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Influence of cytarabine and cyclophosphamide on the disposition kinetics of cyclosporin A after bone marrow transplantation

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Abstract The blood concentration of cyclosporin A (CyA) often gradually increases or is unstable in the early period of immunotherapy in bone marrow transplantation patients. In the protocol for bone marrow transplantation, pretreatment with cytarabine (Ara-C) and cyclophosphamide (CPA) is employed. We examined the influence of Ara-C and CPA on the disposition kinetics of CyA, in using rats to define the mechanism for the observation. Rats were intravenously administered daily with Ara-C (120 mg/kg/day) or CPA (60 mg/kg/day) intravenously for 2 days and were then intravenously given CyA (10 mg/kg). The blood concentration of CyA after intravenous administration of CyA at 1 day after the last administration of CPA was significantly lower and the total clearance was significantly larger than those in the vehicle control rats, while the blood concentration and the pharmacokinetic parameters of CyA were unchanged after Ara-C treatment. The expression of *mdr1a*, *mdr1b*, and *CY3A2* mRNAs, and the levels of the corresponding proteins in the liver were increased after the CPA treatment. These CPA-induced changes were almost fully reversed to the control levels by 2 weeks. Thus, our results indicate that the decrease of blood CyA concentration induced is a consequence of the induction of P-glycoprotein and CYP3A in the liver by the CPA treatment , and these changes are reversed within 2 weeks after the transplantation.

Keywords Cyclosporin A · Cyclophosphamide · Drug interaction · Disposition kinetics · CYP3A2 · P-glycoprotein

Introduction

We have frequently experienced that the blood concentrations of cyclosporin A (CyA) increases gradually or is unstable in the early period of the immunotherapy in many patients who have received bone marrow transplantation. It is a potentially serious problem that the blood concentration-time course of CyA or tacrolimus (FK506) shows a wide variation, even in individual patients, and consequently therapeutic drug monitoring is essential to the success of the transplantation, as well as for the prevention of graft versus host disease (GVHD) and other side effects. However, little is known about the mechanisms of the variation.

Bone marrow transplantation patients are pretreated with various steroid hormones and anti-tumor agents as a part of the protocol for immunotherapy. We previously reported that the blood concentrations of CyA [1] or FK506 [2] decrease in rats treated with dexamethasone (DEX). In that paper, we clarified that the decrease is caused by the induction by DEX of P-glycoprotein (P-gp) and CYP3A in the liver and intestine, and

the changes are reversed within 2 weeks after cessation of DEX treatment. However, it is possible that other combined drugs, such as anti-tumor agents, also affect the disposition kinetics of immunosuppressants. Therefore, in this study, we employed a rats model to examine the influence upon CyA disposition kinetics of cytarabine (Ara-C) and cyclophosphamide (CPA) used in the immunotherapy protocol before bone marrow transplantation. Based on our results and published data, we discuss the mechanisms of the changes in the blood concentration of CyA.

Materials and methods

Materials

Sandimmun® injection (cyclosporin A, CyA), Cylocide®injection (cytarabine, Ara-C), and Endoxan® injection (cyclophosphamide, CPA) were purchased from Novartis Pharma Co. Ltd. (Tokyo, Japan), Nippon Shinyaku Co. Ltd. (Kyoto, Japan), and Shionogi Pharmaceutical Co. Ltd. (Osaka, Japan), respectively. Oligonucleotide primers were custom-synthesized by Amersham Pharmacia Biotech (U.K.). Primary antibody (goat anti-rat CYP3A2 antibody) and secondary antibody (biotinylated anti-goat IgG) were purchased from Daiichi Pure Chemicals Co. Ltd. (Tokyo, Japan) and Vector Laboratories, Inc. (CA, USA), respectively. Other reagents were purchased from Sigma Co. (St. Louis, MO).

Animal experiments

All animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Kanazawa.

Animal experiments were carried out in the same way to the protocol for immunotherapy in our hospital (Fig. 1).

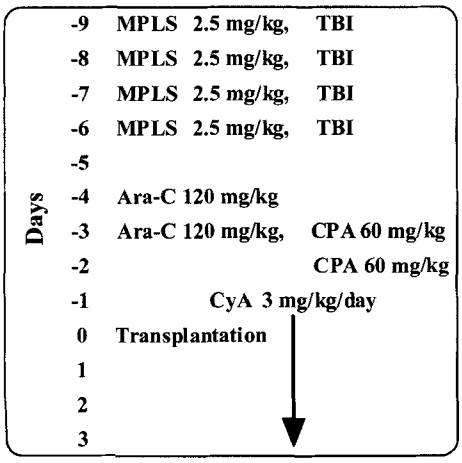

Fig. 1 Treatment protocol for bone marrow transplantation patients (*MPLS* methylprednisolone; *Ara-C* cytarabine; *CPA* cyclophosphamide; *CyA* cyclosporin A; *TBI* total body irradiation [300 cGy])

Male Wistar rats weighing 280–300 g (Japan SLC Co., Hamamatsu, Japan) were intravenously injected daily for 2 days with a solution of Ara-C (120 mg/kg/day) or CPA (60 mg/kg/day). The control rats were injected with saline alone. A 100-μl aliquot of CyA in distilled water (10 mg/kg) was injected via the femoral vein at 72 h after the last treatment with Ara-C. The solution of CyA was injected via the femoral vein at 48 h or 14 days after the last treatment with CPA. Blood samples (200 μl each) were collected at designated time intervals from the jugular vein under light ether anesthesia.

Measurement of blood concentration of CyA

Blood concentration of CyA was measured with a TDx analyzer using a commercial kit according to the manufacturer's instructions (Dainabot Co. Ltd., Tokyo, Japan). The TDx assay is a fluorescence polarization immunoassay (FPIA) reagent system for the measurement of CyA in whole blood [3]. The measurement range of blood concentration was 25–1500 ng/mlL. The cross-reactivities with the metabolites of CyA were 19.4% for M1 and less than 5% for other metabolites.

Reverse transcriptase-polymerase chain reaction (RT-PCR) assay

Total RNA was isolated from the liver and intestine by using an Isogen Kit (Wako, Osaka). Synthesis of cDNA from the isolated total RNA was carried out using RNase H- reverse transcriptase (GIBCO BRL, Rockville, MD). Reverse transcription (RT) reactions were carried out in 40 mM KCl, 50 mM Tris-HCl (pH 8.3), 6 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM each of dATP, dCTP, dGTP, and dTTP, 10 units of RNase inhibitor (Promega, Madison, WI), 100 pmol of of random hexamer, total RNA and 200 units of the Moloney murine leukemia virus reverse transcriptase (Gibco-BRL, Berlin, Germany) in a final volume of 50 μl at 37 °C for 60 min. Polymerase chain reaction (PCR) was carried out in a final volume of 20 μl, containing 1 μl of RT reaction mixture, 50 mM KCl, 20 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 10 μM each of the mixed oligonucleotide primers, and 1 unit of Taq DNA polymerase (Gibco-BRL). Primers used for rat *mdr1a* were 5′–AGA AAC AGA GGA GCG CCA TT–3′ and 5′–GAA TTC AAC TTC AGG ATC CG–3′ (511 bp) ([4]), those for rat *mdr1b* were 5′–ACT GAG CTT CGA GGT GAA GA–3′ and 5′–CAG AGC TGA TGT CGC TTC AT–3′ (451 bp) [4], those for rat *CYP3A2* were 5′–AGT AGT GAC GAT TCC AAC ATA T–3′ and 5′–TCA GAG GTA TCT GTG TTT CCT–3′ (252 bp) [5], and those for rat β-actin were 5′–TTC TAC AAT GAG CTG CGT GTG GC–3′ and 5′–CTC (A/G)TA GCT CTT CTC CAG GGA GGA–3′(456 bp), as previously reported by Waki et al. [6]. Each cycle consisted of 30 sec at 94 °C, 60 sec at 60 °C, and 75 sec at 72 °C for *mdr1a* and *mdr1b*, 30 sec at 94 °C, 60 sec at 55 °C, and 75 sec at 72 °C for *CYP3A2*, and 30 sec at 94 °C, 60 sec at 58 °C, and 75 sec at 72 °C for β-actin. PCR reaction was run for 26 cycles for *mdr1a*, *mdr1b*, and *CYP3A2*, and for 22 cycles for β-actin, respectively.

Preparation of plasma membrane fraction and microsomes

For the preparation of plasma membrane fraction, the liver was homogenized in 100 mM Tris-HCl, pH 7.4. The homogenate was centrifuged at 1,500 × *g* for 15 min, and the supernatant was then centrifuged at 100,000 × g for 60 min. The pellet was washed, resuspended in Tris buffer, and stored at –80 °C until analysis. Rat liver microsomes were prepared as described previously [7] and stored at –80 °C until analysis. Protein concentrations were measured according to the method of Lowry et al. [8].

SDS-PAGE and immunoblotting

SDS-PAGE and immunoblotting with peroxidase/antiperoxidase staining of the plasma membrane for P-gp and the microsomes for CYP3A2 were carried out essentially as described by Laemmli [9] and Guengerich et al. [10]. The sample protein (100 μg) was electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gel and transferred onto immobilon PVDF (Millipore Co. Ltd., MA, USA). After having been blocked with 5% skim milk, the filters were incubated overnight with 1 mg/mL primary antibody, goat anti-rat CYP3A2 antibodies (Daiichi Pure Chemicals Co. Ltd., Tokyo, Japan) and C219 (Dako Co., CA, USA), and for 1 h with secondary antibody, biotinylated anti-goat IgG (Vector Laboratories, Inc., CA, USA). Thereafter, the sample was extensively washed with phosphate-buffered saline. The immunopositive band was detected by means of a light-emitting nonradioactive detection system (Amersham International plc, Little Chalfont, Buckinghamshire, UK).

Patients

In twelve patients who had received the bone marrow transplantation, the blood concentration-time courses of CyA at the trough after administration of CyA (3 mg/kg/day, i.v., 2 times a day, daily, over 30 days) varied characteristically for 30 days (Fig. 2). The blood concentrations of CyA in the initial period of administration after the transplantation were lower than the pharmacokinetically predicted concentrations (200–300 ng/mlL) relative to the dosage, and subsequently increased over 20 days in every patient. These patients had received a combination of methylprednisolone (MPLS), cytarabine (Ara-C), and cyclophosphamide (CPA) before the transplantation (Fig. 1).

Data analysis

The pharmacokinetic parameters were estimated according to model-independent moment analysis as described by Yamaoka et al. [11]. The data were analyzed using the Student's *t*-test to compare the unpaired mean values of two sets of data. The number of determinations is noted in each table and figure. A value of $P < 0.01$ was taken to indicate a significant difference between sets of data. The electrophoresis results were analyzed by using computer aimed NIH-Iimage analyzersoftware.

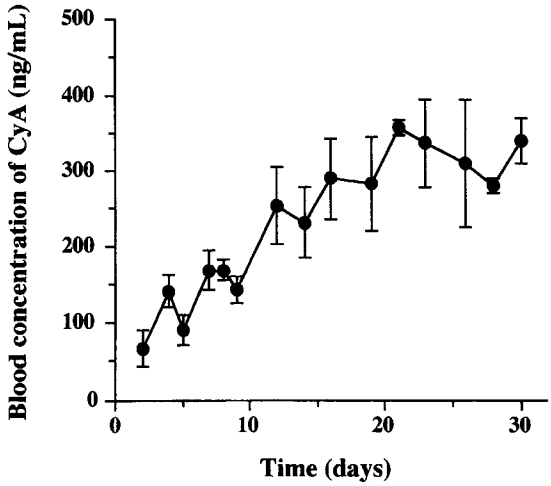

Fig. 2 Blood concentration-time courses of CyA after i.v. administration of CyA (3 mg/kg/day, 2 times a day, daily) in twelve patients received bone marrow transplantation, after pretreatment according to the protocol indicated in Fig. 1. Each point and bar represents the mean ± SD of 3–6 patients

Results

Pharmacokinetics of CyA after Ara-C or CPA treatment in Rats

Figure 3 shows the blood concentration-time courses of CyA after an i.v. administration of CyA (10 mg/kg) to rats pretreated with Ara-C (120 mg/kg/day, i.v.) and/or CPA (60 mg/kg/day, i.v.). CyA was administered to the rats at 2 days after treatment with Ara-C for 2 days, or at 1 day after treatment with CPA for 2 days. The blood

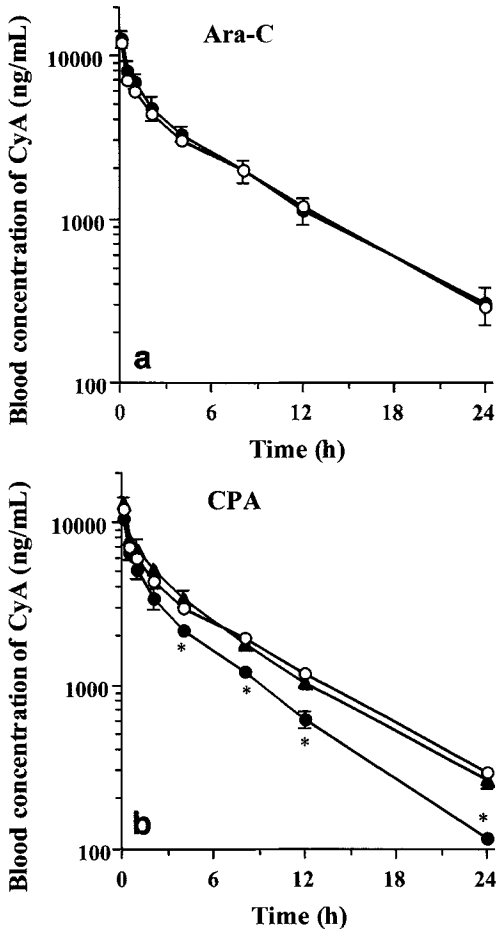

Fig. 3a,b Blood concentration-time courses of CyA after an i.v. administration of CyA (10 mg/kg) in vehicle control rats (○) and in rats treated daily for 2 days with *Ara-C* (120 mg/kg/day, i.v.) or *CPA* (60 mg/kg/day, i.v.). **a** CyA was administered at 2 days (●) after the last administration of Ara-C. **b** CyA was administered at 1 (●) or 14 days (▲) after the last administration of CPA. Each point and bar represents the mean ± SE of four rats. *Significantly different from vehicle control rats at $P < 0.01$

Table 1 Pharmacokinetic parameters of cyclosporin A (*CyA*) after i.v. administration (10 mg/kg). CyA was administered at 2 days after the last administration of Ara-C (120 mg/kg/day i.v.) or at 1 or 14 days after the last administration of *CPA* (60 mg/kg/day i.v., for 2 days). Each value represents the mean ± SD of four rats (*AUC* area under the curve; *MRT* mean residence time; Vd_{ss} volume of distribution at steady state; CL_{tot} total clearance)

Parameters	Vehicle control	Ara-C treatment	CPA treatment	
		2 days	1 day	14 days
AUC (μg× h/mL)	48.7 ± 0.7	51.2 ± 1.5	33.3 ± 2.1*	50.3 ± 1.6
MRT (h)	7.26 ± 0.25	7.21 ± 0.31	5.49 ± 0.58*	6.69 ± 0.36
Vd_{ss} (L/kg)	1.49 ± 0.05	1.41 ± 0.08	1.65 ± 0.13	1.33 ± 0.09
CL_{tot} (mL/h/kg)	205 ± 17	196 ± 32	302 ± 48*	198 ± 38

*Significantly different from vehicle control rats at $P < 0.01$

concentrations of CyA after treatment with Ara-C alone did not differ from those in the vehicle control (Fig. 3a). On the other hand, the blood concentrations of CyA after treatment with CPA were significantly lower than those in the vehicle control, but recovered almost completely to the control level at 14 days after the last CPA treatment (Fig. 3b).

The pharmacokinetic parameters of CyA in rats treated with Ara-C or CPA are listed in Table 1. These parameters of CyA in rats treated with Ara-C showed no significant difference from those in the control rats. In contrast, the area under the blood concentration-time curve from 0 to the infinity (AUC) of CyA at 2 days after the last treatment in rats treated with CPA was significantly lower than that in the control rats. The total clearance (CL_{tot}) of CyA at 2 days after the last treatment was significantly higher than that of the control rats, whereas the distribution volume at the steady-state (Vd_{ss}) was unchanged. All parameters recovered to control levels at 14 days after the last CPA treatment. The blood concentration curves and the pharmacokinetic parameters of CyA in rats treated with the combination of Ara-C and CPA were very similar to those in of rats treated with CPA alone (data not shown).

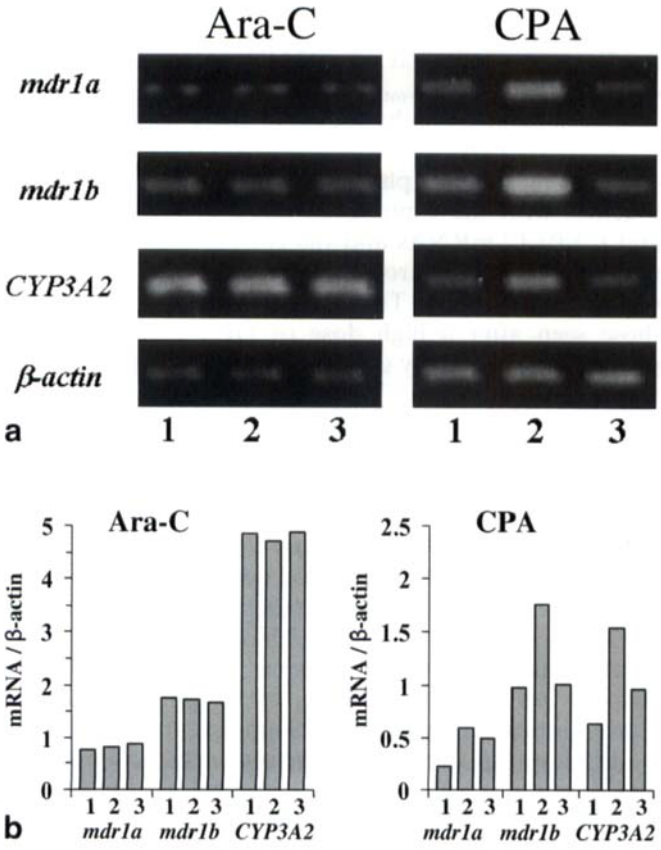

Fig. 4a,b Effects of Ara-C or CPA on the expression of *mdr1a*, *mdr1b*, and *CYP3A2* mRNAs in the liver of vehicle control and Ara-C- or CPA-treated rats. **a** Rats were intravenously given daily with Ara-C (120 mg/kg/day) or CPA (60 mg/kg/day) for 2 days. The sizes of the reverse transcriptase-polymerase chain reaction (*RT-PCR*) products are 511 bp (*mdr1a*), 451 bp (*mdr1b*), and 252 bp (*CYP3A2*). **b** Relative expression of *mdr1a*, *mdr1b* and *CYP3A2* mRNAs. *Lane 1* vehicle control, *lane 2* at 2 days after the last administration of Ara-C or at 1 day after the last administration of CPA, *lane 3*at 14 days after the last administration of Ara-C or CPA

RT-PCR Analysis of *mdr1a*, *mdr1b*, and *CYP3A2* mRNAs in the Liver

Figure 4 shows the effects of Ara-C (120 mg/kg/day, i.v., 2 days) or CPA (60 mg/kg/day, i.v., 2 days) on the expressions of *mdr1a* and *mdr1b* mRNAs for P-gp and *CYP3A2* mRNAs in the liver. In rats treated with Ara-C, the expressions of these mRNAs at 3 and 14 days after the last Ara-C treatment was hardly changed. But, in rats treated with CPA, the expressions of all these mRNAs at 2 days after the last CPA treatment was markedly increased and then reverted to the control levels 14 days after the last CPA treatment.

Western Blot Analysis of P-gp and CYP3A2 in the Liver

Figure 5 shows the protein levels of P-gp and CYP3A2 in the liver after Ara-C or CPA treatment. The levels of the two proteins were unchanged by Ara-C, but were

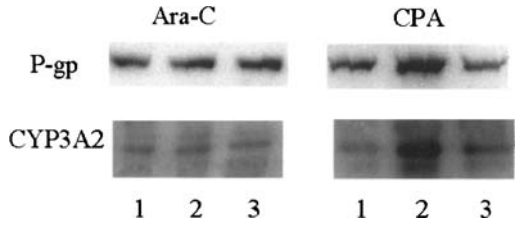

Fig. 5 Western blot analysis of P-gp and CYP3A2 in the liver of vehicle control and Ara-C or CPA-treated rats. Rats were intravenously given daily with Ara-C (120 mg/kg/day) or CPA (60 mg/kg/day) for 2 days. *Lane 1*, vehicle control; *lane 2*, at 2 days after the last administration of Ara-C or at 1 day after the last administration of CPA; *lane 3*, at 14 days after the last administration of Ara-C or CPA

increased by treatment with CPA, and recovered subsequently reverted to the control levels.

Discussion

Generally, the blood concentration of CyA after transplantation must be maintained within the range of about 200–300 ng/mL [12]. However, the attempts to predict the trough concentration of CyA in the initial period after transplantation from the dosage have presented difficulties. We have frequently found that the trough concentration of CyA after transplantation (about 75 ng/mL) is lower than the predicted concentration (about 300 ng/mL) estimated using the normal pharmacokinetic parameters [12] of CyA (Fig. 2). In the immunotherapy protocol for bone- marrow transplantation, CyA is administered together with other drugs such as steroid hormones and anti-tumor agents. Because CyA is a substrate of P-gp and CYP3A [13, 14, 15, 16], possible changes of the blood concentration of CyA should be considered when it is administered in combination with drugs, which may influence the activity and/or expression of P-gp and CYP3A. We have previously demonstrated in in vivo studies that the blood concentration of CyA was lowered as a result of inhibition of intestinal absorption, as well as enhanced metabolism and excretion, due to induction of P-gp and CYP3A in the liver and intestine by DEX [1, 2]. In the immunotherapy protocol for bone -marrow transplantation in our hospital (Fig. 1), a steroid hormone MPLS (2.5 mg/kg, intravenous perfusion) is included to prevent vomiting following total body irradiation. In our previous paper, DEX (75 mg/kg, intraperitoneal injection) enhanced the expression of both P-gp and CYP3A2, but 1 mg/kg DEX (about 5 mg/kg potency equivalent MPLS) induced only P-gp, at least after 4-times intraperitoneal injections [1]. Therefore, because the dose of MPLS (2.5 mg/kg) appeared to be too small to influence the expression of P-gp and CYP3A, we sought other possible other causes of the decrease in CyA concentration.

We examined in this study whether Ara-C and CPA influence the disposition kinetics of CyA in rats. We found that CPA significantly lowered the blood concentrations of CyA and increased the CL_{tot} value, whereas Ara-C did not influence the disposition kinetics of CyA (Fig. 3 and Table 1). Moreover, CPA, but not Ara-C, induced the expressions of *mdr1a*, *mdr1b*, and *CYP3A2* mRNAs and increased the protein levels of P-gp and CYP3A2 in the liver (Figs. 4, and 5). These results suggested that the AUC value of CyA is decreased and the CL_{tot} value is increased as a result of enhanced metabolism by CYP3A2 and enhanced biliary excretion by P-gp.

We found that the pharmacokinetic behavior of CyA, together with the expressions levels of *mdr1a*, *mdr1b*, and *CYP3A2* mRNAs and the corresponding proteins, recovered to the control levels at 2 weeks after the last CPA administration. These findings are very similar to those seen after a high dose of DEX (75 mg/kg), as previously reported by us [1, 2]. The present report is the first to indicate that CPA induces the expression of a drug transporter and a metabolic enzyme.

The laboratory data for hepatic function in rats treated with CPA or Ara-C were not significantly changed compared with those in the control rats (data not shown). Thus, CPA induced P-gp and CYP3A2 in the liver, resulting in lower blood concentrations of CyA during the early period after transplantation. Although these changes were reversed within 2 weeks, successful transplantation without adverse reactions depends strongly on the maintenance of an adequate blood concentration of the immunosuppressant in the early stage of transplantation. Therefore, active monitoring and dose management of CyA are required for at least 2 weeks after CPA treatment in bone marrow transplantation patients.

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