A benzothiophene-carboxamide is a potent inhibitor of IL-1β induced VCAM-1 gene expression in human endothelial cells

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Abstract Vascular endothelial cells respond to cytokines such as IL-1β or TNF-α by undergoing a number of functional alterations. Among these alterations is the induction of cell surface adhesion molecules, including VCAM-1. In this report, we investigated the effects of a 3-alkoxybenzo[β]thiophene-2-carboxamide (BZT) on the cytokine induction of VCAM-1 expression and activation of the transcription factor NF-κB in human endothelial cells. BZT blocked the IL-1β induced cell surface expression of VCAM-1 in human endothelial cells but did not prevent nuclear translocation of NF-κB. This study demonstrates that BZT is a potent inhibitor of VCAM-1 expression in human endothelial cells.

Key words: VCAM-1; Inhibition; Transcription; Nuclear factor κB; Benzothiophene-carboxamide

1. Introduction

Vascular endothelial cells undergo a number of morphological and functional changes in response to cytokines. These changes include the increased synthesis and expression of specific cell surface adhesion molecules including E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) (see [1] for review). At sites of inflammation, recruitment of leukocytes is mediated by the interaction of distinct integrin receptors on the leukocytes with the cell surface adhesion molecules on the vascular endothelial cell.

The intracellular signaling mechanisms by which these adhesion molecule genes are induced in response to cytokine stimulation are beginning to be elucidated. In particular, the induction of VCAM-1 in endothelial cells is directly due to increased transcription rather than mobilization of intracellular pools of protein [2,3]. Recently, the promoter region for VCAM-1 was shown to contain two decameric sequences that are recognized by the nuclear factor κB (NF-κB)/Rel family of transcription factors [4,5]. These κB sites are required for induction of VCAM-1 gene transcription [4,5].

NF-κB is normally found in the cytosol of most cell types as an inactive heterodimer bound to an inhibitor protein, IκBα [6–12]. The IκBα protein is rapidly phosphorylated and subsequently degraded upon stimulation with cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) [13–17]. This allows NF-κB to translocate to the nucleus and modulate gene expression.

Recently, Boschelli et al. [18] described compounds based on the structure of a 3-alkoxybenzo[β]thiophene-2-carboxamide that blocked the adhesion of neutrophils to endothelial cells. In the present report, we investigated the mechanism of action of a 3-alkoxybenzo[β]thiophene-2-carboxamide (BZT) using human endothelial cells. We demonstrated that BZT inhibited the IL-1β induced cell surface expression and promoter activity of VCAM-1 in human endothelial cells. In addition, we show that BZT does not affect IL-1β induced nuclear translocation of NF-κB. Therefore, it appears that IL-1β stimulated translocation of NF-κB is not sufficient for VCAM-1 gene expression in endothelial cells.

2. Materials and methods

2.1. Cells and cell culture

Human umbilical vein endothelial cells (HUVEC) were purchased from Clonetics (San Diego, CA) and used between passages 2 and 6. The human endothelial cell line ECV304 was purchased from ATCC (Rockville, MD). HUVEC and ECV304 were grown in EGM media (Clonetics, San Diego, CA).

2.2. Chemicals and reagents

N-Tosyl-Phe-chloromethylketone (TPCK) was purchased from Sigma (St. Louis, MO). The 3-alkoxybenzo[β]thiophene-2-carboxamide (BZT) was synthesized according to the procedures described by Boschelli et al. [18]. All DNA oligonucleotides were synthesized on an ABI 381A DNA synthesizer (Applied Biosystems, Foster City, CA). IL-1β was purchased from R&D Systems (Minneapolis, MN). Compounds were dissolved in DMSO (Sigma).

2.3. ELISA

HUVEC (≤ passage 6) were plated on gelatin coated 96-well tissue culture dishes (CoStar, Cambridge, MA) and incubated at 37°C overnight. The cells were then pre-treated with the test compound for 30 min followed by a 6 h incubation with the test compound and 0.5 ng/ml IL-1β. Samples were run in triplicate and each experiment was performed a minimum of three times. Following the 6 h treatment, the HUVEC were washed once with PBS. Cells were then fixed by incubation in 3.7% formaldehyde/PBS for 5–10 min. Fixed HUVEC were stored at 4°C overnight in PBS containing 0.02% sodium azide. After the PBS/azide solution was aspirated, fixed cells were incubated in 1% BSA in PBS (dilution/wash buffer) for 1 h at 37°C. This solution was then aspirated and replaced with anti VCAM-1 monoclonal antibody 1G11B1 (Monosan, Uden, The Netherlands) diluted 1:1000. Plates were then incubated for 1–2 h at 37°C. The antibody solution was aspirated and the cells were subsequently washed 3 times with wash buffer. Wash buffer was replaced with a 1:5000 dilution of anti mouse IgG/horseradish peroxidase conjugate (Promega, Madison, WI) and then incubated at 37°C for 1 h. Conjugate solution was

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Abbreviations: ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; HUVEC, human umbilical vein endothelial cells; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; NF-κB, nuclear factor-κB; RT/PCR, reverse transcriptase/polymerase chain reaction; EMSA, electrophoretic mobility shift assay; TPCK, *N*-tosyl-Phe-chloromethylketone.

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aspirated and cells were washed 3 times. TMB/E substrate solution (Chemicon, Temecula, CA) was then applied to the cells. When adequate color had developed, an equal volume of 2 N sulfuric acid was added to stop the reaction. Spectrophotometric readings at wavelength 450 nm were measured in a Molecular Devices (Menlo Park, CA) plate reader.

2.4. Toxicity evaluation

Test compounds at concentrations presented herein were shown to be non-toxic by the following procedure. Viability (< 80% = toxic) of HUVEC treated with experimental compounds was determined using the alamarBlue assay (BioSource International, Camarillo, CA) according to the manufacturer's instructions. HUVEC were cultured in gelatin coated 96-well tissue culture dishes until confluent. Test compounds were evaluated in quadruplicate in EGM/10% alamarBlue reagent. Flourimeter readings (excitation, 530 nm; emission, 590 nm) were taken after a 6 h incubation. A visual inspection of the cells was also performed to ensure that no morphology changes had occurred.

2.5. Reverse transcriptase/polymerase chain reaction (RT/PCR) analyses

All RT/PCR analyses were performed using the GeneAmp RNA/PCR kit (Perkin Elmer, Branchburg, NJ). HUVEC, plated in gelatin coated T25 tissue culture flasks (Corning, Inc., Corning, NY), were treated in the same manner as described previously for the ELISA. Total RNA was isolated using RNAzol B following the procedures of the manufacturer (BioTecX, Houston, TX). Human β-actin primer pairs generated a fragment of 838 base pairs and were purchased from Clontech (Palo Alto, CA). The thermal cycling parameters were as described by Clontech. The plasminogen activator inhibitor type 1 (PAI-1) primers amplified a product of 800 base pairs and contained the following sequences: 5′-CTACTTCAACGGCCAGTGGAAGACTC-3′, 5′-GAGGCCAAGGTCTTGGAGACAGATCT-3′. The PCR primers for human VCAM-1 amplified a product of 1101 base pairs and contained the following sequences: 5′-TTACACATTGATGAAATGGATTCTGT-3′, 5′-AAGGTGAGAGTTGCATTCTCAGAAAG-3′. The amplified PCR products were analyzed by electrophoresis in a 3% agarose gel (3:1 GTG NuSieve, FMC, Rockland, ME: Ultrapure agarose, Life Technologies, Gaithersburg, MD).

2.6. Luciferase assays

Genomic DNA was isolated from HUVEC following the procedure of Cobb et al. [19]. The VCAM-1 promoter region (1811 base pairs) was amplified following the procedures of the GeneAmp kit (Perkin Elmer, Norwalk, CT). The primers used to amplify the fragment were as follows: 5′-GGTACCGGCATTTCTCCAATGTTGCAAGCT-3′ and 5′-GGTACCATAGTGGTTCCAAAACCCTTA-3′. The VCAM-1 promoter fragment (−1717 to +94 base pairs [4]) was cloned into the *Kpn*I site of pGL2 (Promega, Madison, WI). The neo gene was subcloned from pMAMneo (Clontech, Palo Alto, CA) into the *Bam*HI site of pGL2VCAM-1 to generate pGLneoVCAM-1. Positive clones were identified by restriction digest and confirmed by DNA sequence analysis (Sequenase, US Biochemicals, Cleveland, OH). Plasmid DNA was purified using standard procedures [20]. pGLneoVCAM-1 DNA was then transfected into ECV304 cells using Lipofectin (Life Technologies, Gaithersburg, MD) and selected for resistance to geneticin (Life Technologies) at a concentration of 300 μg/ml.

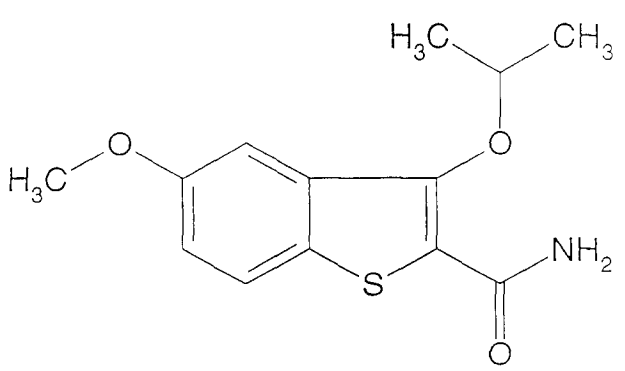

Fig. 1. Chemical structure of BZT.

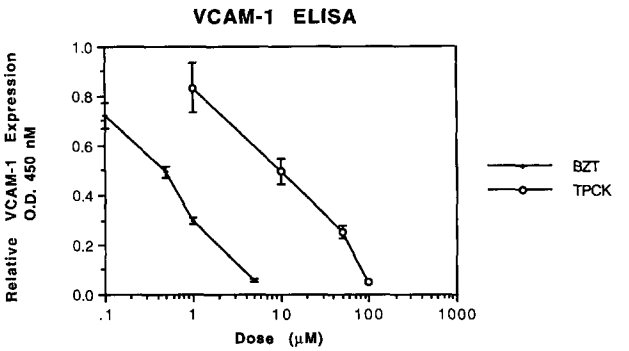

Fig. 2. Inhibition of IL-1β induced cell surface expression of VCAM-1. HUVEC were plated in gelatin coated 96-well plates and were treated with the indicated concentrations of BZT or TPCK for 30 min prior to the addition of 0.5 ng/ml IL-1β. Samples were then incubated for 6 h with IL-1β and the test compound and then treated as described. Samples were performed in triplicate. Results are given as mean ± S.D. VCAM-1 cell surface expression in HUVEC monolayers. No treatment OD_{450nm} = 0.098 (S.D. = 0.061) and 0.5 ng/ml IL-1β OD_{450nm} = 0.809 (S.D. = 0.189). Similar results were observed in two separate experiments.

ECV304 cells stably transfected with pGLneoVCAM-1 were plated in a gelatin coated Falcon 48-well tissue culture plate (Becton-Dickinson, Franklin Lakes, NJ) and allowed to incubate overnight. The cells were then treated in a similar manner as for the ELISA. Following the 6 h incubation, cells were washed with PBS and then lysed with 50 μl lysis buffer (0.1 M KPO_4 (pH 8), 0.1% Triton X-100, 1 mM DTT, 2 mM EDTA). The lysed cells were then placed at −20°C overnight. After thawing, 5 μl of the lysate was added to 100 μl of luciferase assay buffer (Promega, Madison, WI) and immediately analyzed for light production in a Monolight 2010 luminometer (Analytical Luminescence Laboratories, Sorrento Valley, CA). The amount of light emitted (light units) by the cell lysate was measured for 10 s.

2.7. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from 5×10^6 HUVEC as described [21]. Protein concentrations in nuclear extracts were 1–5 mg/ml, as determined by BCA protein assay (Pierce, Rockford IL). An oligonucleotide containing the murine Igκ site (underlined), 5′-CAGAGGGACTTTCCGAGA-3′, was obtained from Operon Technologies, Inc. (Alameda, CA). Analysis of NF-κB protein binding was performed as described [21]. For competition and antibody supershift experiments, binding reactions were incubated with unlabeled double-stranded oligonucleotides or monospecific antibodies for 20 min before the addition of the radiolabeled oligonucleotide. Anti-p65, anti-c-Rel and anti-p50 rabbit polyclonal antibodies were purchased from Santa Cruz Biotechnology.

3. Results

Previous studies have shown that a novel series of compounds inhibited the adhesion of neutrophils to TNF-α stimulated endothelial cells [18]. In the present study, we investigated the effects of one of these compounds, BZT (Fig. 1), on IL-1β induced VCAM-1 gene expression and activation of NF-κB in human endothelial cells.

We first examined the inhibitory effects of BZT on IL-1β induced cell surface expression of VCAM-1 using HUVEC. ELISA assays were performed and the data is presented in Fig. 2. BZT is much more potent in blocking the cytokine induced cell surface expression of VCAM-1 than TPCK, a protease inhibitor that has been shown to block cytokine activation of NF-κB [14].

We next investigated the effects of BZT on the IL-1β induced upregulation of VCAM-1 mRNA in HUVEC. As

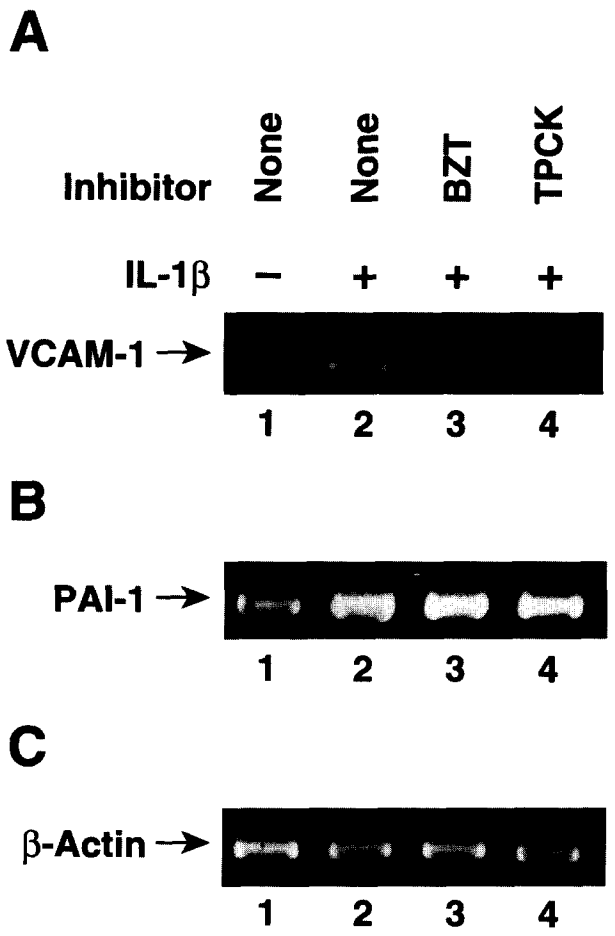

Fig. 3. BZT inhibits VCAM-1 mRNA but not PAI-1 or β-actin in HUVEC treated with IL-1β. HUVEC monolayers were treated with 10 μM BZT or 50 μM TPCK for 30 min prior to the addition of 0.5 ng/ml IL-1β. The cells were then incubated for 6 h with the test compounds plus IL-1β and then total RNA was isolated. 1 μg of total RNA was used for RT/PCR analysis. (A) VCAM-1 specific primers. (B) PAI-1 specific primers. (C) β-Actin specific primers.

shown in Fig. 3, 10 μM BZT and 50 μM TPCK blocked the IL-1β induction of VCAM-1 mRNA. BZT and TPCK did not block the IL-1β induced upregulation of PAI-1 mRNA or the constitutive expression of β-actin mRNA (Fig. 3).

To determine if BZT inhibited VCAM-1 promoter activity, ECV304 cells were stably transfected with pGLneoVCAM-1. ECV304 cells containing pGLneoVCAM-1 exhibited a time- and dose-dependent induction of luciferase activity in response to IL-1β similar to HUVEC transiently transfected with the same reporter gene plasmid (data not shown). Therefore, these cells were chosen to examine the inhibition of the VCAM-1 promoter activity by BZT. As shown in Fig. 4, BZT blocked IL-1β induced VCAM-1 promoter activity. Again, BZT was a more potent inhibitor of VCAM-1 promoter activity than TPCK.

Previous studies have shown that NF-κB is involved in the induction of VCAM-1 gene expression in endothelial cells [4,5]. The effects of BZT on the activation of NF-κB were analyzed using EMSAs. As shown in Fig. 5, BZT did not block IL-1β induced NF-κB nuclear translocation. In contrast, TPCK did block IL-1β induced NF-κB translocation to the nucleus (data not shown). The complex formed using this oligonucleotide containing a prototypic κB site was characterized by competition and antibody supershift experiments. The complex was competed with an unlabeled oligonucleotide containing a wild-type κB site but not with an unlabeled oligonucleotide containing a mutated κB site (Fig. 5). In addition, the complex was specifically supershifted by anti-p50 and anti-p65 antibodies but not by anti-c-Rel antibodies, indicating that it represented binding of p50/p65 heterodimers (Fig. 5).

4. Discussion

In the present study we have investigated the effects of a benzothiophene-carboxamide (BZT) on the IL-1β stimulated induction of VCAM-1 gene expression in human endothelial cells. Previous studies have shown that compounds of similar structure were effective in blocking neutrophil adhesion to TNF-α stimulated HUVEC [18]. Here we present evidence that BZT is a potent inhibitor of IL-1β stimulated induction of VCAM-1 cell surface expression and VCAM-1 promoter activity.

Previous reports have shown that activation of NF-κB is required for cytokine induced VCAM-1 gene expression [4,5]. In this study, EMSAs demonstrated that BZT did not inhibit IL-1β induced nuclear translocation of NF-κB. Our findings are similar to a report by Stuhlmeir et al. [23], which demonstrated inhibition of cytokine induced gene expression of VCAM-1, ICAM-1, and E-selectin without inhibition of NF-κB activation. These data indicate that NF-κB nuclear translocation alone is not sufficient for IL-1β induced upregulation of VCAM-1 in endothelial cells.

We also examined the effects of BZT on IL-1β induced stimulation of PAI-1 mRNA, which is regulated by an NF-κB independent mechanism [22]. BZT did not block the IL-1β induction of PAI-1 mRNA in endothelial cells, indicating that BZT does not inhibit all IL-1β induced intracellular signaling cascades in human endothelial cells.

At least two different mechanisms of action could explain the effects of BZT on endothelial cells. It is possible that the transactivating domain of the p65 subunit NF-κB may be the target of the inhibition. One could envisage that nuclear translocation of NF-κB is not modulated by BZT, but its ability to induce transcription is inhibited. Alternatively, BZT may

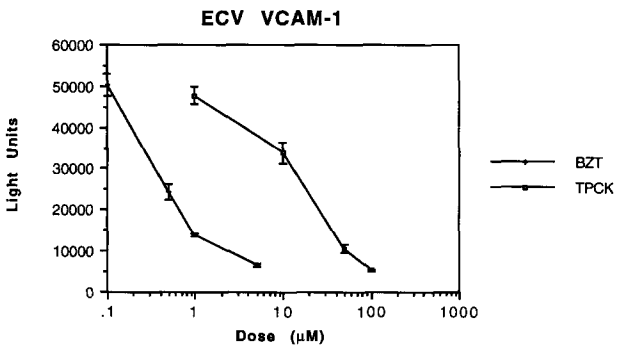

Fig. 4. Inhibition of IL-1β stimulated VCAM-1 promoter activity in endothelial cells by BZT. ECV304 cells stably transfected with pGLneoVCAM-1 were pre-treated with the indicated concentrations of BZT or TPCK for 30 min prior to addition of 0.5 ng/ml IL-1β. Cells were treated for 6 h with IL-1β and the compound. Cells were then lysed and lysates were then analyzed for luciferase activity. Samples were performed in triplicate and tests were performed a minimum of three times.

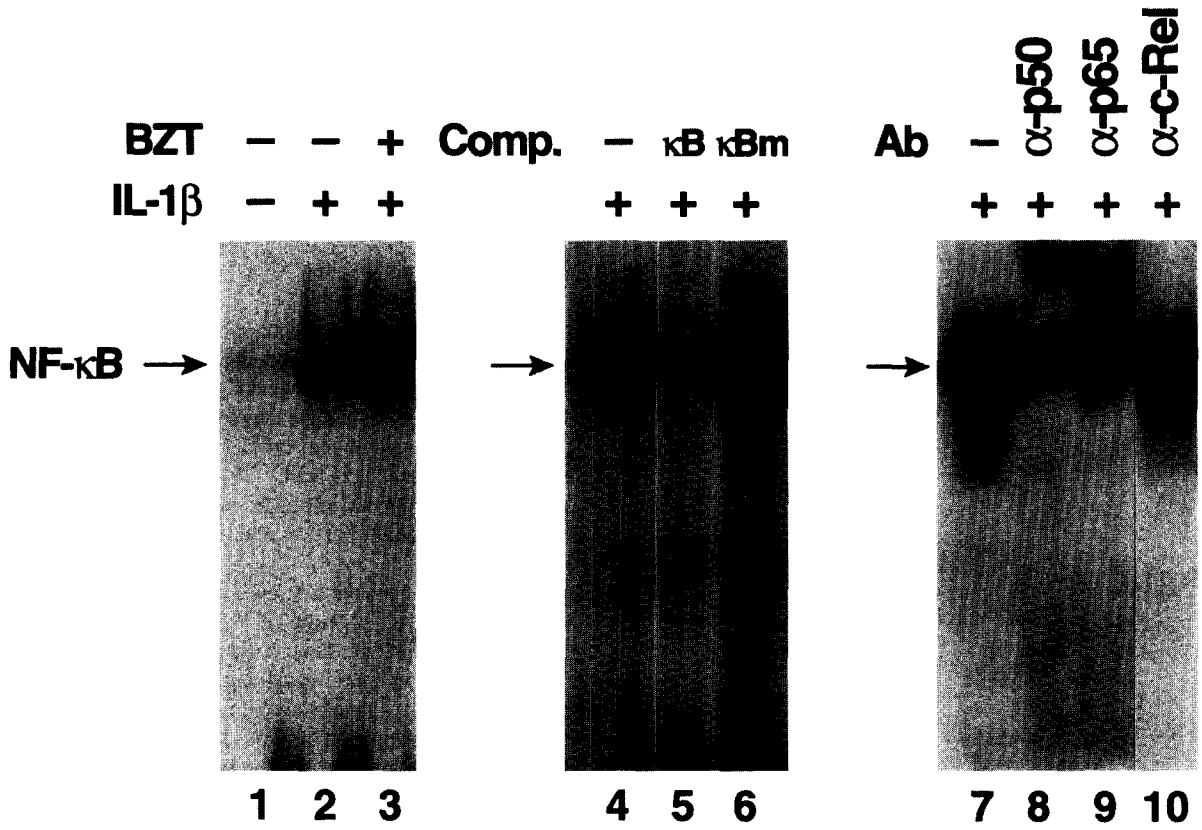

Fig. 5. IL-1β activation of NF-κB in HUVEC. Nuclear extracts were prepared from unstimulated HUVEC (lane 1) and HUVEC incubated for 1 h with 1 ng/ml IL-1β with or without a 30 min pretreatment with 10 μM BZT (lanes 2,3). The presence of NF-κB was determined by EMSA using a radiolabeled oligonucleotide containing a prototype κB site. IL-1β stimulation of HUVEC activated NF-κB (arrow). Competition experiments were performed using 100× molar excess of an oligonucleotide containing a wild-type κB site (GGGACTTTCC) (lane 5) or a mutated κB site (CGGACTTAGA) (lane 6). The protein composition of the complex was determined by antibody (Ab) supershift experiments using anti-p50 (lane 8), anti-p65 (lane 9) and anti-c-Rel (lane 10) antibodies.

block the activation of other transcription factors involved in the IL-1β stimulated induction of VCAM-1 gene expression in endothelial cells such as the interferon regulatory factor 1 [24]. Future studies will investigate the molecular target of BZT.

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