Inhibition of Inflammation by a p38 MAP Kinase Targeted Cell Permeable Peptide

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Abstract: p38 MAPK has been the key therapeutic target for multiple inflammation diseases. However, the clinical applications of p38 inhibitors, most of which target on the ATP binding groove in the kinase, have been held back, largely because of their limited specificity and severe side-effects. An alternative strategy to generate highly selective p38 inhibitor is to block the specific interaction in the p38 signal pathway. Based on the hypothesis that specific binding peptides targeting on the docking groove would interfere the intrinsic interaction between p38 and its partners, we have designed a fusion peptide containing 12aa p38 docking sequence derived from MKK3b and 11aa HIV-TAT transmembrane sequence to form a cell permeable peptide. The peptide specifically binds to p38, and aborts its interaction with upstream kinase as well as downstream substrates, and thus to inhibit p38 phosphorylation and its signaling. Furthermore, the induction and secretion of TNFα and other inflammatory factors by LPS are blocked in peptide treated cells and mice. Finally the peptide has been shown to significantly inhibit ear oedema in mice. Therefore, the peptide holds great potential as an anti-inflammation agent for the treatment of inflammation and its related diseases.

1. INTRODUCTION

Mitogen-activated protein kinases (MAPKs) play a pivotal role in cellular signal transduction processes, and mediate many physiological and pathological pathways [1,2]. p38α MAPK was originally identified as the target of the pyridinyl imidazole class of anti-inflammatory drugs that block the expression of IL-1β and TNFα in bacterial lipopolysaccharide (LPS)-stimulated human monocytes [3]. It is the best characterized and perhaps the most physiologically relevant kinase involved in inflammatory responses [4]. p38α kinase regulates the biosynthesis of diverse inflammatory mediators including cytokine TNF-α, IL-1β, IL-6, IL10, as well as several inducible effector enzymes such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), at the transcriptional and translational level. As a point of convergence for multiple signaling processes that are activated during inflammation, p38α has been regarded as a key potential target for the modulation of cytokine production [5]. In recent years, huge efforts have been made by many companies to develop p38 inhibitors as potential treatments for inflammatory diseases. Based on the structure of original pyridinyl imidazole inhibitor, chemists have developed additional molecules with higher inhibition specificity and potency towards p38 MAPK. It has been identified that the expression of diverse inflammatory mediators such as TNF α, IL-1β, IL-6, IL10, COX-2 and iNOS could be inhibited to certain extend by p38 targeted chemical compounds. Preclinical studies also showed that these inhibitors were efficacious in animal inflammatory disease models including rheumatoid arthritis, ischaemia-reperfusion injury, inflammatory bowel disease, Crohn's disease, psoriasis, chronic obstructive pulmonary disease, asthma, multiple myelomea, atherosclerosis and related cardiovascular diseases [4,6,7].

Despite the significant therapeutic efficiency of p38 inhibitors in inflammation, their clinical applications were held back because of the serious adverse effects on liver and central nervous system (CNS) [8]. Among all the p38 inhibitors reported so far, each of them targets on the ATP binding site in the kinase. Most molecules compete directly with ATP for its access to ATP binding pocket of p38 that is structurally conserved in all catalytically active kinases [8] and makes the specificity of the ATP-competitive inhibitors rather obscure. It has been shown that some p38 inhibitors also had modest inhibition effects on other kinases, which was recognized as the underlying reason for the side effects of these compounds [4,9-12]. In this regard, new inhibitors that target other specific sites on p38 rather than ATP pocket would be non-ATP-competitive, which might have an improved selectivity profiles and thus with a better application perspective.

The specific interaction between proteins makes the cellular signal transduced precisely. In the case of MAPK modules, each has the specific upstream kinases and downstream substrates. For p38, the upstream kinases are MKK3 and MKK6, and specific substrates include downstream kinases such as MK2, MK3 and PRAK, and transcription factors ATF2, CHOP, MEF2A and MEF2C [13]. Structural study showed that similar p38 docking sites exist in these molecules, which confer the specific interaction. Of all the docking sites identified so far, a basic region and a "φ_A-X-φ_B" hydrophobic motif (φ_A and φ_B are hydrophobic residues Leu, Ile or Val) were proved to be the conservative binding se-

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quences. Interestingly, all the docking sites from different molecules share the same binding grooves in p38, flanked by the α_d and α_e and the reverse turn between β_7-β_8. Similar but non-identical grooves are present in other MAP kinases that could contribute to pathway specificity [14-16]. This offers potential inhibition targets for the specific intervention of p38 signal transduction and further for the therapy of related diseases. It has been shown that the endogenous protein-protein interaction would be selectively blocked by introducing peptide that specifically competes with such interaction. In this regard, specific peptide inhibitors have been reported for JNK and ERK [17-19]. Based on the hypothesis that administration of peptide that recognizes p38 docking site could compete with the interaction of intrinsic binding proteins and p38, and subsequently block the p38 signal module specifically, we designed a synthetic cell permeable peptide. It inhibits the p38 signaling by binding to p38, and blocks LPS induced the expression and secretion of proinflammatory factors *in vitro* and *in vivo*. Thus, the peptide has the potential for the therapy of inflammation and its related diseases.

2. RESULTS

2.1. Determination of Small Peptide Binding to p38

The protein-protein interaction is mainly mediated by very specific amino acid sequences that determine the binding specificity. To study the feasibility of small peptides binding to p38, docking site sequences from MKK3, MKK6, MEF2A and MEF2C [14,15] (Table **1**) were selected and tested. For the convenience of the detection, GST-fusion docking sequences were expressed in *E. Coli.* and purified, respectively. In Elisa assay, p38 protein was equally coated on the plate. GST, GST-fusion docking site peptides or GST-fusion NP60 [20] protein were then used for binding assay. The interaction was detected with GST antibody. All the fusion docking site sequences showed high binding activity towards p38 (Fig. **1A**). Of which, GST-MKK3b exhibited the highest affinity. We then performed pull-down assay to further test the interaction *in vitro*. His-tagged recombinant p38 was incubated with GST or GST-fusion docking site sequences, and the complex was pulled down by Ni-NTA Agarose. It showed similarly that GST-fusion docking sites bond to p38, and the docking sites derived from upstream kinases held higher affinity than those derived from downstream substrates (Fig. **1B**). Considering that similar binding groove exist in MAPKs, the binding specificity of the MKK3b peptide was further examined because of its highest binding efficiency towards p38 among the docking sequences we have tested. Flag-tagged JNK, ERK and p38 plasmids were transfected into 293T cells, respectively. Cells were lysed and the lysates were incubated with GST or GST-MKK3b fusion peptides. The protein complexes were then pulled-down by Glutathion Sepherose 4B. The precipitates were sub-jected to SDS-PAGE followed by Western Blotting analysis. MAPKs were detected by anti-flag antibody. As shown in Fig. (**1C**), GST-MKK3b pulled down only p38 MAPK, but not JNK or ERK.

Table 1. Docking Site Peptide Sequences

	Binding Sequence
MEF2A	RKPDLRVVIPPSS
MEF2C	RKPDLRVLIPPGS
MKK3b	GKSKRKKDLRISCNS
MKK6	SKGKKRNPGLKIPKEA

The p38 binding specificity of tested docking site sequences prompts us to address whether they are able to inhibit intrinsic activity of p38 through the competition mechanism. Since the upstream kinases and downstream substrates share the same binding groove in p38, GST fusion docking site sequences from MKK3b, MKK6, MEF2A and MEF2C were used for the test. Upstream kinases or downstream substrates of p38 were incubated with p38 in the presence of GST or GST-fusion peptides for *in vitro* kinase assay. The reaction complex was resolved on SDS-PAGE followed by Western Blotting with anti-phospho-p38 antibody or anti-phospho-ATF2 antibody, respectively. It showed that phosphorylation of p38 by upstream kinase MKK6 was inhibited by docking sequences from upstream kinases (Fig. **1D**) that also inhibited the phosphorylation of ATF2 by p38 (Fig. **1E**). The docking sequences from downstream molecules of p38 showed only weak effect. These results suggest that all the docking sequences could specifically bind to p38, and the sequences from upstream kinases exhibits the inhibitory effect on the interaction between endogenous proteins and p38. MKK3b docking sequence showed the strongest inhibitory effect among tested docking sites.

2.2. TAT-MKK3b Fusion Peptide is Cell Permeable and Selectively Inhibits the p38 Pathway

12aa within MKK3b docking site was selected as inhibitory peptide because of its highest affinity and strongest inhibition activity towards p38. To allow for efficient entry into cells, a transmembrane sequence "YGRKKRRQRRR" from HIV-I TAT protein was linked to the N-terminal of 12aa peptide and thus to form the fusion peptide TAT-MKK3b. Fluorescein-labelled TAT-MKK3b peptide was prepared to monitor the cell permeable efficiency. As shown in Fig. (**2A**), the fluorescent signal was visualized within the cell in 30 min when RAW264.7 cells were cultured in the presence of labeled fusion peptide. The signal kept stable at least for 90 min. To evaluate the binding affinity of fusion peptide, surface plasmon resonance (SPR) was performed. It showed that the TAT-MKK3b fusion peptide bond to p38 effectively with a Kd approximately 21.7 nM (Fig. **2B**), indicating that fusion peptide binding to p38 is relatively strong. TAT peptide alone did not show any binding affinity (data not shown). We next tested the inhibitory effect and specificity of fusion peptide on p38 activity *in vitro*. It showed that the phosphorylation of ATF2 by p38 was efficiently inhibited with the increase of TAT-MKK3b, with the IC_{50}=3.5 μM (Fig. **2C**). The inhibition was p38 specific, since TAT-MKK3b fusion peptide had no effect on the phosphorylation of ATF2 by JNK (Fig. **2D**), and did not affect the phosphorylation of ERK by its upstream kinase even at 20 μM (Fig. **2E**).

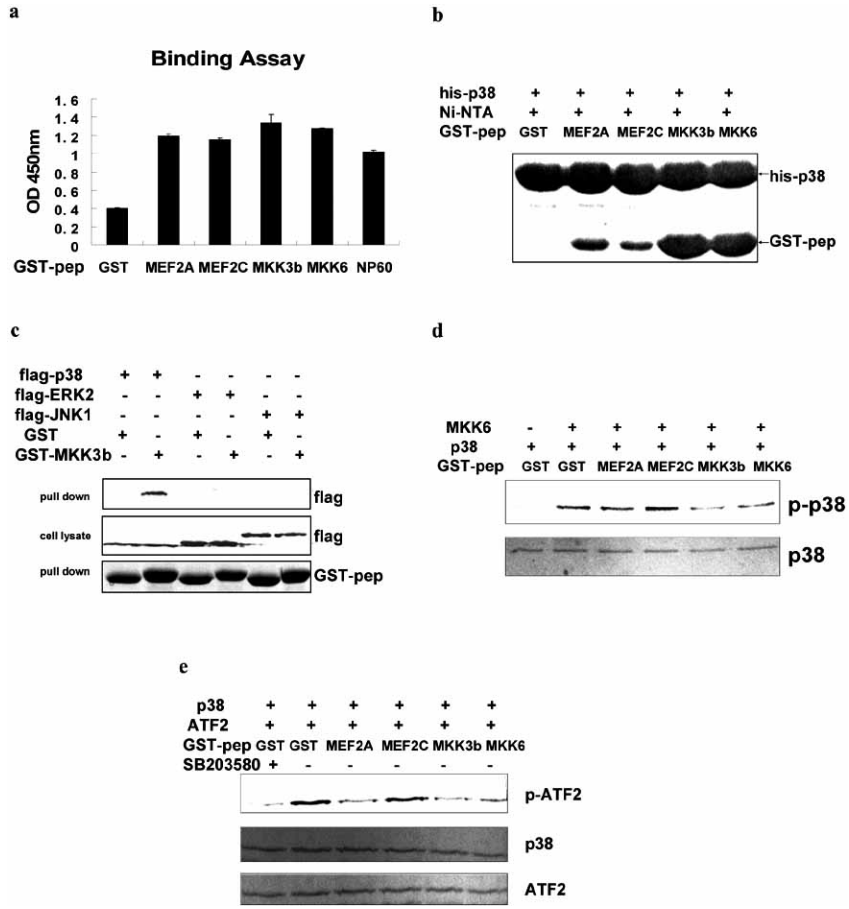

Fig. (1). Determination of small peptide binding to p38. (A) GST-fusion docking site of MEF2A, MEF2C, MKK3b and MKK6 binds to p38.1 μg purified GST, GST-fusion peptides were coated on 96 well plates respectively, and incubated with 0.1 μg purified his-p38. The interaction was determined by Elisa protocol. **(B)** 5 μg purified GST or GST-fusion peptides were incubated with his-p38. The reaction complex was then pulled down with Ni-NTA Agarose, and subjected to SDS-PAGE. Gels were dyed by Coomassie Brilliant Blue G-250. **(C)** GST-MKK3b peptide specifically binds to p38, not JNK or ERK. Flag-tagged p38, JNK1 and ERK2 were over-expressed in 293T cell. 2 μg purified GST or GST-MKK3b were incubated with the 293T cell lysates, and pulled down by Glutathione Sepherose 4B. The precipitation was examined by flag antibody. Gels were dyed with Coomassie Brilliant Blue G-250 for the determination of GST-peptides. **(D)** and **(E)**, Comparison of p38 inhibition effects of GST-fusion peptides. **(D)** MKK6 and p38 were incubated in kinase buffer with equal concentration of GST or GST-fusion peptides. The phosphorylation of p38 was detected with anti-phospho-p38 antibody. **(E)** Similarly, p38 and ATF2 were incubated with GST, GST-fusion peptides or SB203580, and the phosphorylation of ATF2 was detected by anti-phospho-ATF2 antibody.

It has been well known that endotoxin activates MAPKs, including p38, JNK and ERK. We then address whether the fusion peptide inhibits activation of p38 specifically. Cells were exposed to TAT peptide or TAT-MKK3b peptide for 30 min prior to LPS stimulation, and collected after the treatment. Lysates were applied to Western Blotting. It showed that the phosphorylation of p38 induced by LPS was efficiently inhibited in the presence of TAT-MKK3b peptide in a dose dependent way. The inhibition was p38 specific since TAT-MKK3b peptide did not affect the activation of JNK and ERK (Fig. **2F**).

2.3. TAT-MKK3b Fusion Peptide Blocks the Induction and Secretion of Inflammatory Factors in LPS Stimulated RAW264.7 Cells and Mice, and Inhibits Ear Oemeda

Since LPS can activate p38 and further lead to the induction and secretion of proinflammatory factors such as TNFα, IL-1β, IL-6, IL-10, COX-2 and iNOS in macrophages, we next address whether the inhibition of p38 by the fusion peptide could have the effects on the expression of inflammatory factors induced by LPS. RAW264.7 cell was pre-incubated with indicated concentration of TAT, TAT-MKK3b peptide or ATP-competitive inhibitor SB203580 for 30 min, and then subjected to LPS challenge for 4 or 8 hours. The induction of gene expression was determined by RT-PCR. As shown in Fig. (**3A**), LPS induced the expression of TNFα, IL1-β, IL-6, IL-10, COX-2 and iNOS, although RAW264.7 showed different basal level of these gene expressions. The induction was all inhibited in cells treated with TAT-MKK3b peptide. The inhibitory strength is comparable with SB203580. LPS induced secretion of TNFα was also blocked by TAT-MKK3b peptide dose-dependently (Fig. **3B**). TAT peptide did not show significant effect on the inhibition. We next examined the inhibitory effect *in vivo*. Previous experiment showed that serum TNFα could be elevated 1 hour after i.p.

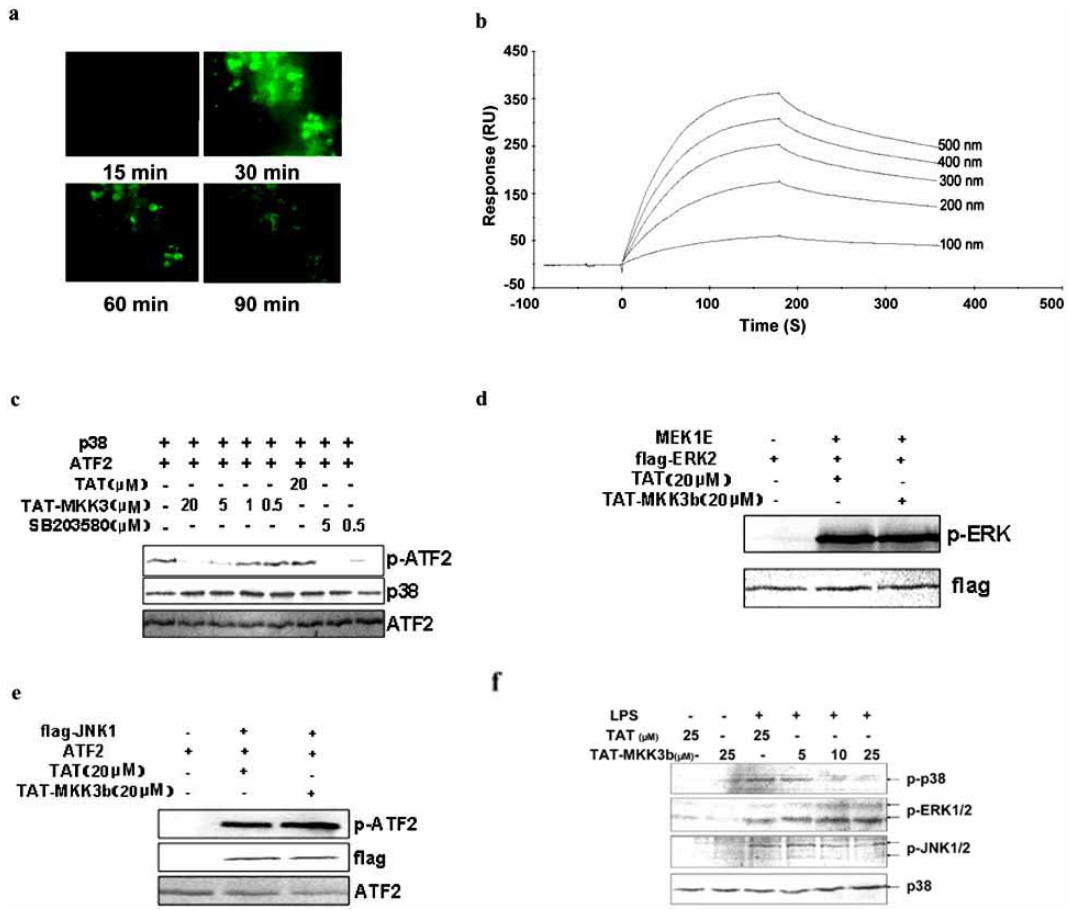

Fig. (2). TAT-MKK3b peptide selectively blocked p38 pathway and LPS induced inflammatory factors *in vitro*. (A) Cell-penetrating efficiency of TAT-MKK3b-peptide. RAW264.7 cells were incubated with 1 μM Fluorescein-TAT-MKK3b peptide for the indicated times and were visualized under confocal scanning laser microscope. **(B)** Determination of the binding affinity of TAT-MKK3b peptide to p38. Surface plasmon resonance experiment was performed as indicated in methods. **(C)**, **(D)** and **(E)** The specificity of the inhibition of p38 activity by fusion peptide *in vitro*. **(C)** 0.2 μg p38 and 2 μg ATF2 were incubated in kinase buffer with indicated concentration of TAT, TAT-MKK3b peptide or SB203580. Phosphorylation of ATF2 was detected by immunoblotting with anti-phospho-ATF2. **(D)** 293T cells were transfected with flag-ERK2 and expressed overnight. Cells were lysed and ERK2 was immunoprecipitated by flag antibody and protein A/G Agarose. ERK2 was then incubated with MEK1E in kinase buffer in the presence of 20 μM TAT or TAT-MKK3b peptide. Phosphorylated ERK2 and total ERK2 were detected by anti-phospho-ERK and flag separately. **(E)** Flag-JNK1 was expressed in 293T cells and immunoprecipitated by flag antibody. JNK1 and GST-ATF2 (1-109) were then incubated in kinase buffer with TAT or TAT-MKK3b peptide. Phosphorylation of ATF2 and total JNK1 were detected by anti-phospho-ATF2 or flag antibody individually. Gels were dyed with Coomassie Brilliant Blue G-250 for the determination of total ATF2. **(F)** TAT-MKK3b peptide specifically inhibited LPS induced p38 phosphorylation. RAW264.7 cells were incubated with indicated concentration of TAT or TAT-MKK3b peptide for 30 min, and stimulated with 1 μg/mL LPS for 30 min. Antibodies for phospho-p38, phospho-JNK, phospho-ERK and p38 were used for the detection of phosphorylation of MAPKs and total p38 protein individually.

administration with 1.25 mg/kg LPS in BALB/C mice[21]. Such an animal model was used. It showed that TNFα serum levels was reduced to 66% in BALB/C mice administrated 30 min prior to LPS injection with 25 mg/kg TAT-MKK3b peptide compared with the TAT treatment (Fig. **3C**). As the same as in Fig. (**3B**), TAT peptide itself did not show significant effect in *in vivo* assay. To further evaluate the feasibility of the fusion peptide as an anti-inflammatory drug, animal model for ear oedema has been established and used for test. It showed that 12.5 mg/kg dose inhibited oedema by 44%, and 25 mg/kg dose inhibited by 62.3%, which is almost the same as Indomethacin did (Fig. **3D**). The transmembrane sequence TAT did not show any inhibitory effect, indicating the inhibition of oedema results from the sequence of MKK3b.

3. DISCUSSION

The central role of p38 MAPK in regulation of induction as well as cellular response of diverse inflammation mediators makes it potential target for anti-inflammatory therapeutics [4,6,8]. Based on the structure of the first p38 inhibitor SB203580, diverse substitution or alteration have been initiated by chemists from multiple pharmaceutical companies in the hope of developing more potent, selective, efficacious and safe p38 inhibitors for clinic application. However, due to the cross-reaction with other kinases, few of these compounds can be used in the therapy because of their serious adverse effects [5,8,11]. We here report a novel potent and cell-permeable peptide inhibitor specifically for p38. It derives from p38 upstream kinase MKK3.

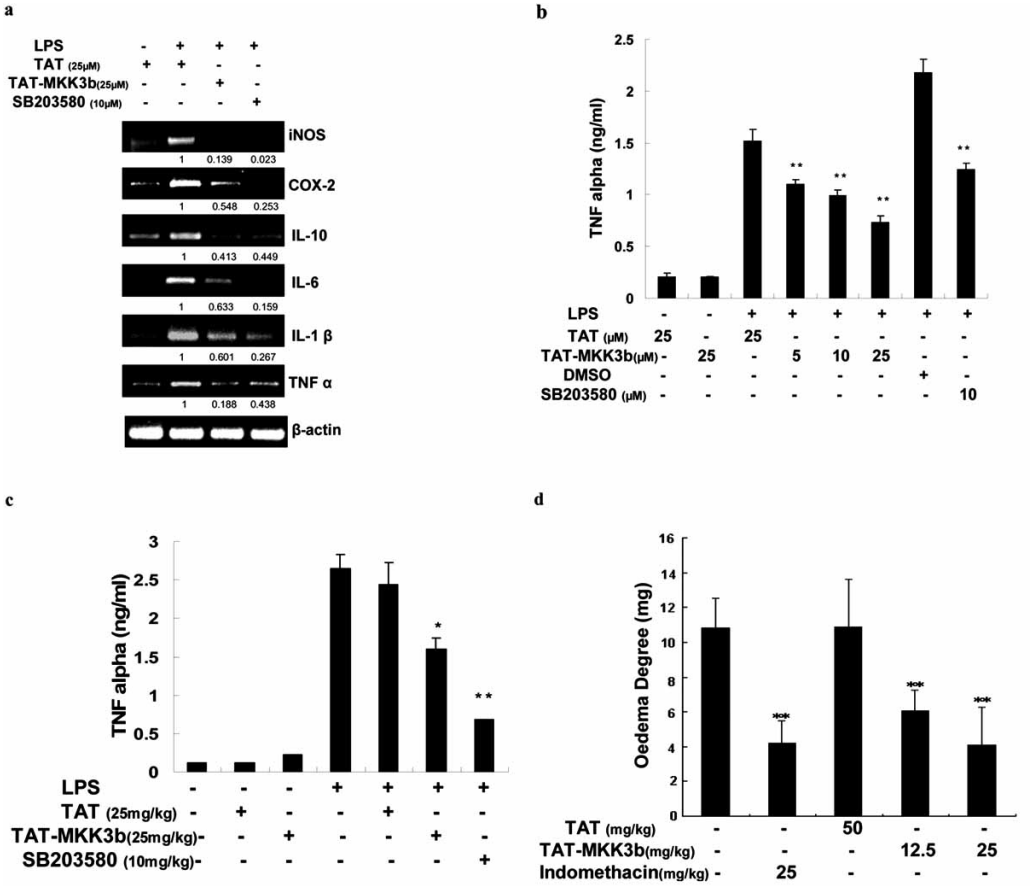

Fig. (3). TAT-MKK3b peptide inhibited LPS induced inflammatory factors in RAW264.7 cells and mice. (A) TAT-MKK3b peptide inhibited LPS induced inflammatory factors. RAW 264.7 cells were incubated with TAT (25 μM), TAT-MKK3b (25 μM) or SB203580 (10 μM) for 30 min, followed by the treatment with 1 μg/mL LPS for 8 hours. The expression of inflammatory factors was detected by RT-PCR, respectively. Densitometric comparison of every gene in relation with β-actin was analyzed with Bandscan V5.0, and the relative value of TAT-MKK3b and SB 203580 treatment against TAT control is shown. **(B)** TAT-MKK3b peptide blocks TNFα secretion in LPS treated RAW 264.7 cells. RAW 264.7 cells were incubated with indicated concentration of TAT, TAT-MKK3b, DMSO or SB203580 for 30 min, followed by the treatment of LPS (1 μg/mL) for 6 hours. Cell medium were collected and TNFα was determined by Elisa Kit. **(C)** TAT-MKK3b peptide blocks the secretion of TNFα in mice. Male BALB/C mice (20±1 g) were i.p. injected with equal volume of sterile water, TAT (25 mg/kg) or TAT-MKK3b (25 mg/kg) for 30 min, followed by i.p. administration with 1.25 mg/kg LPS. 90 mins later, mice were anaesthetised and serum was collect individually. Serum TNFα was determined by Elisa Kit. **(D)** Effect of TAT-MKK3b on xylene-induced ear oedema in mice. Different doses of peptide were i.p injected to mice with ear oedema as indicated. The inhibition rate was evaulated according to the method.

Similar but not identical docking grooves are present in JNKs, ERKs, ERK5 and p38, which makes the pathway specificity. Sequence alignment and crystallographic comparison of docking grooves have shown the difference in binding residues and spatial structure among MAPKs, which partially assure the binding fidelity [14]. However, the specificity of docking-site-mediated interactions is rather limited in some instances. For example, the docking site peptides from MEK1 and MEK2 do not bind to JNK, and not appreciably inhibit JNK-dependent reaction, but the MKK4 docking sites peptide bind to ERK2 and effectively inhibit ERK2-dependent reactions [22]. We have evaluated the specificity and efficacy of docking site peptide from MKK3b as a p38 inhibitor. Results show that the MKK3b peptide does not bind to ERK or JNK, and has no obvious inhibition effect on ERK- or JNK-dependent activity *in vitro* (Fig. **1C**, **2D** and **2E**). In LPS stimulated RAW264.7 cells, it selectively blocks the activation of p38, not the activation of JNK or ERK (Fig. **2F**). These data strongly demonstrate that the peptide derived from MKK3b docking site is a specific inhibitor for p38.

All chemical inhibitors of p38 bind its ATP-binding pocket and inhibit the kinase activity by competing with ATP binding directly or indirectly. Therefore, their inhibition selectivity are rather limited, as similar ATP-binding pocket exist in almost all catalytically active kinases, and this has been recognized as the underlying reason for the adverse effects of these compounds related to p38 inhibition. Diaryl urea compounds BIRB796, for example, the most potent one among the well known p38 inhibitors, can inhibit all the p38 isoforms with picomolar affinity and low nanomolar inhibitory activity in cell culture. However, it is found to affect the kinase activity of JNK2, RSK1, Lck [23]. The clinical studies have been stopped at phase II, because it causes liver enzymes elevations [8]. To solve this problem, it is suggested that inhibitors that target specific sites on p38 rather

than ATP pocket would not likely to inhibit other kinases and might have an improved selectivity profiles and thus better application perspective. Based on this notion, MKK3b docking sequence has been selected for the evaluation as a potential inhibitor of p38. By covalent linkage with 11-amino acid HIV-TAT sequence that directs cellular import in cells and animals [24], TAT-MKK3b-peptide holds high efficiency to penetrate into cells (Fig. **2A**) and specifically inhibits LPS induced p38 activation (Fig. **2F**). Furthermore, it blocks the secretion and expression of LPS induced inflammatory factors including TNFα, IL-1β, IL-6, COX-2, iNOS and the anti-inflammatory cytokine IL-10 (Fig. **3A, B**). In endotoxin shocked BALB/C mice, i.p. injected TAT-MKK3b-peptide inhibits the secretion of serum TNFα in LPS stimulated mice (Fig. **3C**). Finally, it inhibits ear oedema in mice (Fig **3D**). The efficacy is almost the same as Indomethacin at dose of 25 mg/kg. TAT peptide does not have any effect on the inhibition activity even at high dose of 50 mg/kg. Although the effective dose of the peptide seems slight higher, it is possible to minimize the dose by peptide modification or using delivery carriers that have been developed rapidly in recent years.

4. CONCLUSIONS

TAT-MKK3b fusion peptide is a novel cell-permeable p38 peptide inhibitor with a potent anti-inflammatory effect *in vitro* and *in vivo*. It targets on the unique docking groove in p38 rather than the ATP-binding pocket, which makes it specific for p38 only and may avoid the adverse effects that have caused clinical attrition of all the reported p38 inhibitors. Thus, TAT-MKK3b peptide represents a new class of p38 inhibitors with improved specificity and selectivity, hence better prospects for clinical application to treat inflammation and inflammation related diseases, such as oedema.

5. EXPERIMENTAL SECTION

5.1. Materials

TAT peptide (YGRKKRRQRRR) and TAT-MKK3b peptide (YGRKKRRQRRRGKGKSKRKKDLRI) were commercially synthesized by GL Biochem (Shanghai) with purity above 95%. Fluorescein-TAT-MKK3b was synthesized by Sangon Company (Shanghai).

5.2. Animal and Treatment

Male 20±1 g BALB/C mice, 8 to 10 weeks old were housed under controlled conditions and a 12-h day/night cycle. Experiments with animals were approved by and conformed to the guidelines of the Peking University Health Science Center for animal experimentation. Mice were i.p. administrated 25 mg/kg TAT, TAT-MKK3b-pep or equal volume sterile water 30 min before i.p. injection of 25 μg/mouse LPS (*E. coli* 055:B5). 90 min later, the animals were anaesthetized by trichloroacetaldehyde monohydrate and serum was obtained from individual mouse. The serum was detected by Elisa Kit for TNF-α (Boster Biological Technology). Xylene-induced ear oedema animal model was made according to the literature [25]. Briefly, six groups of male Kunming mice (17-22 g) (Institute of Laboratory Animal Sciences, CAMS & PUMC, China) in ten each were used. Normal saline was given as a control group. Mice for positive control group were administrated orally a suspension of Indomethacin (Hebei Yongfen Pharmaceutical Co., Ltd, China) in normal saline at a dose of 25 mg/kg. TAT at a dose of 50 mg/kg and TAT-MKK3b at doses of 25, 12.5 mg/kg were administered to the test groups by i.p. injection, respectively. Thirty minutes later, the anterior and posterior surfaces of the right ears of mice both received 20 μL of xylene by topical application. The left ear was considered as control. Two hours after xylene was applied to the right ears, the mice were sacrificed by cervical dislocation and both ears were dissected. 8 mm punches were made in the right and the left ears by the borer. Each ear disc was weighed and the differences in weights of the right and left ear discs of mice were recorded as oedema level.

5.3. Cell Culture and Treatment

RAW264.7 cells and 293T cells were cultured in RPMI 1640 medium and Dulbecco's modified Eagle's medium (DMEM) (Hyclone) respectively, supplemented with 10% fetal calf serum, 10 mM L-Glutamine, penicillin(100 U/mL), and streptomycin (0.1 mg/mL) (Gibco) at 5% CO_2 and 37 ^{o}C. For 293T cell, fresh medium was added 4 hours before transfection, which was performed using the standard calcium phosphate precipitation method [26]. TAT or TAT-MKK3b peptide was added to the medium with the indicated concentration 30 min before LPS (1 μg/mL) stimulation. 30 min after LPS treatment, RAW 264.7 cells were lysed, and total cell extracts were subjected by SDS-PAGE. Phosphorylation of MAPKs was detected by western blotting with phospho-p38, phospho-ERK or phospho-JNK antibodies (Cell Signaling Technology, MA). p38 monoclonal antibody (CST, MA) was also used in the western blotting for the detection of total p38 protein.

For the inhibitory effect of TAT-MKK3b peptide on the induction and secretion of inflammatory factors, TAT, TAT-MKK3b peptide or SB203580 was added into the RAW264.7 cell medium with the indicated concentration 30 min before LPS (1 μg/mL) (Sigma, St. Louis, MO) stimulation. Cells were collected after incubation for 4 or 8 hours. Total RNA was isolated by Trizol Reagents (Invitrogen, Rockville, MA). cDNA was prepared by reverse transcription with SuperScript III (Invitrogen). The primers used for the detection of gene transcripts are as follows: iNOS: forward primer "gtccgaagcaaacatcacat", reverse primer "tctgcctatccgtctcgtc"; COX-2: forward primer "acctctgagatgctcttc", reverse primer "acactctgttgtgctccc"; IL-10: forward primer "acctggtagaagtgatgc", reverse primer "aaggagttgtttccgtta"; IL-6: forward primer "ggaaatcgtggaaatgag", reverse primer "gcttaggcataac gcact"; IL-1β: forward primer "cctgtggccttgggcctcaa", reverse primer "ggtgctgatgtaccagttggg"; TNFα: forward primer "tcgtagcaaaccaccaag", reverse primer "caatgactccaaagtagacc"; β-actin: forward primer "cactgtgcccatctacga", reverse primer "acaggattccatacccaag".

For TNFα secretion assay, RAW264.7 cells were cultured in 48-well cell flask. Before peptide treatment, cell medium was changed into 150 μL fresh RPMI 1640 medium and treated as above. Cell medium were collected after LPS stimulation for 6 hrs and used for the detection of TNFα with Elisa Kit (Boster Biological Technology).

5.4. Elisa Assay for Protein Interaction

1 μg purified, bacterially expressed GST, GST-MEF2A, GST-MEF2C, GST-MKK3b, GST-MKK6 and GST-NP60 [20] were diluted into 100 μL PBS, PH7.6 and coated on 96-well plates for 2 hours at 37 ^{o}C. Non-specific binding was blocked by adding 200 μL of 2% BSA/PBST (0.1% Tween 20 in PBS, PH7.6) for 1 hour at 37 ^{o}C followed by adding 0.1 μg purified his-p38 diluted in 100 μL of 2% BSA/PBST, and incubated at 37 ^{o}C for 2 hours. The detection of the binding activity of p38 was measured as previously described [20].

5.5. Protein Kinase Assay

0.2 μg purified his-p38 and 4 ng MKK6 were incubated with indicated concentration of peptides in 30 μL kinase reaction buffer(25 mM Tris, 10 mM $MgCl_2$, 5 mM β-Glycerphosphate, 0.1 mM Na_3VO_4, 2 mM DTT, 250 μM ATP, PH7.6) for 30 min at 37 ^{o}C. The reaction was then stopped by adding Lammeli buffer. The complex was resolved by SDS-PAGE, and the phosphorylation of p38 was analyzed by western blotting with phospho-p38 antibody. Similarly for p38 kinase activity assay, 2 μg purified GST-ATF2 and 0.2 μg his-p38 were incubated in kinase buffer with peptides. The phosphorylation of ATF2 was detected by western blotting with phospho-ATF2 antibody. Gels were stained with Coomassie Brilliant Blue G-250 for the detection of total proteins loaded.

For ERK2 kinase assay, cell extracts from Flag-ERK2 transfected 293T cell were immunoprecipitated with flag antibody and Protein A/G Agarose, and the precipitates were washed twice with kinase buffer without ATP. Beads were divided equally into three tubes, and the reaction were initiated by adding 0.3 U MEK1E (Upstate) and indicated concentration of TAT or TAT-MKK3b-pep in the presence of 250 μM ATP. The phosphorylation of ERK2 was detected by western blotting with phospho-ERK antibody. For the assay of JNK1 activity, Flag-JNK1 were collected as previously, and initiated by adding 2 μg ATF2 (1-109) and indicated concentration of TAT or TAT-MKK3b-pep in the presence of 250 μM ATP. The phosphorylation of ATF2 was detected by western blotting with phospho-ATF2 antibody.

5.6. Surface Plasmon Resonance (SPR) Experiment

Surface plasmon resonance experiments were performed using the BIAcore3000 system (Biacore AB) to detect the interactions between TAT-MKK3b and p38α, as well as to determine the thermodynamic constants. TAT-MKK3b was diluted in sodium phosphate buffer (pH 7.3) and immobilized onto a BIAcore sensor chip CM5 (Biacore AB) by amine coupling as recommended by the manufacturer. The final TAT-MKK3b concentration was determined to be 40 nM. p38α with various concentrations (100, 200, 300, 400 and 500 nM) were dissolved in the elution buffer containing 10 mM HEPES, 100 mM NaCl, 3 mM EDTA, 0.05% Tween-20 (PH7.4) for binding analysis by SPR. Following analyte binding, surface was regenerated with 1 M NaCl and 50 mM NaOH. The measurements were performed at 25 °C, with a flow rate of 30 μL/min. The apparent equilibrium dissociation constant KD was determined by curve fitting of the 1:1 binding with mass transfer and drifting baseline using BIAevaluation Version 4.1 software (Biacore AB).

5.7. Statistical Analysis

Results were presented as the means ± S.E.M. Data had been statistically analyzed with a one-way ANOVA analysis of variance followed by the LSD post hoc test. Differences between groups with P-values less than 0.05 were considered to be significant; P-values less than 0.01 were considered to be extremely significant.

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ABBREVIATIONS

ATF2	=	Activating transcription factor 2
COX-2	=	Cyclo-oxygenase-2
ERK	=	Extracellular regulated kinase
GST	=	Glutathione S transferase
IL-1 β	=	Interleukin-1 β
iNOS	=	Inducible nitric oxide synthase
JNK	=	c-Jun NH2-terminal kinase
LPS	=	Lipopolysaccharide
MAPK	=	Mitogen-activated protein kinase
MEF2	=	Myocyte enhancer factor 2
MKK	=	MAP kinase kinase
SPR	=	Surface plasmon resonance
TAT	=	HIV-1 transactivator protein
TNFα	=	Tumor necrosis factor α

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