Novel analgesics targeting brain-derived neurotrophic factor for neuropathic pain

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ABSTRACT Brain-derived neurotrophic factor (BDNF) is necessary for the development, growth, and maintenance of nerve cells. BDNF is expressed in the dorsal root ganglion (DRG); binds to the Tropomyosin receptor kinasas B (TrkB) receptor, which has a tyrosine kinase domain, in the spinal cord; and plays an important role as a pain modulator. BDNF expression is increased in various types of pain, including acute pain, neuropathic pain, and cancer pain. Activation of the BDNF–TrkB pathway transmits pain information. In order to inhibit the BDNF–TrkB pathway, by sequestering BDNF, we constructed a cDNA expression plasmid encoding the extracellular region of rat TrkB fused to enhanced green fluorescent protein (EGFP). When the expression plasmid vector was administered to rat models of neuropathic pain, induced by spinal nerve ligation, statistically significant relief of pain was observed in terms of a 50% paw-withdrawal threshold using the von Frey test. The expression of TrkB-EGFP mRNA was detected in L5 lumbar vertebral nerves by quantitative reverse transcriptase polymerase chain reaction. To verify the pain-suppressive effect of the expression vector, truncated TrkB protein, without EGFP, was purified, and administered to pain model rats. A statistically significant suppressive effect of the truncated TrkB protein on neuropathic pain was observed 2 days after administration. The pain-suppressive effect of the truncated TrkB protein was more effective than that of the TrkB-Fc chimera protein and lasted longer than that of the TrkB antagonist ANA-12. Our results suggested that the truncated TrkB cDNA expression vector and truncated TrkB protein could be used as molecular targeted drugs in patients with neuropathic pain.

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INTRODUCTION Various factors participate in the perception of neuropathic pain; these include neurotrophic factors 1-3). Brain-derived neurotrophic factor

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(BDNF), a member of the neurotropic factor family, is necessary for nervous system development, growth, and maintenance, and plays an important role as a pain modulator. In the peripheral nervous system, BDNF is expressed in the dorsal root ganglion (DRG) under painful conditions, from which it is secreted, and then binds to the Tropomyosin receptor kinase B (TrkB) receptor, a member of the tropomyosin kinase receptor family, on the posterior horn of the spinal cord; this modulates pain transduction from peripheral nociceptors.

The precise mechanism by which BDNF transmits pain information remains unclear. When BDNF binds to TrkB, the activation of the BDNF–TrkB pathway can evoke membrane depolarization through rapid activation of the sodium ion channel NaV1.9, which has been linked to pain in humans. The tyrosine kinase domain of TrkB is activated, leading to activation of phosphoinositide 3-kinase (PI3K), extracellular signal-regulated kinase (ERK), and phosphoinositide phospholipase C (PLCγ). In previous reports, neuropathic pain was increased by administration of BDNF to mice, and neuropathic pain was decreased in rats and mice by inhibition of the BDNF–TrkB pathway.

Various treatments are provided for patients with neuropathic pain; however, some treatments are not effective, and neuralgia often persists. Because there are few effective drugs, the use of strong opioids has increased, which may have contributed to the recent opioid crisis. Therefore, we have focused on developing new therapeutic drugs to decrease the usage of opioids. If BDNF, a modulator of the pain, could be restricted in patients with intractable pain, the BDNF–TrkB pathway would be inhibited, and neuropathic pain might be improved.

In a previous study, in order to inhibit the BDNF–TrkB pathway by sequestering BDNF, we had previously constructed a cDNA expression plasmid, pCMVscript-eTrkB-EGFP, encoding an extracellular region of rat TrkB (from methionine 1 to histidine 429) fused with EGFP, in our previous study. After the plasmid was transfected in HEK293 cells, we confirmed that the eTrkB-EGFP protein was secreted from the transfected cells, and that the protein could bind to BDNF, by using a pull-down assay implementing S-protein agarose beads, as well as by western blotting. Administration of these vectors exerted an analgesic effect in rat models of cancerous osteocopic pain. In the present study, we showed that administration of the vector can also suppress neuropathic pain in a rat model. To verify the function of the gene products from the vector, we purified the protein composed of only the extracellular domain of TrkB (hereinafter referred to as "eTrkB") and showed that administration of the eTrkB protein can suppress the pain in neuropathic pain model rats. The vector and protein might be considered as molecular targeted drugs for intractable chronic pain, including neuropathic pain.

**MATERIALS AND METHODS**

**Materials**

ANA-12 (N-[2-[[hexahydro-2-oxo-1H-azepin-3-yl]amino]carbonyl]phenyl]-benzo [b] thiophene-2-carboxamide), which is an antagonist of TrkB, was purchased from Sigma–Aldrich (SML0209; St Louis, MO, USA). The TrkB-Fc chimera protein was purchased from R&D systems (688-TK-100; Minneapolis, MN, USA).

**Construction of the eTrkB gene expression vectors**

As a vector for DNA administration into rats, pCMVscript-eTrkB-EGFP, encoding only the extracellular domain region of TrkB fused to enhanced green fluorescent protein (EGFP), was constructed in previous our study. Briefly, we extracted mRNA from the brain tissue of rats, and synthesized cDNA by reverse transcription. We amplified the eTrkB cDNA region, encoding the extracellular domain of TrkB, by PCR, using the FW
primer (5'-GGA TCC GCC ATG TCG CCC TGG CCG AGG TG-3', including a BamHI restriction enzyme site) that targets the translation initiation region and the RV primer (5'-GAA TTC ATG CTC CCG ATT GGT TTG GTC-3', including an EcoRI restriction enzyme site) that targets the region upstream of the transmembrane region. The PCR product was cloned into pBluescript KS at the EcoRV site, and was confirmed by sequencing analysis. The eTrkB cDNA was digested by BamHI and EcoRI, and inserted between the BamHI and EcoRI sites of the pCMVscript-EGFP vector. Sequencing analysis confirmed a fusion gene encoding eTrkB, EGFP, a FLAG-tag, and an S-tag, in-frame. This plasmid, pCMVscript-eTrkB-EGFP, was administered to neuropathic pain model rats.

As a vector for producing and purifying proteins for protein administration into rats, the plasmid pCMVscript-eTrkB encoding the extracellular domain region of TrkB, without EGFP, was constructed. Briefly, the truncated extracellular domain region of rat TrkB, eTrkB, was produced by DNA synthesis (TaKaRa-Bio, Shiga, Japan); it encoded 425 amino acids of TrkB, from the first methionine to threonine 425. The 3' end of the cDNA was fused to a FLAG-tag, S-Tag, and Histag, in-frame, and was cloned into the pCMVscript plasmid.

**Purification of eTrkB protein and BDNF-binding assay**

pCMVscript-eTrkB (a vector for protein purification) was transfected into HEK293 cells by using Lipofectamine2000 (Thermo Fisher Scientific, Tokyo, Japan). The medium was changed to serum-free OPTI-MEM medium (Thermo Fisher Scientific) on the next day, and the culture medium (1 L) was collected at 72 h after transfection. Protease inhibitor (10 μl; P1860, Sigma), 500 ng of BDNF protein (ab9794, mature type; Abcam, Cambridge, UK) and S-protein agarose beads (50 μl; Novagen, Madison, WI, USA) were added to 1 ml of the culture medium; the samples were mixed for 2 h at 4°C, and the solution was centrifuged at 5,000 rpm for 3 min. After washing, the S-protein agarose complex was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and blotted onto a polyvinylidene fluoride (PVDF) membrane. Anti-FLAG mouse antibody (F3165, Sigma) and anti-TrkB mouse antibody (#4603; Cell Signaling Technology, Danvers, MA, USA), for detecting truncated eTrkB protein fused with Tag peptides, and anti-BDNF rabbit antibody (ab9793; Abcam), for detecting BDNF, were added. Membranes were then incubated with anti-mouse IgG-HRP antibody (CST; #7076) and Western Lightning Plus-ECL (PerkinElmer Japan, Kanagawa, Japan), and chemiluminescence was detected using the LAS-1000 (FUJIFILM). After we confirmed that the eTrkB protein retained the BDNF-binding activity, the eTrkB protein was purified from culture medium (1 L) using Ni-NTA column (QIAGEN, Hilden, Germany), and eluted with 250 mM imidazole (pH 8.0).

**Animal treatment**

All experiments in this study were performed according to the protocol approved by the Institutional Animal Care and Use Committee of Kawasaki Medical School (approval number 15-0111).

Wister rats (7 weeks old) were subjected to a pain evaluation using von Frey filaments, as described below (day 0). Thereafter, neuropathic pain model rats were generated. After incising the back under general anesthesia with sevoflurane, the L6 transverse process was identified and removed, and the left L5 lumbar nerve was ligated by silk thread of 5-0 at a position more peripheral than the posterior root ganglion\(^{14}\).

One week later (day 7), rats were again subjected to pain evaluation with von Frey filaments. A dorsal
A skin incision was made from L3 to L6 under general anesthesia with sevoflurane; the lumbar level was identified from the vertebral spinous process and pelvis. A 23-G needle was inserted into the spinal cord’s subarachnoid space from the muscular fasciae of L5/6, and a sterilized catheter (polyethylene tube, inner diameter: 0.2 mm, outer diameter: 0.5 mm) for administration of treatment was inserted into the spinal cord’s subarachnoid space through the needle.

For DNA administration, the vector DNA was pretreated with transfection reagent GenomONE (HVJ Envelope VECTOR KIT; Ishihara Sangyo Kaisha, Ltd., Osaka, Japan), and the vector DNA mixture (1 μg/μL, 10 μL) was administered through the catheter.

For administration of protein and other reagents, purified eTrkB protein (100 ng/3 μL), heat-inactivated eTrkB protein (95°C, 4 min) (100 ng/3 μL), ANA-12 (100 ng/5 μL), TrkB-Fc protein (100 ng/5 μL), and TrkB-Fc protein (1 μg/5 μL) were administered through the catheter. Saline (20 μL) was added through the catheter after the dose administration. Rats were subjected to pain evaluations for 1 week afterwards, and then sacrificed under general anesthesia with sevoflurane.

**Pain evaluation**

For pain evaluation, the von Frey test was performed using von Frey filaments (Tactile Test Aesthesio, Muromachi Kikai Co., Tokyo, Japan), and the 50% paw withdrawal threshold (PWT) was calculated as the pain threshold\(^{15}\). The pain evaluation test was performed on days 0, 2, 5, 7, 9, 12, and 14 (day 0 indicates the von Frey test performed immediately before the nerve ligation operation, and day 7 indicates the test immediately before treatment administration). As a pain evaluation test after administration of ANA-12, the von Frey test was performed at 4 h (day 7.5) and at 24 h (day 8) after ANA-12 administration.

**Real-time PCR**

After the pain threshold was evaluated at day 14, rats were sacrificed, and L5 spinal cords were resected under general anesthesia with sevoflurane. RNA was extracted with an RNeasy Lipid Tissue Mini Kit (Qiagen), treated with DNase I, and reverse-transcribed into cDNA using a Ready-to-Go T-primed First-Stand kit (Amersham Biosciences, Little Chalfont, UK). To verify the mRNA expressed from the plasmid vector administered to the rats, eTrkB-EGFP mRNA and internal control RPL-27 (ribosomal protein L27) mRNA were analyzed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) on a StepOne Plus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with the RPL-27 FW-primer 5'–GAA TTG ACC GCT ATC CCA GA–3' and RV-primer 5’–TCG CTC AAA CTT GAC CT–3' (product size: 230 bp), and GFP FW-primer 5’–CGA CAA CCA CTA CCT GAG–3' and RV-primer 5’–GAA TTT AGC GGT TT–3' (product size: 178 bp).

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism7, jmp13, and SPSS. The von Frey test results were analyzed using the Wilcoxon rank-sum test, which was corrected using the Bonferroni method for days 7, 9, 12, and 14. Gene expression results based on qRT-PCR were evaluated using the trend test (SPSS Ver. 18). A value of \( p < 0.05 \) was considered statistically significant.

**RESULTS**

**Effect of eTrkB expression vector on neuropathic pain**

In the present study, we administered this expression vector to a rat model of neuropathic pain. First, we generated a rat model of neuropathic pain by spinal nerve ligation (day 0)\(^{14}\), and divided the rats into two groups. One week later, the 50% PWT was measured using the von Frey test in rats in both...
groups, immediately before DNA administration (day 7). After confirming that there was no difference in the 50% PWT between the two groups, the rats were administered the pCMVscript-eTrkB-EGFP vector or the empty vector pCMVscript-EGFP (day 7). The pain threshold was tested on days 0, 2, 5, 7, 9, 12, and 14.

The effect of eTrkB vector administration on neuropathic pain is shown in Fig. 1. On day 7 (before vector administration), the 50% PWT was 2.34 vs. 2.85 in the eTrkB vector group (n = 6) vs. empty vector group (n = 5), respectively (p = 1.00), suggesting that there was no significant difference between the two groups prior to vector administration. On day 9 (2 days after DNA administration), the 50% PWT was 9.22 vs. 1.86 (eTrkB vector group vs. empty vector group, p = 0.032), suggesting statistically significant alleviation of pain in the eTrkB vector-administered group. On day 12 (5 days after DNA administration), the 50% PWT was 5.69 vs. 4.72 (eTrkB vector group vs. empty vector group, p = 1.00), suggesting that there was no significant difference.

**Confirmation of the eTrkB gene expressed from the expression vector**

L5 lumbar vertebral nerves were resected from the neuropathic pain model rats before and after the vector administration (days 7, 9, 12, and 14, each n = 4); mRNA was extracted and analyzed by qRT-PCR using EGFP primers and internal control gene primers (Fig. 2). The mRNA of the eTrkB-EGFP gene was not detected before vector administration (day 7), but was detected strongly at 2 days after vector administration (day 9). The expression of the eTrkB-EGFP gene was significantly decreased at 5 and 7 days after administration (days 12 and 14) (Trend test, p = 0.03) (Fig. 2).

**Effect of eTrkB protein on neuropathic pain**

To verify the pain-suppressive effect of the eTrkB-EGFP expression vector, we aimed to purify the eTrkB protein, lacking EGFP, and to administer it to neuropathic pain model rats. As a vector for producing the eTrkB protein, a plasmid was constructed that encoded the TrkB extracellular region (from the 1st to 425th amino acid), fused
Fig. 2. Confirmation of the eTrkB-EGFP gene expressed from the expression vector. Spinal cords were dissected from rats before and after eTrkB-EGFP vector administration (days 7, 9, 12, and 14, each n = 4), mRNA was extracted and reverse-transcribed. The eTrkB-EGFP mRNA expression was analyzed by quantitative reverse transcriptase polymerase chain reaction with EGFP primers, normalizing against the expression of the internal control RPL-27 mRNA. The mRNA of the eTrkB-EGFP gene was detected strongly 2 days after vector administration (day 9). RPL-27, ribosomal protein L27; *, Trend test p = 0.03.

Fig. 3. BDNF-binding activity of eTrkB protein. A plasmid encoding the extracellular domain of TrkB, fused in-frame to FLAG-Tag, S-Tag, and His-Tag (without EGFP), was constructed, and transfected into human HEK293 cells. (A) The eTrkB protein secreted into the culture medium was analyzed by western blotting using anti-TrkB and anti-FLAG antibodies. Lane 1, empty vector; lane 2, pCMVscript-eTrkB. (B) The plasmid was transfected to HEK293 cells, BDNF was added to the culture medium collected from the culture dishes. The BDNF-binding activity of eTrkB protein secreted into culture medium was examined using a pull-down assay with S-protein agarose beads and western blotting with the anti-BDNF antibody. Lane 1, empty vector; lane 2, pCMVscript-eTrkB.
Fig. 4. Effect of eTrkB protein on neuropathic pain. (A) The effect of eTrkB protein administration on neuropathic pain model rats was evaluated in the eTrkB protein administration group (n = 9) and control group (saline administration, n = 6). The protein (100 ng) was administered on day 7 after evaluation by the von Frey test. On day 9, a statistically significant suppressive effect of eTrkB protein on neuropathic pain was observed (*, p = 0.007). (B) The effect of heat-inactivated eTrkB protein administration on pain model rats was evaluated in the protein administration group (n = 9) and control group (saline administration, n = 6). The heated protein (100 ng) was administered on day 7 after evaluation using the von Frey test.

to a FLAG-Tag, S-Tag, and His-Tag in frame in pCMVscript plasmid. After transfection of the plasmid into human HEK293 cells, the western blotting and pull-down assay showed that the eTrkB protein was secreted into the culture medium and bound to BDNF (Fig. 3). Then, the protein was purified from the culture medium, and administered to rat models of neuropathic pain, similar to the vector administration experiment.

The effect of protein administration on neuropathic pain was evaluated in the eTrkB protein administration group (n = 9) and the control group (saline administration) (n = 6) (Fig. 4A). On day 7 (before protein administration), the 50% PWT was 1.56 vs. 1.01 (eTrkB protein group versus control group, p = 1.00), suggesting that there was no
significant difference before the administration. On day 9 (2 days after administration), the 50% PWT was 10.73 vs. 1.19 (eTrkB protein group vs. control group, \( p = 0.007 \)), suggesting that the eTrkB protein had a significant suppressive effect on neuropathic pain. On day 12 (5 days after administration), the 50% PWT was 4.78 vs. 1.32 (eTrkB protein group vs. control group, \( p = 0.179 \)), suggesting that there was a trend for a suppressive effect in the administration group.

**Confirmation of the suppressive effect of eTrkB by heat-inactivation**

To verify the suppressive effect of eTrkB protein on neuropathic pain, the eTrkB protein was heated at 95°C for 4 min. On day 7, we administered the heat-denatured protein to the pain model rats, and evaluated their pain threshold (\( n = 9 \)). On day 9, the heat-denatured eTrkB administration group did not show any significant difference from the control group (Fig. 4B). This result confirmed the suppressive effect of eTrkB protein on neuropathic pain.

**Effect of TrkB-Fc protein on neuropathic pain**

It has been reported that a fusion protein of human TrkB and immunoglobulin, TrkB-Fc, showed a suppressive effect on pain in a sciatic nerve ligation model mouse7). Therefore, we evaluated the effect of TrkB-Fc protein on model rats of neuropathic pain induced by spinal nerve ligation. After generating pain model rats, an equal amount of TrkB-Fc protein (100 ng/5 \( \mu l \)) was administered on day 7 (\( n = 6 \)), and their pain threshold was evaluated. However, there was no significant difference between the TrkB-Fc administered group and the control group (Fig. 5A). We then administered a 10-fold increased amount (1 \( \mu g/5 \mu l \)), and confirmed a suppressive effect of the TrkB-Fc protein on neuropathic pain in the rat model (Fig. 5B).

**Effect of ANA-12 on neuropathic pain**

ANA-12 is known to inhibit the BDNF–TrkB pathway as an antagonist of TrkB21). We administered ANA-12 (100 ng/5 \( \mu l \)) to pain model rats on day 7 after spinal nerve ligation (\( n = 7 \)). In comparison with the control group, there was no significant difference prior to administration: 0.74 vs. 0.78 (ANA-12 group vs. control group, \( p = 1.00 \)) (Fig. 6). At 4 h after ANA-12 administration (day 7.5), a significant difference was observed; the 50% PWT was 11.83 vs. 1.01 (ANA-12 group vs. control group, \( p = 0.013 \)). On the next day (day 8), a tendency for the pain-suppressive effect was observed in the ANA-12 administration group, but there was no statistically significant difference between the groups; the 50% PWT was 3.47 vs. 1.19 (ANA-12 group vs. control group, \( p = 0.070 \)). It was 1.60 vs. 1.32 (ANA-12 group versus control group, \( p = 0.88 \)) at 2 days after the administration (day 9).

**DISCUSSION**

BDNF has been reported to participate in the experience of various types of pain\(^1\),\(^16\)–\(^18\) and its expression is increased in various pain models, including acute pain and neuropathic pain models\(^1\),\(^16\),\(^17\), and in a tibia cancer pain model\(^18\). Its specific receptor is TrkB\(^19\), and BDNF acts as a modulator of pain by binding to TrkB\(^20\). Some molecules in the BDNF–TrkB signal pathway are considered to be targets for analgesic drugs.

In the present study, we showed an improvement in the pain threshold after administration of a TrkB cDNA expression vector that encodes only an extracellular region of TrkB (eTrkB) into the subarachnoid space of the spinal cord in rat models of neuropathic pain induced by spinal nerve ligation. The mRNA expression of TrkB-EGFP was observed 2 days after administration, by qRT-PCR. This result supports the results of an evaluation by the von Frey test in which the pain threshold showed statistically significant improvement 2 days after the
vector administration. In our previous study, using rat models of cancerous osteocopic pain, we showed improvement in the pain threshold by administering this vector into the subarachnoid space of the spinal cord of cancer pain model rats\textsuperscript{13}, and confirmed that TrkB-EGFP was expressed from the vector in the posterior horn of the spinal cord, using immunohistochemical analysis.

In clinical practice, administrating an expression vector into the subarachnoid space of the spinal cord may pose risks, such as neuropathy. Therefore, we produced the eTrkB protein that was composed of only an extracellular region of TrkB, and administered it into the subarachnoid space of the spinal cords of neuropathic pain model rats. The pain threshold was improved by eTrkB protein administration. In comparison, no pain alleviation was observed when the heat-inactivated eTrkB protein was administered in the same manner. These results suggested that the eTrkB protein functions

![Graph showing effect of TrkB-Fc protein on neuropathic pain](image)

Fig. 5. Effect of TrkB-Fc protein on neuropathic pain. (A) The effect of TrkB-Fc chimeric protein administration on neuropathic pain model rats was evaluated in the protein administration group (n = 6) and control group (saline administration, n = 6). The protein (100 ng) was administered on day 7 after evaluation using the von Frey test. (B) TrkB-Fc chimeric protein (1 μg) was administered in a model rat of neuropathic pain, induced by spinal nerve ligation.
in improving the pain threshold. Furthermore, the results of eTrkB protein administration might explain the mechanism underlying the pain-suppressive effect of the eTrkB expression vector in neuropathic pain model rats.

Yajima et al. reported that thermal hyperalgesia and tactile allodynia of model mice subjected to sciatic nerve ligation were suppressed by repeated intrathecal injection of a human TrkB-Fc chimera protein, which sequesters BDNF. Here, we evaluated the effect of TrkB-Fc protein in a rat model of neuropathic pain induced by spinal nerve ligation as a comparative experiment. When we administered a single dose of a 10-fold excess amount of TrkB-Fc (1 μg), as compared with the eTrkB protein amount (100 ng), a suppressive effect of TrkB-Fc protein was observed in a neuropathic pain model rats. However, the suppressive effect was not observed with a single dose of TrkB-Fc (100 ng). This may indicate that the binding activity of human TrkB (TrkB-Fc) and rat TrkB (eTrkB), to BDNF differ. Repeated injection of TrkB-Fc protein (100 ng) may be effective in pain model rats. Our results showed that a single administration of eTrkB protein has a suppressive effect on pain, even when using a smaller amount than that of TrkB-Fc protein.

ANA-12 is small molecule that antagonizes TrkB, inhibits processes downstream of TrkB, and prevents activation of the receptor by BDNF. Some studies have reported that ANA-12 improved depression. Improvement in the pain threshold by ANA-12 has not been reported previously. A suppressive effect of ANA-12 on pain model rats was observed 4 h after administration. However, on the next day (day 8), although there was a tendency for a pain-suppressive effect, there was no statistically significant difference. Because there was an improvement in the pain threshold 2 days after eTrkB protein administration (day 9), a single dose of eTrkB protein into the subarachnoid space of the spinal cord appears to be long-acting, as compared with ANA-12.

In conclusion, in neuropathic pain model rats, the pain threshold was improved by administration of a TrkB extracellular domain-encoding cDNA expression vector and of eTrkB protein. This protein had an analgesic effect that was higher than that of TrkB-Fc protein of the same amount, and
persisted for a longer time than that of ANA-12. Furthermore, the eTrkB vector was shown to have a pain-suppressive effect in a cancer pain model in our previous report. The eTrkB expression vector and the protein might be considered for use as new therapeutic reagents for intractable chronic pain, including neuropathic pain and cancer pain. In the future, clinical application, we intend to push forward a study including dosage methods.

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