Inhibitory effect of BMP-2 on the proliferation of breast cancer cells

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Abstract. Bone morphogenetic proteins (BMPs) are involved in diverse biological processes, including cell proliferation, differentiation and apoptosis. Results from the MTT assay revealed that BMP-2 significantly inhibited the proliferation of MDA-MB-231 and MCF-7 breast cancer cells. The flow cytometric analysis demonstrated that BMP-2 caused G1 arrest and promoted apoptosis. An increase in p21 and cleaved caspase-3 in the two cell lines was detected by western blot analysis, which may be responsible for the suppression of cancer cell proliferation caused by BMP-2. BMP-2 protected MDA-MB-231 and MCF-7 cells from generating xenograft tumors in nude mice. Thus, BMP-2 may be considered as an inhibitor of breast cancer at the early stages of disease.

Introduction

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β (TGF-β) superfamily that play important roles in the majority of morphogenetic processes during development. BMPs are able to induce the formation of bone at non-bony sites in adult animals by influencing the differentiation of mesenchymal progenitor cells along the cartilage lineage pathway (1,2). BMPs act not only on osteoblasts and chondrocytes, but also on numerous other cell types, including neuronal cells (3).

BMP-2 signaling involves two types of transmembrane serine/threonine kinases; type I (BRI) and type II (BRII) receptors (4-8). These two types of receptors are required to form a functional complex to initiate further signaling events. BRIs phosphorylate Smad1, Smad5 and Smad8 (R-Smads), which then assemble into heteromeric complexes with Smad4 (Co-Smad), and translocate into the nucleus to regulate the transcription of target genes (9,10). In addition, BMP receptors initiate other signaling pathways, distinct from the Smad pathway, resulting in the activation of p38 MAPK and JNK (11-13).

It has been revealed that BMP-2 is able to inhibit the proliferation of smooth muscle cells, primary glomerular mesangial cells and adrenocortical tumor cells (14-16). In breast cancer cell lines or tumor tissues, BMP-2 expression is often decreased (17,18). Ghosh-Choudhury *et al* studied the function of BMP2 and reported that BMP-2 led to G1 arrest by inducing the expression of p21 to inhibit the proliferation of breast cancer cells (19,20). However, a number of different lines of evidence have shown that BMP-2 inhibits apoptosis (21,22) rather than inhibiting the proliferation of breast cancer cells (23). The effect of BMP-2 on breast cancer is complicated and unclear, and therefore requires further study.

In this study, BMP-2 significantly inhibited the proliferation of the breast cancer cell lines MDA-MB-231 and MCF-7, by promoting cell cycle G1 arrest and apoptosis, and also inhibited the formation of cancer xenografts in nude mice *in vivo*.

Materials and methods

Cell culture and animals. Human breast cancer cell lines MDA-MB-231 and MCF-7 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). MDA-MB-231 cells were maintained in Leiboviz's L15 medium and MCF-7 cells were maintained in DMEM medium, both supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, Logan, UT, USA) in humidified air at 37˚C. Anti-β-actin, anti-phospho-Smad1/5/8 and anti-cleaved-caspase-3 were obtained from Cell Signaling Technology (Beverly, MA, USA) and anti-Smad1/5/8, HRP-goat anti-rabbit conjugate and HRP-goat anti-mouse conjugate were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). BALB/c nude mice (4-5 weeks) were obtained from the Experimental Animal Research Centre of Zhongshan University and were maintained in a specific pathogen-free laboratory. All procedures involving animals were performed in accordance with the institutional animal welfare guidelines of the Experimental Animal Research Center of Sun Yat-Sen University.

Quantitative polymerase chain reaction (qPCR). Total cellular RNA was extracted from cell cultures using a TRIzol

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Table I. Primers used for real-time PCR.

Genes	Forward	Reverse
β-actin	ATTGCCGACAGGATGCAGA	GAGTACTTGCGCTCAGGAGGA
BMPR1b	AATGCCACCATTGTCCA	CTAGGCAACCAGAAGTGACCACAG
BMPR1a	AGTGTCTCCAGTCAAGCTCTGGGTA	CCATCTCTGCTGCTGCGCTCATTTA
BMPR2	TGCAGATGGACGCATGGAA	CGGCAAGAGCTTACCCAGTCA
Smad4	CAGCACTACCACCTGGACTGGA	CTGGAATGCAAGCTCATTGTGAA
p21	TGAGCCGCGACTGTGATG	GTCTCGGTGACAAAGTCGAAGTT

BMPR, bone morphogenetic protein receptor.

reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions, and resuspended in 50 μl of water treated with 0.1% DEPC (Sigma-Aldrich, Seelze, Germany). RNA concentration was determined using a BioPhotometer (Eppendorf Scientific, Hamburg, Germany). Reverse transcription of total RNA (2 μg) primed with an oligo(dT) oligonucleotide was performed using a SuperScript™ III First-Strand Synthesis SuperMix reverse transcription kit (Invitrogen) according to the manufacturer's instructions. qRT-PCR was performed using an IQ SYBR-Green mix in an iCycler PCR machine (Bio-Rad, Hercules, CA, USA). β-actin was used as the housekeeping control gene. The mean value of 3 wells was calculated, and each experiment was repeated 3 times. The primers (Invitrogen, Shanghai, China) designed for the respective genes are shown in Table I.

Western blot analysis. To determine the effect of BMP-2 on the activation of Smad1/5/8, MDA-MB-231 and MCF-7 cells

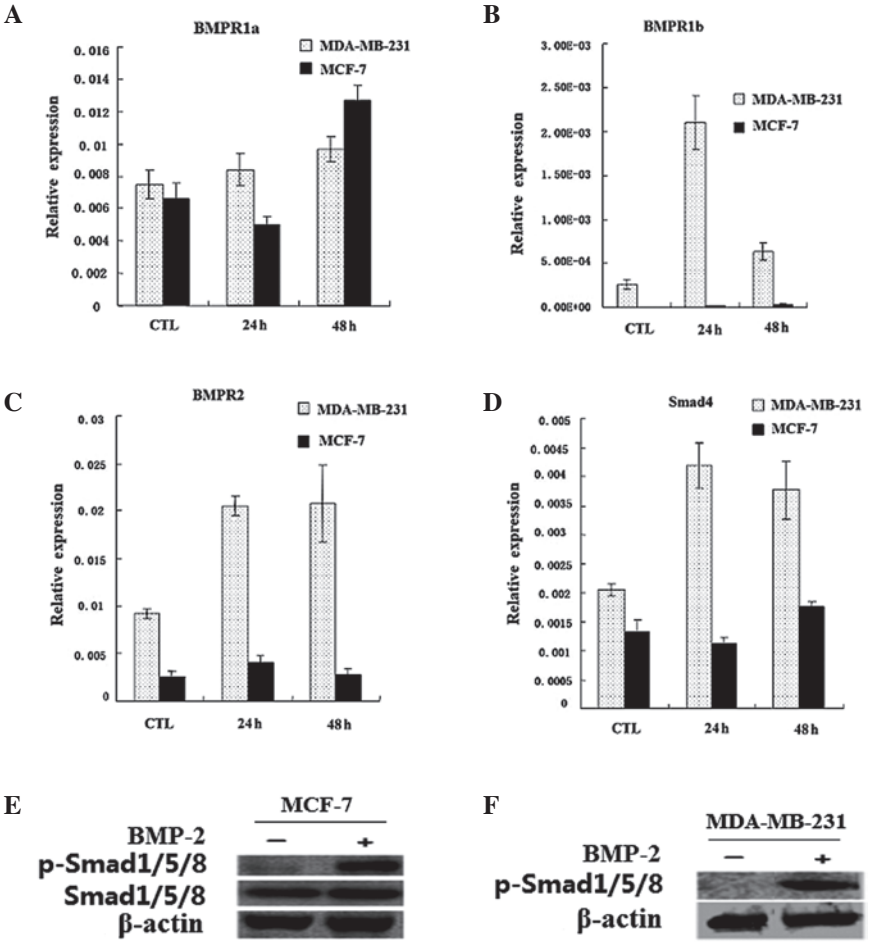

Figure 1. Effects of BMP-2 on the BMP pathway of MCF-7 and MDA-MB-231 cells. (A-D) Quantitative real-time PCR analysis of BMPR1a, BMPR1b, BMPR2 and Smad4 mRNA in MCF-7 and MDA-MB-231 cells. The relative expression levels of these genes are shown in the indicated cells treated with 0 or 20 μg/l BMP-2. (E-F) Western blot analysis of the activation of the BMP signal pathway in MCF-7 and MDA-MB-231 cells. β-actin served as the internal control. BMP, bone morphogenetic protein.

were starved in serum-free medium overnight to knock down the endogenous level of phosphorylated kinases. Cells were pretreated with 20 μg/l BMP-2 for 15 min, and then solubilized by incubation for 15 min at 4˚C in cell lysis solution (Upstate Biotechnology, Inc., Waltham, MA, USA) containing a protease inhibitor cocktail (Roche). The protein concentration of the soluble extracts was determined using a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Separation of 50 μg of total protein was performed on 12% SDS-polyacrylamide gels and was transferred to a polyvinylidene difluoride membrane before immunoblotting with primary antibodies. Equal loading of protein samples was verified with antibodies (dilution, 1:3000) against Smad1/5/8, pi-Smad1/5/8 and β-actin. The specific proteins were detected using an enhanced chemiluminescence detection system (Thermo Fisher Scientific).

MTT assay. MDA-MB-231 and MCF-7 cells were used to detect the antitumor effects of BMP-2. Cells were cultured in 96-well plates (~5,000 cells per well) for 24 h. Cells were then starved with their respective medium plus 1% dialyzed fetal calf serum (A15-107; PAA Laboratories, Linz, Austria) for 24 h. The experiment included a control group and BMP-2 groups (2.5, 5, 10, 20 or 30 μg/l). After 48 h of BMP-2 induction, cell growth was measured using an MTT assay. Absorbance was recorded at 570 nm, with a reference at 630 nm serving as the blank. The effect of BMP-2 on cell viability was assessed as the percentage of cell viability compared with the control cells, which were arbitrarily assigned 100% viability. The mean value of 5 wells was calculated, and each experiment was repeated 3 times.

Flow cytometry. Human breast cancer cell lines MDA-MB-231 and MCF-7 in the logarithmic phase of growth were incubated with 20 μg/l BMP-2 for 24 h. For cell cycle analysis, samples ($1x10^6$ cells) were fixed and permeabilized by the addition of 1 ml of ice-cold 70% ethanol for 15 min on ice. Following washing, the cells were resuspended in 125 μl of 1.12% (w/v) sodium citrate containing 0.2 mg/ml RNase (Roche) and incubated for 15 min at 37˚C. Propidium iodide (PI; 50 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) was added to the cells for 30 min at room temperature in the dark. The cells were then stored at 4˚C until they were assayed by flow cytometry (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA). Cell cycle analysis was performed using ModFit LT software (Verity Software House, Inc., Topsham, ME, USA).

Quantification of apoptotic cells. Apoptosis induced by BMP-2 in MDA-MB-231 and MCF-7 cells was determined by flow cytometry using the Annexin V-conjugated Alexa Fluor 488 (Alexa488) Apoptosis Detection kit (Millipore), according to the manufacturer's instructions. Following overnight serum starvation, cells were treated with BMP-2 (20 μg/l) for 48 h. The cells were then collected, washed in PBS and incubated with Alexa488 and PI for cell staining in the dark at room temperature for 10 min. The stained cells were analyzed by FACS using a FACSCalibur instrument (BD Biosciences) equipped with CellQuest 3.3 software.

Nude mice xenograft model of breast cancer cell lines. BALB/c 4- to-6-week-old female mice, weighing 18-22 g, were randomly divided into 4 groups. Two groups were intravenously injected with $1x10^7$ MDA-MB-231 cells, and the other two groups were intravenously injected with $1x10^7$ MCF-7 cells. The mice (n=6 per group) were treated with either PBS (vehicle) or 20 μg/l BMP-2 via the tail vein daily for 14 days. After the animals were sacrificed, the tumors were weighed.

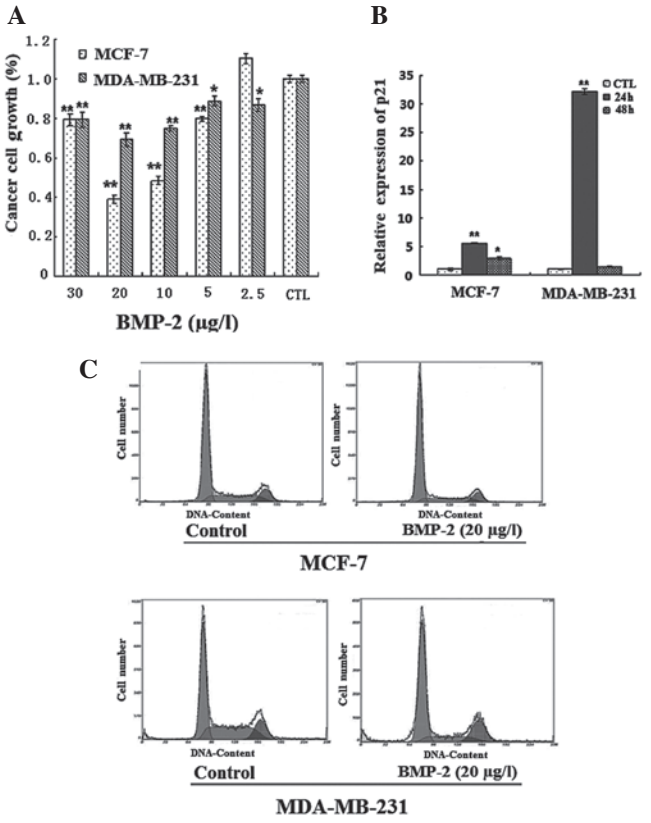

Figure 2. Cell growth *in vitro*. (A) Effects of BMP-2 on the proliferation of MCF-7 and MDA-MB-231 cells revealed by the MTT assay. Cells were treated with 2.5, 5, 10, 20 or 30 μg/l BMP-2 for 48 h. Growth of the two cell lines was inhibited differently. (B) p21 induction folds were detected in MCF-7 and MDA-MB-231 cells by qRT-PCR after treatment with BMP-2 (20 μg/l) for 24 h. Results shown are representative of three separate experiments yielding similar results. (C) MCF-7 and MDA-MB-231 cells were treated with BMP-2 for 24 h. Cells were collected for cell cycle distribution analysis by flow cytometry. BMP, bone morphogenetic protein.

Statistical analysis. Statistical analysis was performed using the Statistical Package for Social Sciences 13.0 (SPSS). Data were presented as the mean ± SEM. The Student's t-test (two-tailed) was used to compare the two groups for independent samples assuming equal variances among all experimental data sets. $P<0.05$ was considered to indicate a statistically significant difference.

Results

BMP-2 signaling pathway assay in MDA-MB-231 and MCF-7 cells. The mRNA expression levels of BMPR1a, BMPR1b, BMPR2 and Smad4 in MDA-MB-231 and MCF-7 breast cancer cell lines are shown in Fig. 1A-D. The expression of the genes increased to varying degrees in the MDA-MB-231 cells when induced by BMP-2 for 24 or 48 h. However, the level of

Table II. BMP-2 inhibits tumor formation in the MDA-MB-231 and MCF-7 breast cancer cells in nude mice.

Group	Tumor weight (g)						Mean (g)	P-value
MDA-MB-231								
Control	0.3126	0.2755	0.539	0.2963	0.3252	0.2422	0.3318	0.004
BMP-2	-	-	-	-	-	0.3602	0.0600	
MCF-7								
Control	0.8612	0.4201	0.3815	0.2831	0.6200	0.2480	0.4690	0.026
BMP-2	0.0814	0.2292	0.3655	0.1021	0.2974	-	0.1793	

BMP, bone morphogenetic protein; -, no tumor.

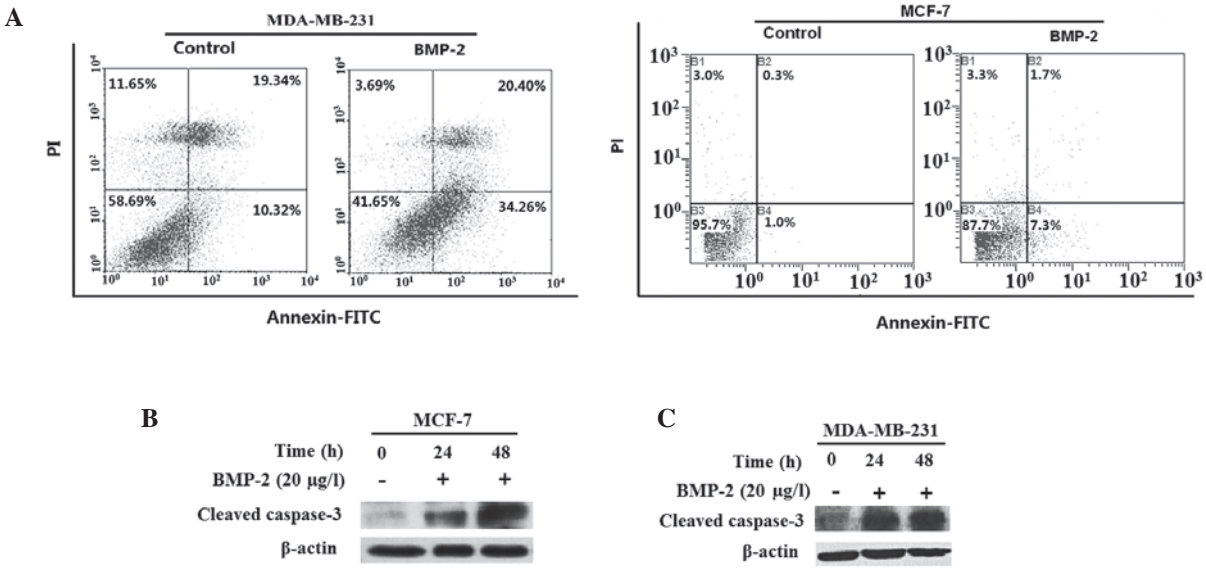

Figure 3. Cell apoptosis *in vitro*. (A) Apoptosis of MCF-7 and MDA-MB-231 cells incubated with BMP-2 (20 μg/l) for 48 h was detected by flow cytometry. (B-C) Expression of cleaved caspase-3 was detected in the MCF-7 and MDA-MB-231 cells by the western blot analysis following treatment of BMP-2 (20 μg/l) for 24 and 48 h. BMP, bone morphogenetic protein.

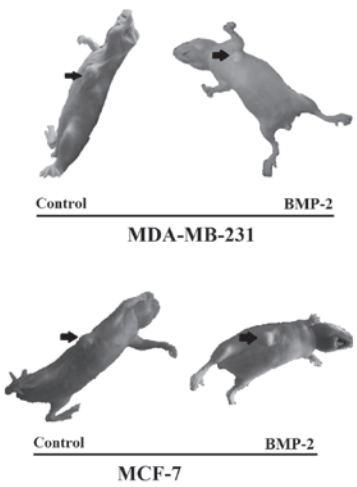

Figure 4. Detection of xenograft tumors in MCF-7 and MDA-MB-231 breast cancer cells in Balb/c (nu/nu) nude mice. The formation of tumors was detected as described in Materials and methods. The inhibitory effect of BMP-2 on the transplanted tumors was distinct in the MCF-7 and MDA-MB-231 groups. BMP, bone morphogenetic protein.

the genes was lower in the MCF-7 cells; only BMPR1a was distinctly upregulated by BMP-2 following 48 h.

Results of the western blot analysis showed that 20 μg/l BMP-2 is able to activate the BMP signaling pathway, which led to the rapid phosphorylation of Smad1/5/8 (Fig. 1E-F). This finding indicated that the BMP signaling pathway remained intact in the MDA-MB-231 and MCF-7 cells.

Inhibitory effect of BMP-2 on the proliferation of breast cancer cells. Cells were induced with 2.5, 5, 10, 20 or 30 μg/l BMP-2 for two days. Results of the MTT assay indicated that the strongest inhibition of BMP-2 on the proliferation of MDA-MB-231 and MCF-7 cells was 30 and 60%, respectively, following the addition of 20 μg/l BMP-2 (Fig. 2A). This finding may be due to the more rapid growth rate of MDA-MB-231 cells compared to MCF-7 cells.

Effect of BMP-2 on the breast cancer cell cycle. The flow cytometric analysis demonstrated that cells treated with

20 μg/l BMP-2 for two days remained in the G1 phase from 46.7 to 62% in the MDA-MB-231 cells and 63.7 to 70.8% in the MCF-7 cells, respectively. This finding indicated that BMP-2 effectively inhibited cell proliferation by initating G1 arrest (Fig. 2C). BMP-2 was also able to induce a rapid expression of p21 (Fig. 2B), which was in accordance with results by Ghosh-Choudhury *et al* which revealed that the increase in p21 led to G1 arrest (19,20).

Pro-apoptotic function of BMP-2 on breast cancer cells. In MDA-MB-231 and MCF-7 cells, BMP-2 was able to promote apoptosis following the addition of 20 μg/l BMP-2 for two days. The early apoptotic cells increased from 10.3 to 34.3% in MDA-MB-231 cells (Fig. 3A) and from 1.0 to 7.3% in MCF-7 cells. In addition, there were ~20% of necrotic cells in the MDA-MB-231 cells. We also observed that the expression of cleaved caspase-3 in the two cell lines was upregulated (Fig. 3B and C).

Inhibitory effect of BMP-2 on the tumorigenesis of MDA-MB-231 and MCF-7 breast cancer cells in the Balb/c (nu/nu) nude mice xenograft model. Balb/c (nu/nu) nude mice were administered with breast cancer cells and xenograft tumors were allowed to form for 14 days. In the MDA-MB-231 group, subsequent tumor analysis demonstrated that 5/6 mice in the control subgroup generated tumors, while only 1/6 mice in the BMP-2 treatment subgroup generated tumors (Table II). In the remaining 5/6 mice, the cancer cells transplanted were necrotic and pulp-like (Fig. 4). We also found in the MCF-7 group that the mean tumor weights of the control subgroup and BMP-2 treatment subgroup were 0.4690 and 0.1793 g, respectively, demonstrating that BMP-2 was also an inhibitor of tumor formation in MCF-7 cells.

Thus, BMP-2 significantly inhibited the formation of tumors in MDA-MB-231 and MCF-7 cells in nude mice (Fig. 4).

Discussion

As previously reported, BMPs described as either growth stimulators or growth inhibitors appear to depend on dosage, type of cells or tissue and tumor microenvironment (24,25). In this study, BMP-2 was found to inhibit cancer cell growth *in vitro* and *in vivo* by inducing G1 arrest and apoptosis in MDA-MB-231 and MCF-7 human breast cancer cell lines. The inhibitory effect on cell growth appears to be due to the G1 phase arrest caused by BMP-2-induced p21 upregulation. p21 is able to bind to cyclin E/CDK2 and inhibit CDK2 activity, thereby preventing cells progressing from G1 to S phase (26). Furthermore, the observed increase in cell apoptosis may be correlated with the upregulation of the caspase signaling pathway by BMP-2.

In this study, the expression of BMPRs and Smad4 in MDA-MB-231 cells was higher than that in MCF-7 cells. Furthermore, there was evident auto-enhancement of BMP signaling following the induction of BMP-2 in MDA-MB-231 cells. These results may explain the reason for the inhibitory effect of BMP-2 on tumor formation in MDA-MB-231 cells being stronger than that in MCF-7 cells *in vivo*.

To the best of our knowledge, this is the first study demonstrating that BMP-2 was able to inhibit MDA-MB-231 and MCF-7 tumor formation in nude mice. Although certain lines of evidence have demonstrated that BMP-2 promotes the metastasis of breast cancer (23,27-28), such an event was not observed in this study. It is worth mentioning that in this study the formation of breast tumors was terminated after two weeks, which may have been an insufficient period of time to observe the metastasis of breast xenograft tumors.

In conclusion, BMP-2 was an inhibitor of early tumor development through the induction of G1 arrest and apoptosis.

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