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A novel retro-inverso peptide is a preferential JNK substrate-competitive inhibitor

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ABSTRACT

A novel 18 amino acid peptide PYC98 was demonstrated to inhibit JNK1 activity toward c-Jun. We observed a 5-fold increase in the potency of the retro-inverso form, D-PYC98 (a D-amino acid peptide in the reversed sequence) when compared with the inhibition achieved by L-PYC98, prompting our further evaluation of the D-PYC98 inhibitory mechanism. *In vitro* assays revealed that, in addition to the inhibition of c-Jun phosphorylation, D-PYC98 inhibited the JNK1-mediated phosphorylation of an EGFR-derived peptide, the ATF2 transcription factor, and the microtubule-regulatory protein DCX. JNK2 and JNK3 activities toward c-Jun were also inhibited, and surface plasmon resonance analysis confirmed the direct interaction of D-PYC98 and JNK1. Further kinetics analyses revealed the non-ATP competitive mechanism of action of D-PYC98 as a JNK1 inhibitor. The targeting of the JNK1 common docking site by D-PYC98 was confirmed by the competition of binding by TIJIP. However, as mutations of JNK1 R127 and E329 within the common docking domain did not impact on the affinity of the interaction with D-PYC98 measured by surface plasmon resonance analysis, other residues in the common docking site appear to contribute to the JNK1 interaction with D-PYC98. Furthermore, we found that D-PYC98 inhibited the related kinase p38 MAPK, suggesting a broader interest in developing D-PYC98 for possible therapeutic applications. Lastly, in evaluating the efficacy of this peptide to act as a substrate competitive inhibitor in cells, we confirmed that the cell-permeable D-PYC98-TAT inhibited c-Jun Ser63 phosphorylation during hyperosmotic stress. Thus, D-PYC98-TAT is a novel cell-permeable JNK inhibitor.

1. Introduction

Protein phosphorylation is a critical post-translational modification that rapidly controls protein properties (such as activity, stability and localization) and protein–protein interactions (Cohen, 2002). As these biochemical changes are coordinated to impact on cellular events and processes, including proliferation, survival, death, and movement, there has been continued interest to understand the full repertoire of protein kinases, their substrates and their regulation (Manning et al., 2002b; Hunter, 1995; Johnson and Hunter, 2005). More than 500 mammalian protein kinases that catalyze protein phosphorylation through their transfer of the γ-phosphate of ATP to their specific substrate proteins have been defined by bioinformatics interrogation of the genome (Manning et al., 2002a,b). The mitogen-activated protein kinase (MAPK) family, within the CMGC group of serine/threonine kinases, includes the c-Jun N-terminal kinases (JNKs) and the p38 MAPKs, that have been primarily considered to be stress-activated and contribute to processes of cell death and inflammation, as well as the extracellular signal-regulated kinases (ERKs) that have been commonly associated with growth factor actions, cell survival and growth (Marshall, 1995; Davis, 1994).

The JNKs are encoded in mammals by three genes, with *jnk1* and *jnk2* showing widespread expression but *jnk3* expression being restricted primarily to the brain, heart, and testes (Martin et al., 1996; Mohit et al., 1995). Attention has been directed toward improving the understanding of the pathways leading to JNK activation as well as the regulation of the range of different JNK targets that dictate cellular outcomes (Bogoyevitch and Kobe, 2006). Importantly, as studies in *jnk* knockout mice have shown improvements in disease pathologies such as stroke and diabetes (Yang et al., 1997; Hirosumi et al., 2002), the JNKs have continued to be considered attractive therapeutic targets through the development of JNK inhibitory molecules.

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The inhibitors of JNKs can be broadly divided into two major groups. The first group, ATP-competitive inhibitors, is typified by the first commercially available inhibitor SP600125 (Bennett et al., 2001). Despite the evidence of its effects to inhibit multiple kinases (Bain et al., 2007; Fabian et al., 2005), SP600125 continues to be used in studies that interrogate the possible contributions by JNKs to activities such as neuronal injury following focal ischemia and reperfusion (Gao et al., 2005), impaired glucose homeostasis (Benzler et al., 2013) as well as altered angiogenesis (Takahashi et al., 2013; Bogoyevitch et al., 2010). The intense interest to develop and improve JNK inhibitors is exemplified by the studies reporting a range of different inhibitor chemotypes. Specifically, the aminopyridine-based JNK inhibitor VIII (N-(4-amino-5-cyano-6-ethoxypyridin-2-yl)-2-(2,5-dimethoxyphenyl) acetamide) shows minimal off-target effects on other kinases tested (Szczepankiewicz et al., 2006), CC-930, an aminopurine JNK inhibitor, is currently in Phase II clinical trial for the treatment of idiopathic pulmonary fibrosis (Krenitsky et al., 2012), a tri-substituted thiophene is a JNK3 inhibitor (Bowers et al., 2011), and novel quinazolines are potent and brain-penetrant JNK inhibitors (He et al., 2011). In some instances, these JNK inhibitors preferentially target JNK1, JNK2 or JNK3 despite the conservation of the ATP-binding site across these isoforms (Song et al., 2011).

The second group of JNK inhibitors, protein substrate-competitive inhibitors, is typified by the small peptide derived from the JIP1 scaffold protein, referred to as JIP1pep or TIJIP (Barr et al., 2002). The structure of the JNK1-TIJIP complex has highlighted the engagement of a docking site some distance from the ATP-binding site of the JNK1 protein (Heo et al., 2004). Targeting this site with small molecules, such as the thiadiazoles (De et al., 2010) or the lignan (−)-zuonin A (Kaoud et al., 2012a,b), has also proven a successful strategy to achieve non-peptide JNK inhibitory molecules. As predicted from extensive biochemical studies, recent structural studies have shown peptides from additional substrates such as ATF2 and Sab engage this docking site of JNK1 (Garai et al., 2012; Laughlin et al., 2012). Overall, there have been increasing interests to identify and exploit new vulnerabilities of the JNKs in the development of novel inhibitors for further therapeutic applications.

In our studies examining JNK inhibitors, we previously identified a novel JNK inhibitory peptide PYC71N that utilized a substrate–inhibitor complex mechanism for inhibition (Ngoei et al., 2011). Our further characterization of PYC71N revealed its potency to inhibit JNK activity toward a range of substrates *in vitro*, however a stabilized retro-inverso form of TAT-PYC71 (*i.e.* a form of the peptide composed of D-amino acids in the reverse sequence to maintain sidechain topology similar to that of the original L-amino acid PYC71 peptide (Fischer, 2003) and conjugated to the TAT cell-permeable peptide sequence (Jarver and Langel, 2006; Bogoyevitch et al., 2002)) was a relatively poor inhibitor of JNK-mediated phosphorylation of c-Jun in cells (Ngoei et al., 2011). With an ultimate aim to identify a novel JNK inhibitor with improved stability in a cellular context, we report our characterization of a peptide originally identified from yeast two-hybrid screening of a biodiverse gene fragment library (Watt, 2006), herein referred to as PYC98. Further investigation of a D-amino acid retro-inverso form of this peptide, D-PYC98, revealed a more potent JNK inhibitory activity than the original L-amino acid sequence. Whilst D-PYC98 inhibited all JNK isoforms, we also noted the striking differences in potency toward inhibition of phosphorylation of different substrates, as well as actions to inhibit the related stress-activated p38α MAPK. Importantly, a cell-permeable, TAT-conjugated form of D-PYC98 inhibited JNK-mediated phosphorylation of c-Jun in cells. Taken together, our studies reveal the novel peptide D-PYC98-TAT as a promising new lead in the development of D-amino acid containing peptide inhibitors of stress-activated MAPKs.

2. Experimental procedures

2.1. JNK1α1, JNK2α2 and related MAPKs

The preparation and purification of full-length wild-type JNK1α1 or JNK2α2 as glutathione S-transferase (GST) fusion proteins using the Sf9 cell/baculovirus system have been described previously (Ngoei et al., 2011). For active JNK1, we included the co-expression of active mutant forms of MKK4 and MKK7 (Ngoei et al., 2011). We also expressed a common docking site double mutant, JNK1 R127A and E329A (JNK1 ERA) (Heo et al., 2004) to evaluate contributions by these residues. GST was removed by cleavage with PreScission Protease (GE Healthcare) prior to further use. The purified recombinant kinases, JNK3α1, ERK2 and p38α, were purchased from Upstate Cell Signaling Solutions/Millipore.

2.2. Expression and preparation of recombinant JNK substrates

The JNK substrates (c-Jun(1-135), ATF2(19–96), Elk1 (307–428), DCX(1–366), SCG10(38–179), and stathmin/Op18(1–149)) were expressed in *Escherichia coli* as GST-fusion proteins and purified using glutathione-sepharose (Ngoei et al., 2011). Protein concentrations and purity were determined using the Bradford Protein Assay and Coomassie-staining of protein samples separated by SDS-PAGE, respectively.

2.3. Peptide sequences and synthesis

The sequences of the JNK inhibitory peptides used in this study are summarized in Table 1. L-PYC98 and its D-amino acid retro-inverso form D-PYC98, were synthesized by Mimotopes (Clayton, Victoria, Australia) as were the derivatives of these peptides used for interaction analyses by surface plasmon resonance (with an N-terminal aminohexanoic acid-biotin residue; b-L-PYC98 and b-D-PYC98, respectively) and in studies for the evaluation of JNK inhibition in cells (with a C-terminal extension to include the cell-permeable TAT-sequence in its D-amino acid retro-inverso form; D-PYC98-TAT). The JNK inhibitory peptide, TIJIP (Barr et al., 2002), was synthesized by Proteomics International (Perth, Western Australia) and TAT-TIJIP was synthesized by Auspep (West Melbourne, Victoria). To minimize error and ensure accuracy, peptides were dispensed by liquid transfer by the manufacturers. The purity of all peptides was determined to be ≥95% by high performance liquid chromatography (HPLC) and mass spectrometry. All peptides were dissolved in dimethyl sulphoxide (DMSO) at room temperature and concentrations confirmed by UV spectrometry. All peptides remained soluble in subsequent assays.

2.4. In vitro activity assays

The activities of JNK isoforms JNK1α1, JNK2α2 and JNK3α1, as well as the related MAPKs p38α and ERK2 were assayed (35 μL final reaction volume) toward recombinant GST-fusion protein substrates (10 μg of transcription factors: c-Jun(1–135), ATF2(19–96), or Elk1(307–428), or microtubule-regulatory proteins: DCX (full length, 1–366), SCG10(38–179) or stathmin/Op18(1–149)). L- or D-PYC98 (0–100 μM as indicated) were included in the reaction buffer (20 mM HEPES, 20 mM β-glycerophosphate, pH 7.6, supplemented with 20 mM $MgCl_2$, 25 μM sodium orthovanadate and 100 μM dithiothreitol). After pre-incubation (5 min) of JNK with its protein substrate and inhibitors, each reaction was initiated by the addition of ATP (5 μM ATP, 1 μCi [γ-^{32}P] ATP) followed by incubation for 20 min at 30 °C. To assess the mechanisms of JNK inhibition by D-PYC98, the concentrations of substrates and inhibitor were varied (0.8–8.1 μM GST-cJun (1–135), 2–20 μM ATP, 0–10 μM D-PYC98).