Anti-inflammatory effect of BMP326, a novel benzothiazole derivative: Possible involvement of the NF-κB and MAPKs Signaling Pathways in LPS-induced RAW264.7 macrophages

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Abstract

Background: Benzothiazole and its derivatives have been extensive studied due to their versatile biological properties and pharmaceutical applications. We recently found that the BMP326 (1-(1,3-benzothiazol-2-yl)-3-(2-methoxyphenyl)-1H-pyrazol-5-ol), a novel benzothiazole derivative, have anti-inflammatory properties in lipopolysaccharide(LPS)-induced RAW264.7 macrophages and there were no relevant reports previously. Methods and Results: Treatment with BMP326(5, 10 and 20 μM) can significantly inhibit nitric oxide production and down-regulate mRNA expression of nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) but did not cause significant cytotoxicity on RAW264.7 macrophages. It is also observed BMP326 can inhibit LPS-stimulated interleukin (IL)-6, interleukin(IL)-1β, tumor necrosis factor (TNF)-α production depending on its dosage. The gene transcription levels of IL-6, IL-1β and TNF-α were reduced under BMP326 exposure in LPS-treated RAW264.7 cells. In addition, we explored the inhibitory mechanisms of BMP326 on the production of pro-inflammatory mediators. The results showed that BMP326 inhibited nuclear factor kappa B (NF-κB) activation by reducing the phosphorylation of p65 and IκBα. Moreover, the phosphorylation of p38 MAPK (p38), extracellular signal-regulated kinases 1/2 (ERK1/2), and c-Jun N-terminal kinase (JNK) in RAW264.7 cells which are stimulated with LPS were suppressed in a dose-dependent manner. Conclusions: In summary, these results suggest that BMP326 exerts anti-inflammatory properties via suppression of the NF-κB and MAPKs signaling pathways.

Αντι-ινφλαμματορψ εφφεςτ οφ ΒΜΠ326, α νοελ βενζοτηιαζολε δεριατιε: Ποσσιβλε ινολεμεντ οφ τηε ΝΦ-κΒ ανδ ΜΑΠΚς Σιγναλινγ Πατηωαψς ιν ΛΠΣ-ινδυςεδ ΡΑΩ264.7 μαςροπηαγες

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Results: Treatment with BMP326(5, 10 and 20 μM) can significantly inhibit nitric oxide production and down-regulate mRNA expression of nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) but did not cause significant cytotoxicity on RAW264.7 macrophages. It is also observed BMP326 can inhibit LPS-stimulated interleukin (IL)-6, interleukin(IL)-1β, tumor necrosis factor (TNF)-α production depending on its dosage. The gene transcription levels of IL-6, IL-1β and TNF-α were reduced under BMP326 exposure in LPS-treated RAW264.7 cells. In addition, we explored the inhibitory mechanisms of BMP326 on the production of pro-inflammatory mediators. The results showed that BMP326 inhibited nuclear factor kappa B (NF-κB) activation by reducing the phosphorylation of p65 and IκBα. Moreover, the phosphorylation of p38 MAPK (p38), extracellular signal-regulated kinases 1/2 (ERK1/2), and c-Jun N-terminal kinase (JNK) in RAW264.7 cells which are stimulated with LPS were suppressed in a dose-dependent manner.

Conclusions: In summary, these results suggest that BMP326 exerts anti-inflammatory properties via suppression of the NF-κB and MAPKs signaling pathways.

Keywords: BMP326; LPS-induced RAW264.7 macrophages; NF-κB ; MAPKs;

Background

Inflammation is an essential biological protective mechanism against various foreign infection or physical damage. The key features are redness, heat, swelling, pain and impaired function [1].Macrophages as the vital immune cells play an important role in host defense through producing inflammatory mediators [2]. However, the chronic inflammation characterized by prolonged overproduction of inflammatory mediators may aggravate several system disorder and diseases such as rheumatoid arthritis[3], cancer[4], bacterial sepsis[5],type 2 diabetes[6], hearing loss[7], heart failure[8] and autoimmune diseases[9]. Anti-inflammatory drugs are widely used in the clinic, but many drugs are notoriously known for their side effects or individual differences among patients. Therefore, it is imperative to explore a novel low toxicity and effective anti-inflammation drug to relieve patient pain.

Lipopolysaccharide (LPS), the structural component of Gram-negative bacteria's outer membrane, can initiate the inflammatory cascades via Toll-like receptors 4 (TLR4) in macrophages. LPS stimulated RAW264.7macrophages that producing inflammatory cytokines as an inflammatory model that is extensively used [10]. Nuclear factor-κB (NF-κB) activation and Mitogen-activated protein kinases (MAPKs) pathway has been recognized as the classical signaling pathway that involved in the inflammatory response. MAPKs (Mitogen-activated protein kinases) contain three families (extracellular signal-regulated kinase [ERK]1/2, p38, and c-Jun N-terminal kinase [JNK]).Some or even all of them are involved in regulating of the pro-inflammatory cytokine production[11].Furthermore, activated NF-κB as one of the most important regulators reacts to the extra-and intra-cellular stimuli through up regulating the expression of various pro-inflammatory mediators[12]. There have been many reports about these two prototypical pro-inflammatory signaling pathways [13, 14].

Benzothiazole is an aromatic heterocyclic compound which has been researched wildly due to its extensive pharmaceutical applications. Benzothiazole and its derivatives show a wide range of biological activities, such as anticancer[15-17], anti-inflammatory[18-20], immunosuppressive[21,22], antitubercular[23], antidiabetic[24], antimicrobial[25], analgesic[26], antiviral[27], antimalarial [28], anticonvulsant[29]and other activities[30].Some drugs derived from benzothiazole have been used in clinical treatment of various diseases, e.g ethoxzolomide serves as diuretic. Frentizole is used as an antiviral as well as immunosuppressive agent. Tiaramide is an Anti-inflammatory agent. Zopolrestat is an important drug with anti diabetic effect[31]. Meanwhile, some Benzothiazole derivatives like Pramipexole and Riluzoleare still in clinical trials[32]. To discover new derivatives of benzothiazole with anti-inflammatory activity, a series of benzothiazole compounds were synthesized and screened. We found that BMP326 is one of the most effective and no related

preceding research is observed.

In this study, we indicate the anti-inflammatory effects and investigate the possible mechanism underlying the action of BMP326in LPS-induced RAW264.7 macrophages. It inhibited the production of inflammation medium (NO, IL-6, IL-1β, TNFα) and decreased the gene transcription levels of iNOS, IL-6, IL-1β and TNF-α in LPS-treated RAW264.7 cells. The mechanism of its anti-inflammatory effects of BMP326 is involved in the NF-ϰB and MAPKs signaling pathways. Potentially, BMP326 may be used as a lead compound for design and development of new anti-inflammatory drugs for therapeutic use in inflammatory diseases.

Materials and methods

Reagents and Antibody

BMP326 (Fig.1a) was purchased from ChemBridge Corp (San Diego, CA, USA). We dissolved it in dimethyl sulfoxide (DMSO) and stored in -80 at a concentration of 40mM. Lipopolysaccharide (LPS, E.coli O55:B5) was purchased from Sigma–Aldrich and which was dissolved in PBS as a 1μg/ml stock solution. The following rabbit monoclonal antibodies for western blot assay were purchased from Cell Signaling Technology: anti-phospho-p65, anti-p65, anti-phospho-IϰBα, anti- IϰBα, anti-phospho-p38MAPK, anti-p38, anti-phospho-Erk1/2, anti-Erk1/2, anti-phospho-JNK, anti-JNK, anti-GAPDH and HRP-goat anti-Rabbit IgG. The antibodies for ELISA assays were purchased from eBioscience, include mouse TNF-α, IL-1β, IL-6 and HRP-linked antibody.

Cell culture

The murine macrophage cell line RAW264.7 was obtained from Key Laboratory of Biotherapy (Chengdu Sichuan University, China) and cultured in RPMI1640 (SH30809.01B, Hyclone) supplemented with 10% fetal bovine serum (FBS) (SFBS, BOVOGEN) at 37 in a 5% CO2 incubator. Cells were routine sub-cultured after reaching 80%–90% confluence.

Cell viability assay

To evaluate the cytotoxicity of BMP326 on RAW264.7 macrophages, the cell viability was determined by using the Cell Counting Kit (Dojingdo, Kumamoto, Japan). Briefly, Raw264.7 macrophages were seeded in 96-well plates at a density of 1×10^5cells/well and incubated at 37overnight. Then the cells were treated with or without BMP326 (0-40μM) at different concentrations for 24 h. For additional incubation, CCK-8(10 μl/well) solution was added to the wells and incubated for another 4 h. The absorbance was measured at a wavelength of 450 nm using SpectraMax M5 microplate reader (Sunnyvale, CA, USA).

Measurement of NO production

TheGriess Reagent Kit (Beyotime, Shanghai, China) was used to detect the NO production according to the manufacturer's instructions. Briefly, Raw264.7 macrophages were seeded in 96-well plates at a density of 1×10^5 cells/well and incubated at 37 overnight. Cells were treated with different concentrations of BMP326 (0-20μM) for 3 h then added LPS (100 ng/ml) for another 24 h. Subsequently culture supernatant was collected and mixed with Griess reagent. The absorbance was measured at a wavelength of 540 nm by using microplate reader. Obtain the output of NO produced through the standard curve.

Quantitative Real-Time polymerase chain reaction

RAW264.7 macrophages were seeded in 6-well plates at a density of 1×10^6 cells/well and incubated at 37 overnight. Cells were pretreated with BMP326(0-20μM) for 3 h prior to adding 100 ng/ml LPS for another 24 h. Total RNA were extracted by Trizol Reagent (TaKaRa, Japan) according to the manufacturer's protocol. Concentration and purity of total RNA were measured with NanoDrop 2000 Spectrophotometer (Thermo scientific, Wilmington , DE, USA).1μg total RNA was reverse transcribed to cDNA following the PrimeScript RT reagent kit (TaKaRa). Universal Probe Library and FastStart Universal Probe Master (ROX) were purchased from Roche for RT-PCR analyses. The relative genes expressions level were quantitated by the CFX Connect Real-Time System (Bio-Rad Laboratories, USA). All the sequences of primers used in

this study were synthesized by Sangon Biotech (Shanghai, China), the sequences information are listed in Supplemental Table 1.

Enzyme-linked immunosorbent assay (ELISA)

RAW264.7 macrophages were seeded in 96-well plates at a density of 1×10^5 cells/well and incubated at 37 overnight. Cells were pretreated with BMP326 at different concentrations (0-20μM) for 3 h prior to adding 100 ng/ml LPS for another 24 h. IL-6, IL-1β and TNF-α secretion levels were detected in culture supernatant by commercial ELISA kits (Multi Sciences, China) according to the manufacturer's instructions. Ultimately, the absorbance was determined with a microplate reader at 492 nm. Three replicates were conducted for each sample.

Western Blot Analysis

RAW264.7 macrophages were seeded in 6-well plates at a density of 2×10^6 cells/well and incubated in low serum medium (1640+1%FBS) at 37 overnight. The cells were pretreated in the absence or presence of BMP326 (0-20μM) for 2hr and then stimulated with or without LPS (100ng/ml) for another 30min. Discarded the medium and rinsed cells with the ice-cold PBS before added Lysis buffer with phosphatase and protease inhibitors (Thermo Scientific, USA). Whole cell lysates were centrifuged at 13,000 g for 15 min at 4°C. Protein concentrations were measured by the BCA Protein Assay kit (BioTeke, China). Equal amounts of protein(30μg) were denatured in 5×SDS loading buffer at 100@C for 10 min. Different proteins were separated on 30% SDS-polyacrylamide gels and transferred onto polyvinylidene fluoride (PVDF) membrane (Millipore, USA). After blocking for 1 h in 5% skim milk, the membranes were incubated with specific primary antibodies overnight at 4. The dilution ratio was 1:000 except rabbit anti-GAPDH for 1:3000. After washing 3 times with TBST buffer, the membranes were incubated with the secondary antibodies (1:3000) for 1 h at room temperature following washing 3 times with TBST buffer again. The protein bands were revealed by chemiluminescence (Millipore, USA) and detected by ChemiDoc XRS+ (Bio-Rad, USA).

Statistical analysis

Data are expressed as means ± SD, Statistical analyses was performed using GraphPad Prism 6 (San Diego, USA).Student's t-tests were performed to determine differences between the BMP326 treatment group and control groups. $P < 0.05$ was considered as statistically significant.

Results

The cytotoxic effects of BMP326 on RAW264.7 cell viability

To confirm the optimal condition of LPS on RAW264.7 macrophages, NO production were detected at 24h after treated with different concentrations of LPS, and 100ng/ml is the optimal stimulate condition (Fig 1b). The CCK-8 assay was performed at 24 h after treatment with the indicated concentrations of BMP326 (0 to 40μM) and 100ng/ml LPS to determine the effect of BMP326 on RAW264.7 cell viability. BMP326 at 40μM showed obvious cytotoxic effects but BMP326 (0-20μM) did not cause significant cytotoxicity on RAW264.7 macrophages (Fig 1c). Therefore, LPS (100ng/ml) and BMP326 (0-20μM) were conducted for the following experiments in this study.

Effect of BMP326 onNO production and the mRNA expression level of iNOS and COX-2 in LPS-stimulated RAW264.7 cell lines

As an initial indicator of the anti-inflammatory effects of BMP326 on LPS stimulated RAW264.7 cells, iNOS and its mediated pro-inflammatory mediator COX-2 induction and NO production were determined using the qRT-PCR and Griess reagent separately[33, 34].Compared with LPS stimulation only,BMP326 markedly inhibited iNOS mRNA expression, NO production and COX-2 mRNA expression level also reduced in concentration dependent manner (Fig. 2a, 2b and 2c).These results showed that BMP326 has a preliminary anti-inflammatory effect in this inflammatory model.

Εφφεςτ οφ ΒΜΠ326 ονπρο-ινφλαμματορψ μεδιατορ ΤΝΦ-α, ΙΛ-6, ανδ ΙΛ-1β προδυςτιον ιν ΛΠΣ-στιμυλατεδ ΡΑΩ264.7 ςελλς

To further determine whether BMP326 has anti-inflammatory activity, we detected several critical pro-inflammatory cytokines including IL-6, IL-1β and TNF-α which are involved in multiple inflammatory pathways[35]. Both the mRNA and protein expression levels of these pro-inflammatory mediator were significantly increased in LPS-stimulated RAW264.7 cells. But pre-treatment of the BMP326 inhibited the mRNA expression levels of IL-6, IL-1β and TNF-α (Fig. 3a, 3b and 3c). As our prediction, similar results showed that the secretion of TNF-α, IL-6and IL-1β in culture supernatant were considerably decreased by BMP326 in a dose-dependent manner (Fig. 3d, 3e and 3f). These results confirm that BMP326 has effective anti-inflammatory properties in LPS-induced RAW264.7 cells.

ΒΜΠ326 μιγητ ηαε αντι-ινφλαμματορψ εφφεςτς τηρουγη τηε ΝΦ-κΒ πατηωαψ

Previous studies have showed that NF-κB as a well-known classical transcription factor involved in regulating the expression of inflammatory mediators[36]. NF-κB as an inactive complex exists within the cytoplasm of unstimulated normal cells. p65 subunit is an important component of NF-κB which generally bound to the IκBα family. The IκB kinase complex (IKK), as an important upstream kinase to phosphorylate IκBα and then IκBα degradation from p65 when cells are stimulated by LPS. Activated NF-κB p65 translocate to nuclei and trigger the transcription of downstream proinflammatory signaling[37]. As shown in the previous results, we detected the highest level of phospho-NF-κB p65 protein occurred at 30min after LPS stimulating. However, BMP326 markedly attenuated the LPS-induced phospho-NF-κB p65 and phospho-IκBα expression in a concentration-dependent manner compared with LPS-stimulated along (Fig.4a and 4b). These results suggest that the anti-inflammatory effect of BMP326 in LPS stimulated RAW264.7 cells might related to the NF-κB pathway.

BMP326 might have anti-inflammatory effects through the MAPK pathway

Multiple studies have found that MAPK signaling pathway plays critical role in LPS-stimulated RAW264.7 macrophages[38]. So we proceeded to analyze whether BMP326 inhibited inflammatory cytokines via the MAPK signaling pathways. Compared to the blank group, the highest level of phosphorylation p38MAPK, JNK, ERK1/2 were detected at 30min after LPS stimulating (Previous research results showed). However, pretreatment with BMP326 in different concentration can reversed the phosphorylation levels of p38 MAPK, JNK and ERK and resulted in 61.7%, 57.9% and 80.6% of inhibition respectively (Fig.4d, 4e and 4f). These results indicate that BMP326 inhibits inflammatory cytokines expression through MAPK signaling pathways in LPS-induced RAW264.7 macrophages.

Discussion

Chronic inflammation threatens human health which is characterized by tissue infiltration by macrophages and lymphocytes [39]. Among the efforts to identify novel candidates for the treatment of inflammatory diseases, we investigated the anti-inflammatory effects of BMP326 - a novel benzothiazole derivative in LPS-induced RAW264.7 macrophages. Our results demonstrated that BMP326 with the high anti-inflammatory activity could effectively suppress the inflammatory cytokines production. And preliminary molecular mechanisms underlying these effects involved in NF-κB and MAPK signaling pathways in RAW264.7 macrophage cells.

NO over production during a variety of inflammatory diseases as well as the macrophages stimulated by LPS. iNOS as one isoform of nitric oxide synthase (NOS) family plays a crucial part in production of NO[40]. Therefore, a good strategy to control the inflammation is to reduce the NO production through suppression of iNOS gene expression. In our results, BMP326 significantly inhibited iNOS mRNA expression and NO production but without cytotoxicity in 20μM (Fig1, Fig2A,2B). Cyclooxygenase (COX) involved in the prostaglandin biosynthesis and plays an important role in the development of inflammation. This enzyme exists as COX-1 and COX-2 isomers.COX-1 constitutively expressed in most tissues and organs and responsible for maintaining homeostasis of physiology include gastric and renal integrity whereas COX-2

induces inflammatory conditions. Many traditional Nonsteroidal anti-inflammatory drugs (NSAIDs) are non-selective inhibitors for both COX-1 and COX-2, thereby accompanied by side effects such as gastrointestinal erosion and renal damage [41, 42].So an ideal strategy for the reduction of inflammation is to screen selective COX-2 inhibitors. Our results displayed that BMP326 can effectively reduce the COX-2 mRNA expression in a dose-dependent manner but it has no effect on the expression of COX-1(Fig2C, 2D). Pro-inflammatory cytokines released is the typical manifestation in the early stages of inflammation, and played important roles in development of the inflammatory process. As macrophages express IL-6 when they are induced by microbes or LPS and then regulate production of TNF-α and IL-1β[43]. TNΦ-α is another early responsive pro-inflammatory cytokines which leads to the activation of the downstream inflammation-related signaling pathways[44].IL-1β as a key proinflammatory cytokines produced by the inflammasome, which involved in a number of inflammatory diseases[45]. Increasing evidences suggest that inhibited the production of inflammatory cytokines is becoming a therapeutic strategy. As shown by our results, LPS significantly induced the mRNA expressions and cytokine secretions of IL-1β, IL-6, and TNF-αcompared with the control group, but BMP326 inhibited the expression of pro-inflammatory cytokines in a dose-dependent manner (Fig3A-F). Consequently, BMP326 can be used as a relatively safe and effective anti-inflammation drug.

Then we explored the potential signaling pathway. Our research utilized Raw264.7 macrophages which are stimulated with LPS as the inflammatory model to explore the anti-inflammatory effect of BMP326. LPS as the outer membrane structural component of Gram-negative bacteria can be recognized by TLR4 in macrophages and then activating the downstream signaling pathways[46]. Many studies have shown that nuclear factor-ϰ-gene binding (NF-ϰB) and mitogen activated protein kinase (MAPK) signaling pathways play a crucial part in inflammation regulation. Activated NF-ϰB translocated to the nucleus and bind to specific DNA binding site then promotes the transcription of inflammatory genes including iNOS, COX-2, IL-1β, IL-6, TNFα[38].Τηε ΜΑΠΚ φαμιλψ ινχλυδες τηρεε μεμβερς (ΕΡΚ, π38, ανδ ΘΝΚ). ΛΠΣ-ινδυχεδ πηοσπηορψλατιον οφ τηεσε ΜΑΠΚς ινχρεασεδ τηε εξπρεσσιον οφ ιΝΟΣ, ΅ΟΞ-2, ανδ ινφλαμματορψ χψτοκινες ιν ΡΑΩ264.7 χελλς[47]. Ααιλαβλε ρεσυλτς ινδιχατεδ τηατ ΛΠΣ ινδυχεδ πηοσπηορψλατιον οφ ΙϰΒ-α, P65, JNK, P38 and ERK are significantly reduced after pretreatment with BMP326. Admittedly, it is unclear whether there are other regulatory mechanisms for BMP326 to exert anti-inflammatory effects. Further experiments are needed to verify this.

Conclusions

Until now, the anti-inflammation effect of BMP326 has not been explored . We firstly demonstrated that BMP326 exerts anti-inflammatory properties and clarified the potential mechanism(Fig5). Therefore, these results suggest that BMP326 may be used as a lead compound for design and development of new anti-inflammatory drugs for therapeutic use in inflammatory diseases.

Declarations

Ethics approval and consent to participate: Not applicable

Consent for publication: Not applicable

Availability of data and materials: All data generated or analysed during this study are included in this published article [and its supplementary information files].

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Competing Interests: The authors declare that they have no competing interests.

Acknowledgements: Not applicable

Figures

Fig.1 Effects of BMP326 on the viability of RAW264.7 macrophages. (a) Chemical structure of BMP326.

(b)RAW264.7 macrophages were treated with different concentrations of LPS(0-1000ng/ml) for 24 h, Griess Reagent Kit detected that 100ng/ml LPS is the optimal stimulate condition for following researches. (c) RAW264.7 macrophages were pretreated with BMP326 (0-20 μM) for 3 h and were stimulated with or without LPS (100 ng/ml) for another 24 h. The cells viability was determined by CCK-8 assays. #P < 0.05 vs. Control group; **P < 0.01 vs. LPS alone.

Fig.2 BMP326 inhibited LPS-induced iNOS gene expression and NO synthesis in RAW264.7 macrophages. RAW264.7 Macrophages were pretreated with or without various concentrations of BMP326(0-20 μM) for 3 h before LPS(100 ng/ml) stimulation for another 24 h.(a) The culture medium was used for measuring the level of NO by Griess reagents.(b,c,d) The mRNA levels of iNOS, COX-1 and COX-2 were measured by qRT-PCR.Values are referred as mean ± SD,#P < 0.05 vs. Control group,*P < 0.05 vs. LPS alone, **P < 0.01 vs. LPS alone.

Fig.3 BMP326 inhibited the inflammatory cytokines secretion and expression of mRNA in LPS-induced RAW264.7 macrophages. RAW264.7 macrophages were treated as described before (a,b,c). The mRNA levels of IL-6 , TNF-α and IL-1β were measured by qRT-PCR. (b) The effects of BMP326 on secretion of IL-6 , TNF-α and IL-1βwere measured by ELISA. Values are referred as mean ± SD, ##P < 0.01 vs. Control group,*P < 0.05 vs. LPS alone, **P < 0.01 vs. LPS alone.

Fig.4 Effects of BMP326 on the NF-κB and the MAPK signaling pathways in LPS-induced RAW264.7 macrophages. Cells were pretreated with the indicated concentrations(0-20 μM) of BMP326 for 3 h and then stimulated with LPS (100ng/mL) for another 30 min. Protein lysate were harvested and analyzed by western blot with specific antibodies.(a) Protein levels of phospho-p65 were measured.(b)Protein levels of phospho-IκBα were measured. Protein levels of phospho-ERK(c),phospho-JNK(d) and phospho-p38(e) were measured. GAPDH was used as an internal standard control and blot bands densitometry were analyzed with Image J software. Values are referred as mean ± SD, ##P < 0.01 vs. Control group,*P < 0.05 vs. LPS alone, **P < 0.01 vs. LPS alone.

Fig.5 The possible molecular mechanism underlying the anti-inflammatory effect of BMP326. BMP326 suppressed the production of inflammatory mediators and pro-inflammatory cytokines through inhibition of NF-κB and MAPKs signaling pathway in LPS-stimulated RAW264.7 cells.

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