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Subcellular daunorubicin distribution and its relation to multidrug resistance phenotype in drug-resistant cell line SMMC-7721/R

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Abstract

AIM: **To investigate the correlation between subcellular daunorubicin distribution and the multidrug resistance phenotype in drug-resistant cell line SMMC-7721/R.**

METHODS: **The multidrug resistant cell line SMMC-7721/R, a human hepatocellular carcinoma cell line, was established. Antisense oligonucleotides (AS-ODN) were used to obtain different multidrug resistance phenotypes by inhibiting the expression of mdr1 gene and/or multidrug resistance-related protein gene(mrp) using Lipofectamine as delivery agent. Expression of mdr1 and mrp genes was evaluated by RT-PCR and Western blotting. Intracellular daunorubicin (DNR) concentration was measured by flow cytometry. Subcellular DNR distribution was analyzed by confocal laser scanning microscopy. Adriamycin (ADM) and DNR sensitivity was examined by MTT method.**

RESULTS: **Low level expression of mdr1 and mrp mRNAs and no expression of P-Glycoprotein(P-gp) and multidrug resistance-related protein (P_{190}) were detected in parental sensitive cells SMMC-7721/S, but over-expression of these two genes was observed in drug-resistant cell SMMC-7721/R. The expression of mdr1 and mrp genes in SMMC-7721/R cells was down-regulated to the level in the SMMC-7721/S cells by AS-ODN. Intracellular DNR concentration in SMMC-7721/S cells was 10 times higher than that in SMMC-7721/R cells. In SMMC7721/S cells intracellular DNR distributed evenly in the nucleus and cytoplasm, while in SMMC-7721/R cells DNR distributed in a punctate pattern in the cytoplasm and was reduced in the nucleus. DNR concentration in SMMC-7721/R cells co-transfected with AS-ODNs targeting to mdr1 and mrp mRNAs recovered to 25 percent of that in SMMC7721/Scells. Intracellular DNR distribution pattern in drug-resistant cells treated by AS-ODN was similar to drug-sensitive cell, and the cells resistance index (RI) to DNR and ADM decreased at most from 88.0 and 116.0 to 4.0 and 2.3, respectively. Co-Transfection of two AS-ODNs showed a stronger synergistic effect than separate transfection.**

CONCLUSIONS: **P-gp and P_{190} are two members mediating MDR in cell line SMMC7721/R. Intracellular drug concentration increase and subcellular distribution change are two important factors in multidrug resistance (MDR) formation. The second factor, drugs transport by P-gp and P_{190} from cell nucleus to organell in cytoplasm, may play a more important role.**

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INTRODUCTION

Multidrug resistance (MDR) remains a significant obstacle for cancer chemotherapy. The MDR observed in many cell lines is most commonly accompanied with overexpression of one or both of the members of the ATP-binding cassette superfamily of transport proteins, P-glycorprotein (P-gp) and multidrug resistance-related protein(Mrp, P_{190})[1-7]. P-gp or P_{190} acts as an energy-dependent outward transport pump, removing drugs from the cytoplasm and from the plasma membrane, thereby decreasing intracellular drug accumulation[8-12].

Human hepatocellular carcinoma drug-resistant cell line SMMC7721/R showed a strong multidrug resistance to DNR and other anthracycline, and overexpression of P-gp and P_{190} was observed in this cell line. Previous studies suggested that subcellular drug distribution contributing to cells drug resistance may be mostly mediated by P-gp and/or P_{190} in many other cell lines[13-20]. But there is no direct evidence suggesting the role of these two pump proteins in MDR of SMMC7721/R. In order to understand MDR phenotype and mechanism in SMMC7721/R, based on previous studies of antisense technology related to mdr1 gene and mrp gene, we used laser scanning confocal microscopy to evaluate the intracellular distribution of DNR and then explored the correlation of intracellular drug(DNR) transportation and distribution with multidrug resistance phenotype.

MATERIALS AND METHODS

Cell lines and culture conditions

Human hepatocellular carcinoma cell line SMMC-7721 was provided by Cancer Research Institution, West China Hospital of Sichuan University. Drug-resistance cell line was established by the stepwise selection with increasing concentration of ADM as previously described[21]. The ADM gradually increased from 0.005 μmg/ml to 0.1 μmg/ml.

Cells were grown in RPMI 1640 medium supplemented with 10 % fetal calf serum in a 5 % CO_2 atmosphere at 37 ℃.

Materials

Phosphorothioate antisense oligonucleotides(AS-ODN): targeting to mdr1 start codon region (AS-ODN/mdr1): 5' -CCA TCC CGA CCT CGC GCT CC-3' [22], targeting to mrp coding region (AS-ODN/mrp): 5' -TGC TGT TCG TGC CCC CGC CG-3' [23]. Control oligonucleotide (AS-ODN/nonsense) was a 20-mer nonsense phosphorothioate oligonucleotide. All oligonucleotides were synthesized by Life Technologies Inc, USA. Lipofectamine, TRIzol, RT-PCR kit, and primers were also purchased from Life Technologies Inc. Lumi-Light^{PLUS} Western Blotting Kit was purchased from Boehringer Mannheim, German. Antibodies against mdr1/P-gp and mrp/P_{190} were from Santa Cruz, USA. DNR was purchased from Minalo Inc, Italy.

Treatment of cells with AS-ODNs

The experimental protocols were similar to those previously described[22,24]. Briefly, cells(5×10^5) were seeded in a 25 ml flask at 1×10^5 cells/ml and grown to 75 % confluence. Cells were transfected with 1.5 nmol of AS-ODN with 50 ml of Lipofectamine. Cells were harvested at different times after transfection for analysis.

Detection of drug sensitivity of cells by MTT[25,26]

Cells were exposed to drug at 37 ℃ for 2 h, then were washed and seeded (50 000 cells/ml) in 96-well microplates for 72 h. MTT (20 μl, 2.5 mg/ml) was added to each well for 3 h. Medium was discarded and 150 μl of DMSO were added to each well. Optical densities were measured at 490nm (A490). The tumor cells living ratio (TCL) was determined according to the formula: $TCL=A490_{experient}/A490_{control}\times100$ %. The 50 % inhibitory concentration (IC_{50}) was calculated according to concentration-TCL curve. Resistance index (RI) was calculated using the formula: $RI=IC50_{drug\ resisant\ cells}/IC50_{parent\ cells}$.

Measurement of mdr1 and mrp mRNAs by RT-PCR

Primers mrp: 5' -TGA AGG ACT TCG TGT CAG CC-3' , 5' -GTC CATGAT GGT GTT GAG CC-3' ; mdr1: 5' -GGC TCC GAT ACA TGG TTT TCC-3' , 3' -TTC AGT GCG ATC TTC CCA GC-5' . β_2-microglobulin(β_2M): 5' -ACC CCC ACT GAA AAA GAT GA-3' ,5' -ATC TTC AAA CCT CCA TGA TG-3' .

β_2M expression was used as control for the amount of RNA used. Total RNA from cells was extracted using TRIzol. The effect of AS-ODN was studied after 24 h pre-incubation with AS-ODN and Lipofectamine. Mrp, mdr1, and β_2M RNA transcripts were detected using RT-PCR as described before[27,28]. An aliquot of each reaction mixture was then analyzed by electrophoresis on 2 % agarose gel. Densitometry was performed using UVP gel image analysis system (BIO-RAD, USA) and the ratio between the target and control PCR products was determined by dividing the densitometric volume of the target band by that of the control band.

Measurement of cell expression of P-gp and P_{190} with western blotting

The expression of the two proteins was detected according to the manufacturer' s instructions of Lumi-Light^{PLUS}Western Blotting Kit. The concentrations of the primary antibodies against P-gp and P_{190} were 1 μg/ml and 2 μg/ml, respectively. The concentrations of the secondary antibodies were both 0.4 μg/ml.

Observation of intracellular DNR distribution by confocal laser scanning microscopy (CLSM)

The experimental procedures were similar to those previously described[29-31]. Cells (1×10^5) were seeded to 960 mm^2 petri dish with a slide inside and incubated in a 5 % CO_2 atmosphere at 37 ℃. After cells had reached 75 % confluence, normal medium was replaced with serum-free RPMI 1640 medium and cells were incubated with DNR at 2 μg/ml for 1 h. The effect of AS-ODN was studied after 72 h pre-incubation with AS-ODN and Lipofectamine. After two washes with PBS and addition of drug free medium cells grown on slides were examined with CLSM (MRC-1024ES, BIO-RAD Inc., USA). Cover-slips were mounted on slides, supported and sealed on lacquer tiers to prevent compression and drying out. Intracellular drug fluorescence was observed using the 488 nm laser line for excitation and the filter that allows measurement of emitted light above 515 nm.

Detection of intracellular DNR concentration by flow cytometry (FCM)

Cells (1×10^5) were seeded into 25 ml flask and grown to 95 % confluence. They were then dissociated with pancreatin and suspended in serum-free medium. DNR was added to a final concentration of 2 μg/ml and incubated for 1 h at 37 ℃. After two washes with PBS each sample was divided into 3 tubes to be analyzed by FCM[32] (Elite ESP, Coulter Inc., USA). Results were expressed as the ratio of fluorescence intensity values between each experimental sample and control sample of SMMC7721/R cells without treatment.

RESULTS

Drug sensitivity

As shown in Table 1, the parental sensitive cells SMMC7721/S were highly sensitive to DNR and ADM. However, the drug resistant cells SMMC7721/R were 88-fold resistant to DNR and 116-fold to ADM, respectively, when compared with SMMC7721/S cells. Resistance index(RI) to DNR and ADM in SMMC7721/R cells co-transfected of AS-ODN/mdr1(0.5 μmol/L)and AS-ODN/mrp(0.5 μmol/L) decreased from 88 and 116 to 4 and 2.3, respectively. Treatment with separate AS-ODN/mdr1 or AS-ODN/mrp showed a lesser reversal effect on MDR than co-transfection of these two AS-ODNs. Nonsense oligonucleotides did not affect the drug resistance in SMMC7721/R cells.

Table 1 Effect of AS-ODN on drug sensitivity of cells ($\bar{x}\pm s$)

Cells	IC_{50}(mg/L)		RI	
	DNR	ADM	DNR	ADM
SMMC-7721/S	0.003±0.0006	0.004±0.0008	1.0	
SMMC-7721/R	0.264±0.0094	0.463±0.0254	88.0	116.0
AS-ODN/mdrr1	0.094±0.0065	0.079±0.0014	31.4	19.8
AS-ODN/mrp	0.072±0.0002	0.097±0.0009	24.0	24.3
SMMC-7721/R+ AS-ODN/mdr1+mrp	0.012±0.0011	0.009±0.0007	4.00	2.3
SMMC-7721/R+ AS-ODN/nonsense	0.245±0.0110	0.451±0.0187	81.7	112.8

Values(IC_{50}) represent the mean±standard deviation of at least three experiments and value(RI) represent the mean of three experiments. All values were calculated as described in MATERIALS AND METHODS. The concentration of each AS-ODN is 0.5 μmol/L and the treatment time is 72 hours.

Expression of mdr1 geng and mrp gene

As shown by RT-PCR, The amplification products of mrp, mdr1, and β_2M were 256bp, 168bp, and 120 bp, respectively. Over-expression of mrp and mdr1 mRNAs were detected in SMMC7721/R, but low level mRNA expression in SMMC7721/S was observed. The mRNA expression in SMMC7721/R cells treated with AS-ODN decreased to the level of SMMC7721/S (Figure 1).

No P-gp and P_{190} were detected in parental cells SMMC7721/S. Over-expression of P-gp and P_{190} was observed in drug resistant cells SMMC7721/R. Treatment of SMMC7721/R with AS-ODNs inhibited the expression of P-gp and P_{190} (Figure 2).

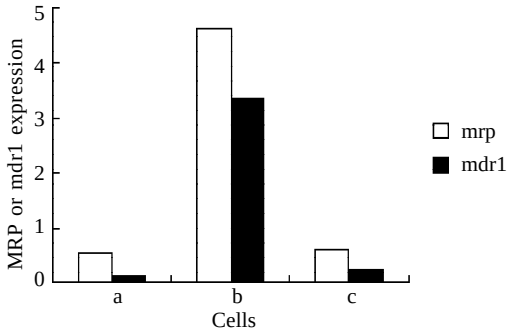

Figure 1 Quantification of PCR. The ratio between the mdr1 or mrp and β_2M gene is expressed as described in MATERIALS AND METHODS. (a. parental cell-SMMC7721/S; b. SMMC7721/R; c. SMMC7721/R incubated with 0.5 μmol/L AS-ODN for24 hours)

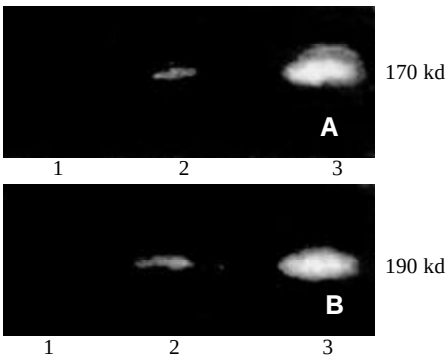

Figure 2 Expression of P-gp and P_{190} analyzed with Wstern blot. (a) immunoblotted with anit-P-gp antibody; (b) immunoblotted with anti-P_{190}antibody. 1. parental cell-SMMC7721/S; 2. SMMC7721/R treated by AS-ODN(0.5 μmol/L, 72 hours); 3. SMMC7721/R.

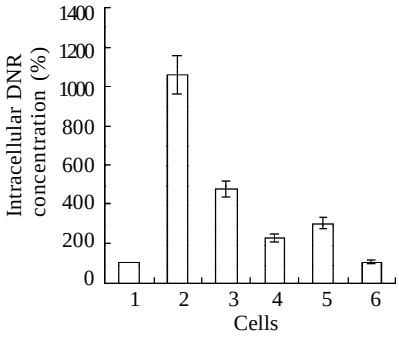

Figure 3 Intracellular DNR concentrations in cells treated with antisense oligonucleotids. The concentration of each AS-ODN is 0.5 mmol/L and the treatment time is 72 hours. Data are the mean¡Àstandard deviation of three independent experiments. (1. SMMC7721/R; 2. SMMC7721/S; 3. SMMC7721/R treated with AS-ODN/mdr1+mrp; 4. SMMC7721/R treated with AS-ODN/mdr1; 5. SMMC7721/R treated by AS-ODN/mrp; 6. SMMC7721/R treated with AS-ODN/nonsense.)

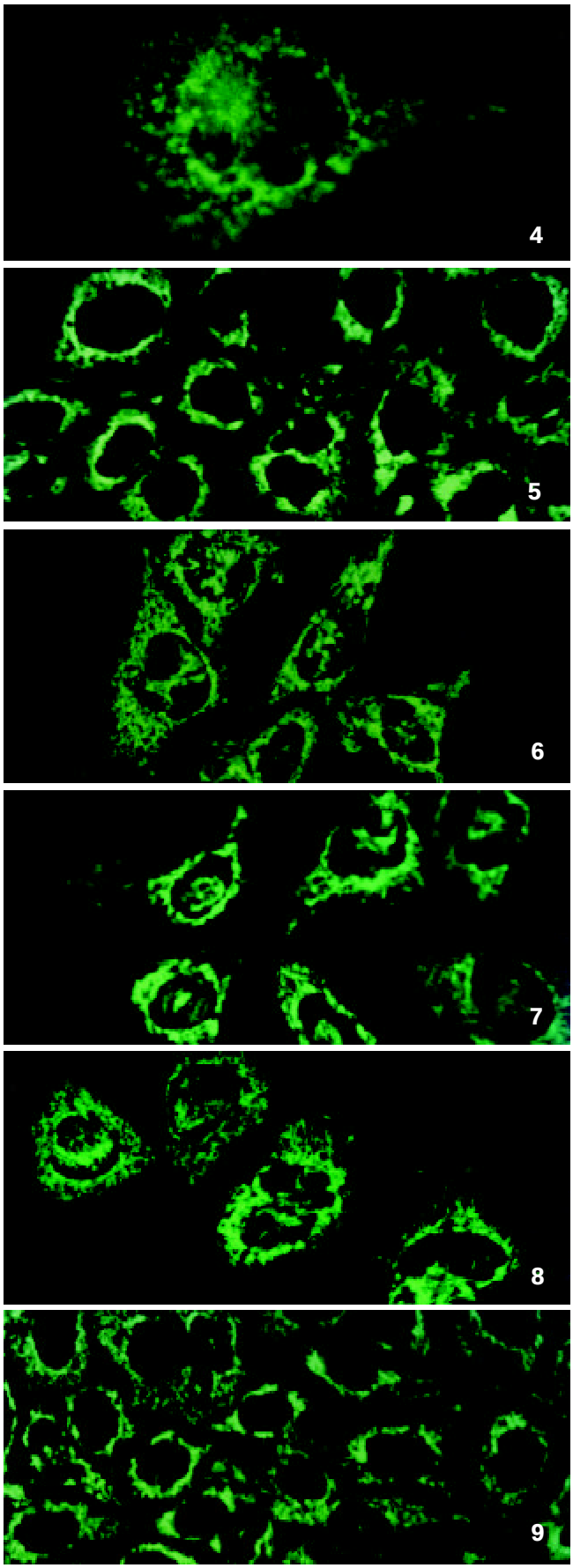

Figure 4 Intracellular DNR distribution in parental sensitive cells SMMC7721/S.

Figure 5 Intracellular DNR distribution in drug-resistant cells SMMC7721/R.

Figure 6 Intracellular DNR distribution in drug-resistant cells SMMC7721/R pre-treated with AS-ODN/mdr1 and AS-ODN/mrp. Each AS-ODN concentration is 0.5 μmol/L and the treatment time is 72 hours.
Figure 7 Intracellular DNR distribution in drug-resistant cells SMMC7721/R pre-treated with AS-ODN/mdr1(0.5 μmol/L) for 72 hours.
Figure 8 Intracellular DNR distribution in drug-resistant cells SMMC7721/R treated with AS-ODN/mrp(0.5 μmol/L) for 72 hours.
Figure 9 Intracellular DNR distribution in drug-resistant cells SMMC7721/R treated with nonsense oligonucleotides(0.5 μmol/L) for 72 hours.

Intracellular accumulation of DNR

Intracellular concentration of drugs in drug-resistant cells reduced to 10 percent of that in parental cells SMMC7721/S. Co-transfection of AS-ODN/mdr1+mrp up-regulated intracellular drug concentration to 4 times of that in SMMC7721/R cells. The up-regulation effect of separate transfection of AS-ODN/mdr1 or AS-ODN/mrp was much less than co-transfection (Figure 3).

Subcellular distribution of DNR

In parental cells SMMC7721/S DNR fluorescence mainly located in the nucleus and was diffusely present in the cytoplasm (Figure 4). In drug-resistant cells SMMC7721/R, DNR fluorescence retained in the perinuclear zone and in peripheral punctate vesicles, with little or no drug in the nuclear zone (Figure 5).

In SMMC7721/R cells incubated separately with AS-ODN/mrp or AS-ODN/mdr1 for 72 h, the subcellular distribution of DNR was similar to parental cells SMMC7721/S, with less intranuclear fluorescence intensity than in SMMC7721/S cells. Cells transfected separately with AS-ODN/mrp or AS-ODN/mdr1 did not show any difference in subcellular DNR distribution(Figure 7 and Figure 8). Co-transfection of AS-ODN/mdr1 and AS-ODN/mrp caused more drugs accumulation in nuclear than that in cells incubated separately with either AS-ODN (Figure 6). Nonsense oligonucleotides did not affect the drug subcellular distribution pattern in drug-resistant cells SMMC7721/R (Figure 9).

DISCUSSION

It has been reported that parental SMMC7721 cells have no or low level expression of mdri/P-gp and mrp/P_{190}. However, over-expression of P-gp and P_{190} was observed in drug-resistant cells SMMC7721/R, which was confirmed in our study.

In order to obtain cell line with different multidrug resistance phenotypes, we made use of the specificity of antisense oligonucleotides in down-regulating gene expression to specifically block mdr1/P-gp or mrp/P_{190} function. Antisense technology is increasingly becoming a reliable tool for manipulation of gene expression and rapidly moving into the therapeutic arena[33-47]. Down-regulation of expression of mdr1 mRNA/P-gp and mrp mRNA/P_{190} by AS-ODN was observed and accompanied with recovery of drug sensitivity in SMMC7721/R cells. Drug-resistant SMMC7721/R cells co-transfected with AS-ODN/mrp and AS-ODN/mdr1 showed 50-fold drug sensitivity to ADM, when compared with drug-resistant cells SMMC7721/R.

The fluorescence emitted by anthracycline DNR, a substrate of P-gp and P_{190}, can be detected by FCM and CSLM[14,17,31,48,51]. There is a good correlation between intracellular drug concentration and cells drug sensitivity[8,16,49,50,52,53]. In our studies, FCM data enforced the opinion that reduction of intracellular drug concentration results in increase of drug resistance in cells. Intracellular DNR concentration in cell line SMMC7721/R decreased to 10 percent of that in SMMC7721/S and meanwhile resistance to ADM and DNR increased by 116 times and 88 times respectively than that of SMMC7721/S. Co-transfection of AS-ODN/mrp and AS-ODN/mdr1 only restored intracellular DNR concentration to 40 percent of that of SMMC7721/S. Inconsistently, the drug resistance to DNR was reduced from 88-fold to 4-fold and to ADM from 116-fold to 2.3-fold when compared with SMMC7721/S. These data indicate that some other factors must play a more important role in MDR mechanism besides the reduction of intracellular drug accumulation. The mechanism focused in recently is modified drug localization[13,51,54]. We used CSLM technology to explore this interesting point.

In our studies, we observed that in cell line SMMC7721/S DNR fluorescence distributed evenly in the nucleus and cytoplasm, while in cell line SMMC7721/R DNR distributed in a punctate pattern in the cytoplasm and was reduced in the nucleus. Transfection of AS-ODN changed the the subcellular DNR distribution pattern in SMMC7721/S cells to that in SMMC7721/R cells. This observation indicates that P-gp or P_{190} not only pumps DNR out of cells, but also transports DNR from nuclear to cytoplasm and into some organelles such as Golgi apparatus. As P-gp or P_{190} probably locates in cell membrane, nuclear membrane,Golgi apparatus,or endoplasmic reticulum[13,54-59], the actions of these two proteins may cause reduction of intracellular and intranuclear drug concentration and drug accumulation in some organelles. The combined effect may prevent the targeting of the drugs to nucleus, which reduces cell death even if the total amount of drugs inside the cells was not dropped significantly. Golgi apparatus is widely believed to be the organelle which holds the drugs[54,59]. Previous studies found that there were some differences in subcellular drug distribution pattern between different cell lines in which MDR phenotype was mediated by P-gp and by P_{190}[49]. However, we did not observe such phenomenon in the present study.

Our studies suggest that after the potent inhibition of P-gp or P_{190} expression by AS-ODN intracellular drug concentration was increased and subcellular drug distribution changed, which leads to the reversal of multidrug resistance in cell line SMMC7721/R. Therefore, we believe that over-expression of P-gp and/or P_{190} is an important mechanism in mediating MDR in cell line SMMC7721/R.

Although co-transfection of AS-ODNs targeting the two genes didn' t enhance the inhibitory effect on expression of P-gp or P_{190} when compared with separate transfection of either AS-ODN, the synergistic effects of the two AS-ODNs on reduction of intracellular or intranuclear drugs and on recovery of cell drug sensitivity were much more prominent than separate transfection. This finding indicates that MDR in SMMC7721/R is mediated at least by both P-gp and P_{190}. The combination of MDR reversal methods against these proteins is effective in drug-resistant cells.

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