly, in kidney obstruction, CC-401 significantly reduced tubular apoptosis and inhibited renal fibrosis as shown by interstitial myofibroblast accumulation and collagen IV deposition. This latter effect was attributed to suppression of gene transcription for the profibrotic factors, tumour growth factor-β1 and connective tissue growth factor [55].

CC-401 or related compounds have also been used in models of liver injury. Thus, the inclusion of JNK inhibitory compounds (CC-401, CC0209766, or CC0223105) in a hepatic warm ischemia/reperfusion injury model significantly improved

survival rates from <40% to 60–100% [56]. This decreased mortality was correlated with improved hepatic histology as these compounds significantly inhibited pericentral necrosis, neutrophil infiltration and apoptosis of both hepatocytes and sinusoidal endothelial cells, with decreased caspase-3 activation and cytochrome *c* release from mitochondria, and lowered levels of lipid peroxidation [56]. As similar beneficial effects were noted following cold ischemic storage of liver tissue followed by its warm reperfusion, benefits would be expected upon the inclusion of these JNK inhibitory compounds in storage and transport solutions used during liver transplantation surgery [57].

To confirm that JNK inhibition is critical for the benefits associated with SP600125 or CC-401 treatment, additional interventions directed towards JNK activity *in vivo* are needed. In the following paragraphs, new classes of ATP-competitive JNK inhibitors are described that will allow the benefits of JNK inhibition as a new therapeutic approach to be further explored.

3. Emerging new classes of small molecule JNK inhibitors

To date, the other small molecule JNK inhibitors recently disclosed in the publicly available scientific literature have not received the same attention as that directed towards SP600125. In this section, ten additional JNK inhibitors are briefly overviewed. A summary of these inhibitors, together with SP600125 and their chemical structures, is provided in Table 1. This summary is listed chronologically by the first published report of each inhibitor. We also present structures for those inhibitors cocrystallised with JNK proteins (Fig. 3). These structures suggest the ATP-competitive nature of these inhibitors.

The small molecule JNK inhibitors include examples from the diaryl-imidazoles (as reported by scientists at Merck [5]), (benzoylaminomethyl)thiophene sulfonamides (Serono [58]), dihydro-pyrrolo-imidazoles (Eisai [59]), (benzothiazol-2-yl) acetonitrile (also known as AS601245; Serono [60]), anilinoindazoles and anilino-bipyridines (Astra Zeneca [61,62]), as well as pyrazoloquinolinones, aminopyridines, pyridine carboxamides and anilino-pyrimidines (Abbott [63–66]). These compounds have largely been discovered by high throughput screening of compound libraries, typically by testing actions in *in vitro* kinase assays against purified JNK. Subsequent structure–activity studies and testing in cell culture models has allowed the refinement of these inhibitors. A notable, different approach has also shown the refinement of p38 inhibitors to increase potency towards JNK activity rather than continuing to re-screen libraries directly for JNK inhibitors [59]. Some of the inhibitors have also been reported to show some selectivity towards JNK1 [67], or JNK3 [58,60–62], but maximal differences were only approximately 35-fold as seen for the anilinoindazoles with higher affinity for JNK3 [61].

It remains critical to evaluate the biological actions of these new JNK inhibitors. The limited reports that have tested these JNK inhibitors in perfused organ systems or *in vivo* have shown mixed results. The therapeutic potential for JNK inhibitors is supported by the findings in models of rheumatoid arthritis [60], as well as cerebral and cardiac ischemia [68,69], and the

undisclosed claims for benefits in models of inflammation and diabetes [64]. In contrast, the haemodynamic effects reported for the 4-aminopyridine carboxamide-based JNK inhibitors suggests that further caution may be warranted [70]. Whether undesired side effects arise from JNK-dependent or -independent inhibitor actions must be addressed. Ideally, the effects of many structurally unrelated JNK inhibitory compounds can be compared to determine JNK-independent actions.

4. Natural product inhibitors of JNKs

The success of chemical library screening in identifying JNK inhibitory molecules raises the possibility that additional JNK inhibitors can be found in other sources. A screen of 100,000 natural extracts revealed an extract from the New Guinea vine, *Gnetum latifolium,* as an *in vitro* JNK3 inhibitor [71]. Further purification revealed the JNK inhibitory components to be latifolians A and B (Table 2). These compounds form part of the 8-benyl-berberine alkaloid structure class distributed across many plant families. The energy-minimised three dimensional structures of these latifolians were determined, together with their IC_{50} values towards JNK3 (Table 2) [71]. Further studies including kinetic and structural analyses, should address whether the latifolians are ATP-competitive JNK inhibitors, whether all JNK isoforms are targeted equally, and how these molecules interact with the JNK proteins. This information could then direct the development of new classes of JNK inhibitors that exploit the essential structural features of these latifolians without their complex structure.

5. Peptide inhibitors of JNKs

5.1. A JNK inhibitory peptide derived from the JNK substrate, c-Jun

As recently reviewed, peptide inhibitors of protein kinases have been derived from direct interacting partners of protein kinases, such as their substrates [72]. A cell-permeable peptide JNK inhibitor has been derived from the δ-domain of the JNK substrate, c-Jun [73]. The sequence of this peptide is shown in Table 3. As the c-Jun δ-domain interacts directly with JNK, this peptide would compete directly with c-Jun substrate binding. This peptide has been used to highlight the complexity of JNK–c-Jun-mediated gene regulation in the response to interleukin-1 [73]. Of interest, differences were observed when the effects of the JNK inhibitory peptide and the ATP-competitive inhibitor, SP600125, were compared [73]. For example, of the interleukin-induced genes, 20 genes were down regulated in the presence of either the c-Jun peptide or SP600125. Of these 20 genes, only 4 were down regulated by both c-Jun peptide and SP600125, 6 genes were affected by c-Jun peptide only and 10 genes were affected by SP600125 only [73]. Whether these differences reflect off-target effects of SP600125, or other differences between these inhibitors such as the mode of action, compartmentalisation or stability of the inhibitors remains to be addressed.
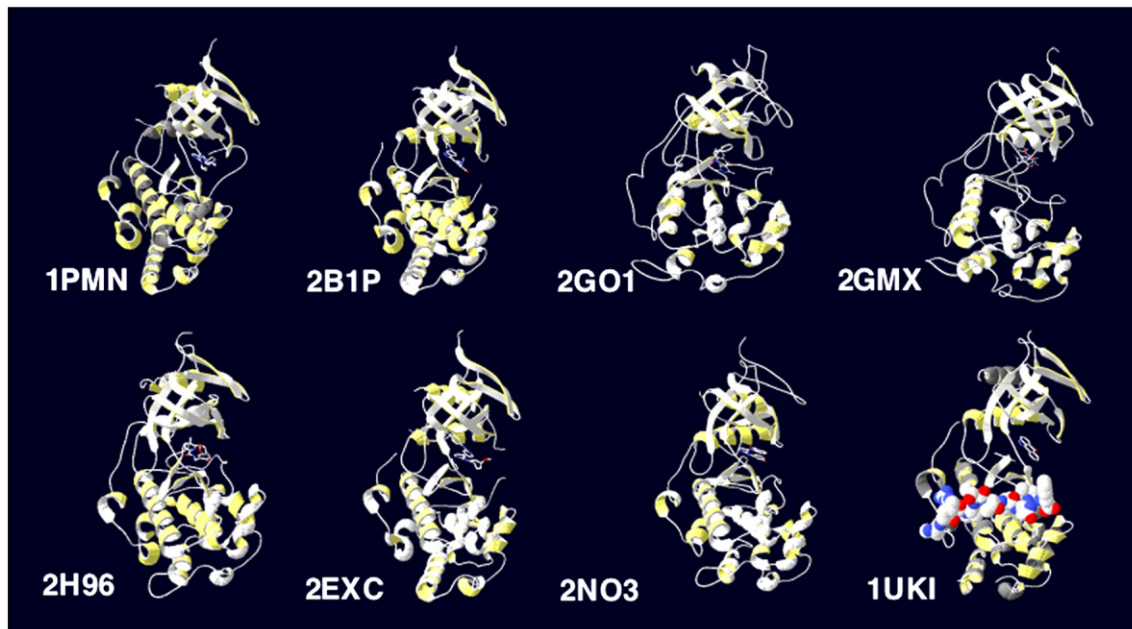
Fig. 3. Structures of complexes of JNK with ATP-competitive inhibitors or the JNK-inhibitory peptide derived from JIP. Crystal structures have been recorded in the PDB for JNK in the presence of a number of different ATP-competitive inhibitors. These include *1PMN*: diaryl-imidazoles (Merck [5]), *2B1P*: anilinoindazoles (Astra Zeneca [61]), *2GO1*: pyrazoloquinolinones, *2GMX*: aminopyridines and *2H96*: pyridine carboxamides (Abbott [64,65,67]), *2EXC*: anilino-bipyridines (Astra Zeneca [62]) and *ZNO3*: anilino-pyrimidines (Abbott [66]). The complex of JNK1 with SP600125 and the JNK inhibitory peptide derived from JIP1 is also indicated in 1UKI.

5.2. Use of JNK inhibitory peptides derived from the JNK pathway scaffold protein, JIP1

Scaffold proteins, known as JNK-interacting proteins or JIPs, form an additional important feature of the JNK pathway. Of note, JIP1 was first described to inhibit JNK by preventing JNK nuclear translocation, but a short conserved sequence was also identified as critical for the JIP1–JNK interaction [74]. Short JIP-derived peptides have been subsequently shown to inhibit JNK activity *in vitro* [75]. These peptides, in their cell-permeable form through their conjugation to the Tat peptide [76–78]), have been used to investigate effects of JNK inhibition in cells and *in vivo*.
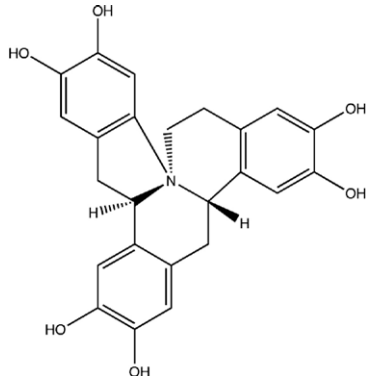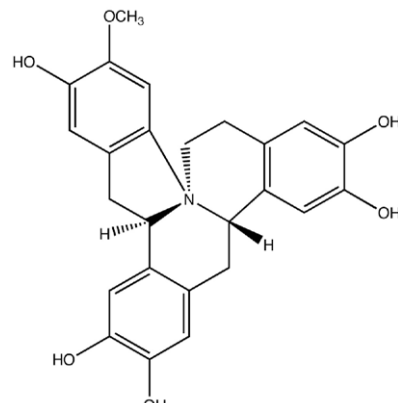
The sequences of the commonly used cell-permeable JNK inhibitory peptides derived from JIP are shown in Table 3. These include the conventional ʟ-amino acid containing peptides TI-JIP, TAT-TIJIP and L-JNKI, as well as the ᴅ-amino acid-containing retroinverso peptide, D-JNKI. These JIP-derived peptides inhibitors have been shown kinetically to act in a protein substrate-competitive manner [79], and by co-crystallisation and mutagenesis studies to bind directly to the putative protein substrate docking domain of JNK [80,81]. More recently, these peptides have been used to evaluate the kinetic mechanism of JNK2 [82]. The results have provided important insights into the biochemistry of JNK including that protein substrate binding is primarily due to the distal contacts in the JNK2 docking groove, that there is minimal allosteric communication between the protein–substrate docking site and the ATP binding site in the active JNK2 catalytic centre, and that phosphorylation proceeds via a random sequential mechanism [82].

A recent review evaluated the studies using the cell-permeable forms of these JNK inhibitory JIP-based peptides [83]. This highlighted the success of these peptides in blocking pancreatic β-cell death [76], cerebral ischemia/stroke [77,84], and hearing loss induced by aminoglycosides and acoustic trauma [85]. The latter has been extended in recent studies [86,87]. Here we restrict our attention to reports on the efficacy of JNK inhibitory peptides appearing in the last 2 years since that review and we begin with recent studies on the effects in neuronal cells.

Neuropathic pain often accompanies nerve damage, but there are few options currently available for its effective treatment. In searching for possible targets for therapeutic intervention in the treatment of pain, it had been noted that spinal nerve ligation resulted in a slow but persistent activation of JNK in spinal cord astrocytes [88]. Intrathecal infusion of D-JNKI to spinal fluid did not alter the basal mechanical threshold prior to injury but prevented mechanical allodynia for more than 10 days [88]. It should be noted that the pain returned when the 14-day infusion protocol ended [88]. Thus, D-JNKI treatment provided only temporary pain relief and additional strategies are needed to identify targets for long-term pain relief.

Consistent with the observed benefits of SP600125 or D-JNKI in ischemia and reperfusion, particularly in the brain, TAT-TIJIP also prevented both apoptotic death and necrotic death of neurons in culture [89,90]. For apoptosis, inhibition of both nuclear and non-nuclear pathways is important [89,90]. For necrosis, the exact JNK-mediated events remain to be defined, but a number of key findings should direct future studies. Specifically, TAT-TIJIP when applied prior to the transient exposure to glutamate that mimics the excitotoxicity that

Table 2
Latifolians A and B as a new class of natural product JNK inhibitors

Inhibitor	Inhibitor Structure	Identification	IC$_{50}$	PDB entry	Reference
Latifolian A		High throughput screening of 100,000 natural extracts for inhibitors of JNK3 activity *in vitro*.	IC$_{50}$ JNK3 = 13 μM	–	[71]
Latifolian B		High throughput screening of 100,000 natural extracts for inhibitors of JNK3 activity *in vitro*.	IC$_{50}$ JNK3 = 10 μM	–	[71]

accompanies stroke, prevented mitochondrial ROS generation, increased cytosolic calcium concentration, and maintained mitochondrial membrane potential [90]. The basic principles of this model involving JNK activation in necrosis are illustrated in Fig. 4. More recently, the use of SP600125 or JNK knockout cells has shown that JNK mediates necrotic death via its sustained activation of poly(ADP-ribose) polymerase-1 (PARP-1) following exposure to ROS [91]. The direct *in vitro* phosphorylation assays [91] suggested that PARP-1 can be added to

growing list of JNK substrates [92]. It will be of interest to test whether JNK inhibitory peptides can inhibit the actions of JNK on PARP-1 or whether other modified peptide antagonists are required.

Continuing these strategies to reduce neuronal cell death, a recent study has shown that D-JNKI is effective in the treatment of Reovirus-induced encephalitis [93]. Infection was achieved by direct injection of high doses of virus into the brain tissue of neonatal rats, with subsequent evaluation of brain pathology

Table 3
Peptide inhibitors of JNK

Protein from which inhibitor derived	Peptide name	Amino acid sequence (residue numbers)	Identification	IC$_{50}$ (and/or K$_i$ when reported)	PDB entry	References
c-Jun	TAT-cJun peptide[a]	<u>Y-G-R-K-K-R-R-Q-R-R-R-G</u>-I-L-K-Q-S-M-T-L-N-L-A-D-P-V-G-S-L-K-P-H-L-R-A-K-N(33–57)	δ-domain of c-Jun	Not determined, but used in cells at 100 μM concentration	–	[73]
JIP1	TI-JIP	R-P-K-R-P-T-T-L-N-L-F(153–163)	JNK interaction domain of JIP1	K$_i$0.39 +/− 0.08 μM*in vitro*	1UKH/1UKI	[75,79]
	TAT-TIJIP[a]	<u>G-R-K-K-R-R-Q-R-R-R-P-P</u>-R-P-K-R-P-T-T-L-N-L-F (153–163)	JNK interaction domain of JIP1	Not determined, but used in cells at 10 μM concentration	–	[78]
	JIP1 peptide	R-P-K-R-P-T-T-L-N-L-F-P-Q-V-P-R-S-Q-D(153–171)	JNK interaction domain of JIP1	Not determined	–	[76]
	LJNKi (XG101)[a]	<u>G-R-K-K-R-R-Q-R-R-R-P-P</u>-R-P-K-R-P-T-T-L-N-L-F-P-Q-V-P-R-S-Q-D (153–171)		IC$_{50}$ ~ 1 μM *in vitro*	–	[76,77]
	DJNKi (XG102)[a, b]	t-d-q-s-r-p-v-q-p-f-l-n-l-t-t-p-r-k-p-r-p-p-r-r-r-q-r-r-k-k-r-g (171–153)		Not determined, but 15–20-fold less potent than L-JNKI	–	[76,77]

[a] Cell-permeable vector sequence is underlined.
[b] Amino acids denoted without capitals are the D-amino acid forms.

and survival. Despite the positive findings with D-JNKI delivered intraperitoneally before or after the viral infection, a number of interesting observations should be further considered. Most notably, the symptoms of myocarditis were not blocked by D-JNKI. Thus, reoviral infection remained lethal due to these cardiac effects. It should be addressed whether JNK activation also underlies this cardiac pathology and whether D-JNKI inhibits JNK activity in the heart. The positive effects of D-JNKI in the heart to reduce ischemia/reperfusion injury and infarct size *in vivo* have been recently reported, but only when delivered prior to the onset of ischemia [94]. In this latter study, D-JNKI when delivered at the time of reperfusion prevented apoptosis and thus limited the cardiac infarct size but, intriguingly, it did not improve functional recovery [94]. The reasons underlying this discrepancy between cardiac cell death in the infarct zone and functional performance of the heart requires further evaluation.

The JNK inhibitory peptides should also allow greater evaluation of the roles of JNK in infection by other viruses. JNK inhibition by L-JNKI resulted in a 2-fold increase in Varicella–Zoster Virus replication in melanoma cells whereas a strong decrease in virus replication was observed after inhibition of p38 MAPK [37]. It should be noted however that a more recent study has shown SP600125 to cause a dose-dependent reduction in Varicella–Zoster virus yield in primary fibroblasts [38]. The reasons for this discrepancy will require further evaluation, but may include the differences in the cell types evaluated as well as the differences in the mechanism of action of ATP-competitive versus ATP-noncompetitive inhibitors [38]. The recent study showing that alterations in the immune response following JNK2 knockout can influence malarial infection [95] suggests that JNK inhibitors may have far greater use in the treatment of a range of infectious diseases.

The benefits of lowering JNK-dependent signalling in diabetes were first seen in JNK gene knockout studies [96,97]. This has been extended with observation that the intraperitoneal administration of JNK inhibitory peptides improved insulin resistance

Fig. 4. Calcium and reactive oxygen species (ROS) as primary drivers of JNK-dependent necrotic cell death. In this model, calcium and reactive oxygen species (ROS) are involved in an amplification cycle leading to cell death. Calcium overload in the mitochondria causes ROS generation, ROS can in turn increase intracellular calcium by directly activating calcium channels or increasing membrane permeability by lipid peroxidation and inactivating calcium removal transporters, JNK inhibitors break this cycle to attenuate the increase in both ROS and calcium.

and glucose tolerance in diabetic mice [98]. JNK inhibitory peptides have also now been tested for their effects on pancreatic islet β-cells. In transplantation, during the isolation process and subsequent clinical transplantation, islets are subjected to severe adverse conditions that impair survival and ultimately contribute to graft failure. Intraportal injection of JNK inhibitory peptides at islet transplantation reduced JNK activity in insulin target organs, prevented islet graft loss immediately after transplantation, and improved islet transplant outcome thus showing the value of JNK inhibition during these procedures [99]. This has been supported by the independent observation that D-JNKI conferred protection against apoptosis induced during the islet preparation and subsequent exposure to IL-1β [100].

Some controversy remains in this area of islet preservation. A recent report suggested that L-JNKI, but not D-JNKI, would provide protection [101]. The toxicity of D-amino acid-containing peptides, with the paradoxical activation of JNK and p38 MAPKs following exposure of islet β-cells to D-JNKI, was suggested to underlie the observed detrimental effects [101]. Further work is needed to characterize these detrimental effects and to define when D-amino acid-containing peptides may be toxic. However, extending the half-life of the JNK inhibitory peptide may not always be necessary for the desired therapeutic effect. For example, L-JNKI limited lung ischemia/reperfusion damage, and so D-amino acid-containing peptides were not necessary in this system [14]. When rapid, acute treatment is desirable, the prolonged *in vivo* half-life offered by D-amino acid-containing peptides may not be required.

Lastly, in considering how these peptide inhibitors may advance to clinical trials, Xigen has reported its Phase I trial of XG-102 (D-JNKI). In addition to demonstrating efficacy of the JNK inhibitory peptides, it will be important to optimise *in vivo* cell-permeable delivery strategies particularly as cytotoxic effects of cell-permeable peptides have been noted [102].

6. Conclusions and perspectives

Despite important advances in recent years in the development of both JNK ATP-competitive and ATP-non-competitive inhibitors, many questions have also arisen. These centre on the controls needed to establish JNK inhibitor specificity, whether JNK isoform-selective inhibitors are possible or desirable, whether other compounds may have off-target effects to inhibit JNK, and what concerns may accompany the chronic use of JNK inhibitors. In concluding this review, we briefly consider these questions that may direct further research efforts to discover and improve JNK inhibitors.

6.1. What controls are needed to establish JNK inhibitor specificity?

The testing of small molecule inhibitors against panels of protein kinases in activity assays in protein-interaction studies has emphasized that off-target effects should always be considered, particularly during the earliest stages of inhibitor/drug development. Although simple concordance between the effects
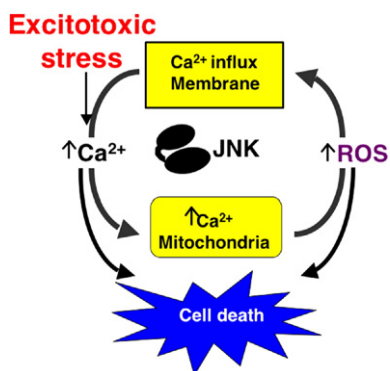
observed with putative JNK inhibitors and the phenotypes of the JNK gene knockout animals may initially support the specificity of inhibitor actions, the use and interpretation of JNK knockout animals can be complicated both by the need to target the different JNK genes and by functional redundancies between the isoforms. A more robust approach has combined genetic and pharmacological approaches to evaluate protein kinase specificity. Modification of the ATP-binding pocket of the protein kinase of interest at the so-called "gate-keeper" residue allows interaction with bulky ATP analogues that may act as either substrates or inhibitors. This approach has aided JNK substrate identification [103], an has been more recently used to inhibit JNK to define JNK2 actions [104] and to determine how JNK activation time courses affect its downstream signalling consequences [105].

6.2. Are JNK isoform inhibitors possible or desirable?

From the phenotypes of JNK1−/−, JNK2−/− or JNK3−/− mice, JNK isoform-selective targeting appears beneficial (see review [3]). Although, high sequence and structure similarity, suggests that this may be difficult to achieve with small molecule inhibitors, *in vivo* RNA interference (RNAi) remains an option that has been recently used to evaluate the specific role for JNK1 in insulin resistance in a mouse model of diet-induced diabetes [106]. Adenoviral delivery of the RNAi resulted in almost complete knockdown of hepatic JNK1 levels, without affecting JNK1 in other tissues examined. Whilst this was accompanied by reduced circulating glucose levels and enhanced insulin signalling *in vitro*, plasma triglyceride levels were elevated [106]. This appeared to be the result of the altered expression of several clusters of genes involved in glycolysis and the triglyceride synthesis pathways. Why earlier studies using JNK inhibitors, the overexpression of dominant-negative JNK mutants, or gene knockout studies (e.g. [98,107,108]) have not observed similar changes remains to be established. The striking differences when comparing small molecule inhibition or genetic ablation approaches have been recently highlighted [109]. Specifically, for JNK, this has been attributed to compensation in the absence of JNK2 leading to enhanced JNK1 signalling [104].

6.3. Might other compounds currently being evaluated against other targets also inhibit JNK?

Inhibitors initially directed towards other targets in the cell may also interfere with JNK actions. A recent example shows the discovery of an anti-hepatitis C virus compound, 4-[2-(5-bromo-2-fluoro-phenyl)pteridin-4-ylamino]-N-3-(2-oxopyrrolidin-1-yl) propyl] nicotinamide, that inhibits vascular endothelial growth factor receptor kinase as well as JNK activities [110]. Similarly, indirubin-3′-oxime, a known inhibitor of cyclin-dependent kinases and glycogen synthase kinase 3-β, also inhibits JNK [111]. This raises questions on the whether actions of indirubin-3′-oxime to inhibit apoptosis are due to its actions on JNK, cyclin-dependent kinases, or glycogen synthase kinase 3-β, alone or in combination. Similarly, the neuropro-

tectant 3-aminopyridine-2-carboxaldehyde thiosemicarbazone (PAN-811), may exert its actions to protect against glutamate toxicity via inhibition of both JNK and p38, or leflunomide may protect again acetaminophen-induced liver necrotic injury through its JNK inhibition [112,113].

6.4. What concerns may accompany chronic use of JNK inhibitors?

The embryonic lethality of the JNK1−/−JNK2−/− mice (see review [3]) has suggested critical roles for JNK in development and homeostasis. JNK has been implicated as critical regulators of neurite formation [114], neuronal axon formation [115], and recently it has been suggested that JNK regulates events associated with both health and degeneration or motoneurons [116]. Furthermore, JNK may play protective roles as demonstrated in thrombin-induced ischemic tolerance in the brain [117], and JNK may aid in regulating circadian rhythms [118]. These roles suggest that chronic JNK inhibition may not be desirable. It will therefore remain a challenge, at least in the short term, to define the range of JNK actions in the cell, as these are likely to be many and diverse. Short-term use of JNK inhibitors remains an attractive option in a number of diseases, and the increasing availability of JNK inhibitors will allow rapid progress in determining inhibitor efficacy.

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