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Hepatobiliary Disposition of 3′-Azido-3′-deoxythymidine (AZT) in the Rat: Effect of Phenobarbitone Induction*

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Abstract—Isolated liver with a recirculating perfusate was used to study 3′-azido-3′-deoxythymidine (AZT) disposition in phenobarbitone-pretreated rats at 68 μM AZT concentration in the reservoir. Clearance of AZT in the livers obtained from control animals was 0·42 ± 0·01 (mean ± s.d.) mL min^{-1}/10 g liver. Over the study period of 105 min, 12·7 ± 2·6% of the dose was excreted in bile and of this 95% was recovered as 3′-azido-3′-deoxy-5′-*O*-β-D-glucopyranuronosylthymidine (GAZT). The amount of GAZT found in the perfusate after 105 min of liver perfusion was < 1% of the AZT dose introduced into the reservoir. Phenobarbitone pretreatment of rats resulted in a 5·5-fold increase of AZT clearance. In addition, the area under the perfusate concentration-time curve ($AUC_{0-105\ min}$) for 3′-amino-3′-deoxythymidine (AMT) and for a catabolite of unknown structure was increased 3- and 10-fold, respectively, and the amount of AZT dose excreted in the bile was nearly doubled. Thus phenobarbitone was capable of stimulating both detoxification of AZT to GAZT and bioactivation of AZT to AMT, a catabolite known to be highly toxic to human bone marrow cells. This induction was the result of enhancement of AZT catabolism rather than its transport into the cells, since on incubation of AZT (0–250 μM) with rat isolated hepatocytes, a linear relationship between concentration and amount taken up by the cells was shown. In addition, the rate of AZT uptake was not influenced by KCN, dinitrophenol, or temperature, which is consistent with a simple diffusion of AZT through the hepatocellular membrane. Rats dosed intraduodenally with [^{3}H]GAZT excreted 5·8 ± 3·3% of the GAZT dose in bile within 4 h after administration. This suggests that enterohepatic recycling is involved in AZT disposition in the rat.

3′-Azido-3′-deoxythymidine (AZT) is a thymidine analogue active against human immunodeficiency virus, a causative agent of acquired immunodeficiency syndrome (Fischl et al 1987).

Preclinical disposition studies of AZT in experimental animals including mice, rats, rabbits, and cats have shown that AZT was largely eliminated unchanged with 3′-azido-3′-deoxy-5′-*O*-β-D-glucopyranuronosylthymidine (GAZT) being a minor urinary catabolite (Good et al 1986; Doshi et al 1989; Hedaya & Sawchuk 1989; Patel et al 1989; Unadkat et al 1989; de Miranda et al 1990; Ahmed et al 1991). In man and in cynomolgus monkeys, GAZT represented the main pathway of AZT elimination, accounting for more than 60% of the dose recovered in urine (Good et al 1986, 1990; Cload 1989). The discrepancy between human and rat in-vivo conversion of AZT to GAZT was explained by the higher catalytic efficiency of the human enzyme when compared with the rat enzyme (Resetar & Spector 1989; Cretton et al 1990).

Considering the above studies, the monkey was assumed to be a good model, whereas the rat appeared to be inappropriate for the study of AZT pharmacokinetics. However, both the rat model and AZT catabolism in monkeys and man had to be re-examined in the light of two new findings. First, was the discovery of the 3′-amino-3′-deoxythymidine (AMT) catabolite on incubation of AZT with rat isolated hepatocytes (Cretton et al 1991a) and second, the finding of Mays et al (1990) that the rat with an exteriorized bile duct excreted more than 10% of the AZT dose as GAZT in the bile. Moreover, Mays et al (1991) found that probenecid pretreatment of rats resulted in decreased GAZT formation and AZT renal excretion, an effect previously observed in man (Kornhauser et al 1989). On the other hand, Cretton et al (1991b) reported the formation of AMT in monkeys, and Stagg et al (1992) showed that the area under the curve (AUC) of AMT in plasma represented nearly 30% of the AUC of AZT after its intravenous administration to patients. AMT was found to inhibit haemoglobin synthesis (Weidner et al 1992) and to be highly toxic to human bone marrow cells (Cretton et al 1991a).

Very recently, we presented a rapid method for the biosynthesis and isolation of GAZT from rat bile from perfused liver. The catabolite so obtained can then be used as an analytical standard (Kukan et al 1993). However, detailed information on AZT disposition in rat perfused liver is still lacking in the literature. Nevertheless, understanding of this process deserves attention because of the usefulness of the rat model for predicting the interactions between AZT and concurrently administered drugs, and the potential extrapolation of the results obtained from the rat to man (Mays et al 1991). Neither the role of reabsorption of GAZT from the rat intestine nor its subsequent elimination by bile has been taken into consideration as yet. Therefore, the objectives of this study were to elucidate the disposition of AZT in rat perfused liver using the recirculating mode of perfusion, to examine the effect of microsomal enzyme induction with phenobarbitone on the disposition of AZT and its catabolites in the rat perfused liver, to determine whether carrier

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mediated mechanisms of uptake play a role in AZT transport into liver cells, and to ascertain whether GAZT can be absorbed after intraduodenal administration to the rat.

Materials and Methods

Materials

[5-^{3}H]AZT C sp. act. 1600 mCi mmol^{-1}, radiochemical purity >98%) was kindly donated by the Institute of Isotopes, Hungarian Academy of Sciences (Budapest, Hungary). Unlabelled AZT was obtained from Lachema (Brno, Czech Republic). AMT was synthesized by Dr Holý at the Institute of Organic Chemistry and Biochemistry (Prague, Czech Republic). The authenticity of AMT was confirmed by comparing mass spectra, using a GC/MS HP 5890 gas chromatograph with an HP 5970 mass selective detector (Hewlett-Packard, Palo Alto, CA, USA). GAZT and [^{3}H]GAZT were prepared by biosynthesis in rat perfused liver as described and confirmed by NMR (Kukan et al 1993). Phenobarbitone sodium was purchased from Fluka (Buchs, Switzerland). Uridine 5′-diphosphoglucuronic acid (UDPGA) and bovine uridine 5′-diphosphoglucuronyltransferase (UDPGT) were purchased from Sigma (St Louis, MO, USA). All other chemicals used were of the highest purity commercially available.

Rat liver perfusion with AZT

Male Wistar rats, 240–270 g, from our breeding station (Dobrá Voda, Slovakia) were used. Three rats were given phenobarbitone in 0·9% NaCl intraperitoneally (80 mg kg^{-1} in 2 mL) on the first day, followed by 0·1% phenobarbitone in their drinking water over five days. The control group received vehicle alone. The rat liver perfusions were carried out essentially according to the procedure described by Bezek et al (1990a), which involved a slightly modified technique of Sies (1978). The livers were perfused from the reservoir (116 mL) containing AZT at the initial concentration 68 μM and 50 μCi [^{3}H]AZT in Krebs–Henseleit buffer. The perfusate recirculated through the liver at 4 mL min^{-1} g^{-1}. During 105 min of perfusion, 0·4 mL samples were taken at intervals. Bile samples were collected into Eppendorf tubes at 5-min intervals. At the end of perfusion, the livers were blotted, weighed and homogenized in 10 mL distilled water. Homogenate (1 mL) was digested in 5 mL 25% KOH in 20% ethanol.

Uptake experiments of AZT with rat isolated hepatocytes

Hepatocytes were isolated according to the two-step procedure of Seglen (1976). Viability and yield were assessed by trypan blue dye exclusion and counting in a Buerker chamber, respectively. Cell viability for all experiments was >90%. The stock cell suspension contained 2×10^7 cells mL^{-1}. Stock cells (0·1 mL) were added to 0·8 mL Krebs–Henseleit buffer, pH 7·4, fortified with glucose (10 mM final concentration) containing HEPES (10 mM final concentration), and preincubated at 37°C for 5 min in a metabolic shaker. After preincubation, 0·1 mL substrate ($1·5 \times 10^6$ d min^{-1} mL^{-1} plus unlabelled drug) was added and incubated for various times to determine the initial velocity of uptake. The period of 10 s was selected as the incubation time with the concentrations of AZT ranging from 0 to 250 μM. To test the effect of metabolic inhibitors and thymidine (each at 1 mM final concentration) and of temperature (4°C), the cells were preincubated for 5 min. The uptake was quantified by a centrifugal filtration technique through a 1-bromododecane layer according to the method of Wosilait et al (1981).

Incubation of AZT with bovine UDPGT

In an attempt to prepare GAZT from AZT, incubation was performed in round bottom flasks (50 mL) placed in a Dubnoff shaker with 120 oscillations min^{-1} at 37°C. Each flask contained [^{3}H]AZT (10^6 d min^{-1} mL^{-1}), unlabelled AZT (final concentration 10 mM), 5 mM $MgCl_2$, 10 mM UDPGA, bovine UDPGT (2 mg mL^{-1} crude powder; 0·004 units mL^{-1}) in Tris-HCl, pH 7·5. Samples were taken at 2, 4, and 6 h after admixture of the substrate.

Kinetics of [^{3}H]GAZT in-vivo

Four rats with surgically implanted bile cannulae, as described by Bezek et al (1990b), were dosed intraduodenally with 15 μCi [^{3}H]GAZT (1 mg/rat) in 1 mL control bile diluted with 0·9% NaCl. Bile samples were collected in scintillation vials. Appropriate samples were taken for determination of total ^{3}H. The radioactivity was measured as given below.

Determination of AZT and its catabolites

Samples (0·2 mL) of the perfusate were lyophilized. The residue was dissolved in 0·2 mL methanol, applied to silica gel TLC plates (UV_{254}, Kavalier Votice, Czech Republic) with carriers of AZT, GAZT, and AMT and developed in 80% acetonitrile in water. Bile samples (10 μL) were applied directly to TLC plates. The R_f values in 80% acetonitrile were: 0·8, 0·48, 0·22, and 0·05 for AZT, GAZT, AMT, and unidentified material, respectively (Kukan et al 1993). After developing the plates, the areas corresponding to AZT, GAZT, AMT, and the unknown were visualized under UV_{254} light, cut out, placed into scintillation vials, and eluted with 1 mL methanol. After addition of 10 mL liquid scintillator (SLS 31 Spolana (Neratovice, Czech Republic)), radioactivity was determined in a liquid scintillation counter (TriCarb 300 CD, Packard Instruments, Downers Grove, IL, USA). Counting efficiency was determined by external standardization.

Determination of total ^{3}H

Perfusate (0·1 mL), bile (10 μL) from rat liver perfusions and 0·2 mL diverted bile from the in-vivo experiment were counted after adding 15 mL SLD 31. Radioactivity was measured as above.

Treatment of data

$AUC_{0\text{-}105\ min}$ for AMT and the unknown were calculated by the trapezoidal rule. Clearance for AZT was calculated as the ratio between the dose introduced into the reservoir and $AUC_{0\text{-}\infty}$, which was determined by the trapezoidal rule and extrapolated to infinity, dividing the last perfusate AZT concentration by the elimination rate constant (k_{el}). The k_{el} value for AZT was estimated by log-linear least-squares fit of the last eight perfusate concentrations. Statistical analysis was by unpaired Student's *t*-test.

Table 1. Effect of phenobarbitone pretreatment on AZT clearance in the rat liver perfused with [^{3}H]AZT.

	AZT clearance (mL min/10 g)	$AUC_{0\ 105\ min}$ (μmol min mL^{-1})		GAZT in perfusate (% dose)	Total ^{3}H in bile (% dose)
		AMT	Unknown		
Control	0·42±0·01	0·90±0·14	0·10±0·04	0·85±0·13	12·70±2·60
Phenobarbitone	2·29±0·31*	2·68±0·16*	1·18±0·46*	2·50±0·60*	21·60±6·10*

Values are the mean ± s.d. of three experiments. *$P<0·01$.

Results

The disposition of AZT and its catabolites in rat perfused liver: effect of phenobarbitone pretreatment

Based on TLC analysis of perfusate and bile samples, AZT was found to be catabolized into at least three catabolites in rat perfused livers obtained from both central and phenobarbitone-induced rats. These catabolites included AMT, GAZT, and unknown material. AMT was the major catabolite in the perfusate accounting for 26·0±5·0 and 51·0±12% (mean±s.d.) of radioactivity in control and phenobarbitone-induced livers, respectively, after 105 min of perfusion, while the amount of GAZT in the perfusate of both types of liver was small (Table 1).

The time courses of AZT, AMT, and the unidentified material in the perfusates are shown in Fig. 1. It can be seen that phenobarbitone pretreatment resulted in an increased elimination of AZT from the perfusion medium, which was accompanied by acceleration of AMT and unknown metabolite formation. The amount of AMT rose gradually during perfusion of the livers. The effect of phenobarbitone pretreatment on clearance of AZT and on AUC for its catabolites is displayed in Table 1. Phenobarbitone proved to be a powerful inducer of AZT catabolism in rat perfused liver since it stimulated AZT clearance more than 5-fold. AUC for AMT was increased 3-fold and for the unknown 10-fold. The amount of radioactivity eliminated via bile was increased 2-fold after induction (Table 1). In control and in induced livers, 95 and 90% of radioactivity, respectively, was recovered as GAZT. The time course of cumulative elimination of GAZT in bile is shown in Fig. 2. Again, it can be seen that phenobarbitone increased the initial rate of excretion of GAZT into bile.

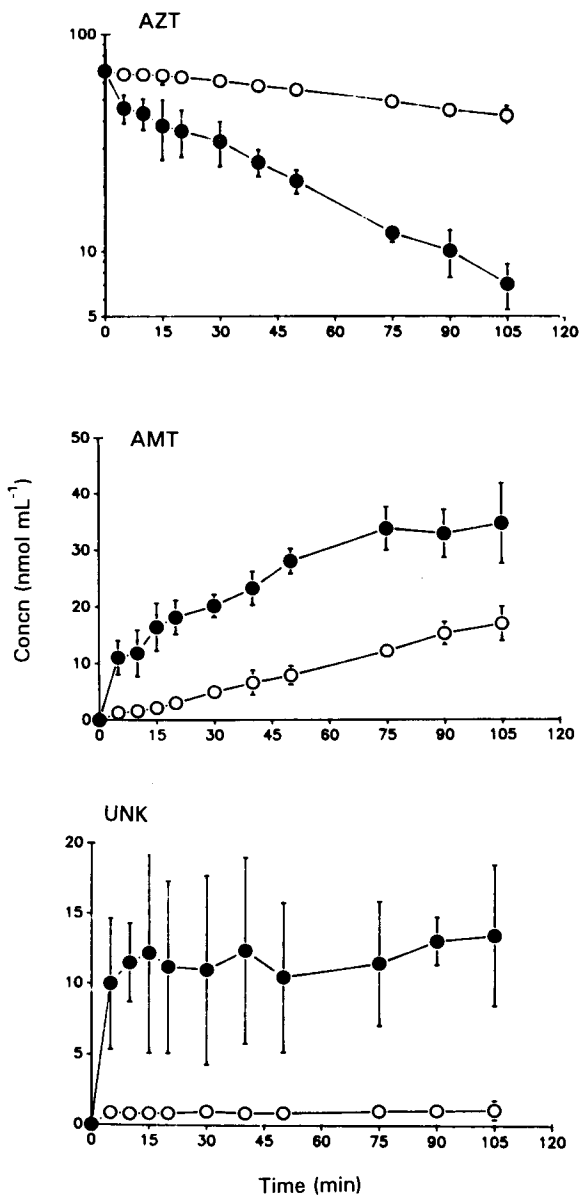


FIG. 1. Effect of phenobarbitone pretreatment on concentration of AZT and catabolites in rat liver perfused with [^{3}H]AZT. ○ Control, ● phenobarbitone-pretreated. Each point represents the mean±s.d. from three determinations. UNK = unknown metabolite.

Uptake of AZT by rat isolated hepatocytes

The relationship between the concentrations of AZT and the amounts of AZT taken up by rat isolated hepatocytes within 10 s incubation was linear (Fig. 3). Neither temperature,

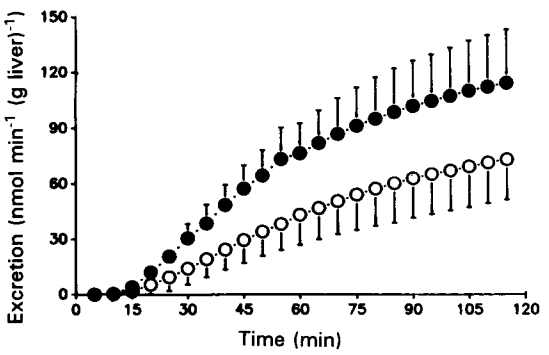
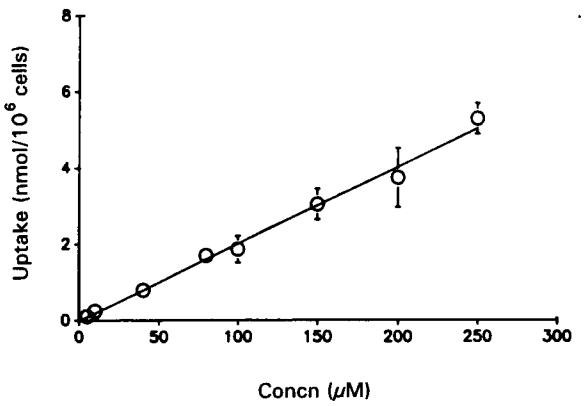


FIG. 2. Cumulative rates of excretion of GAZT by bile of the rat liver perfused with [^{3}H]AZT. ○ Control, ● phenobarbitone-pretreated. Each point represents the mean±s.d. from three determinations.



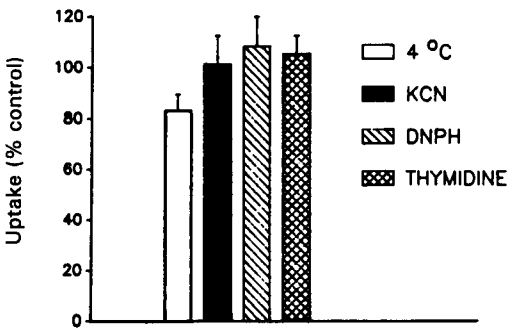


FIG. 3. Uptake of AZT by rat isolated hepatocytes within 10 s at various concentrations of AZT in the medium and the effect of temperature, metabolic inhibitors (KCN and dinitrophenol (DNPH)), and thymidine (each at 1 mM final concn). Each point represents the mean ± s.d. from four determinations.

metabolic inhibitors nor thymidine influenced the uptake of AZT (Fig. 3).

Incubation of AZT with bovine UDPGT

Under conditions found to be appropriate for the human and rat enzyme, an attempt to prepare GAZT by incubation of AZT with bovine UDPGT proved unsuccessful. The analysis of the samples showed that less than 0·4% total ^3H was associated with GAZT during a 6-h incubation. Therefore

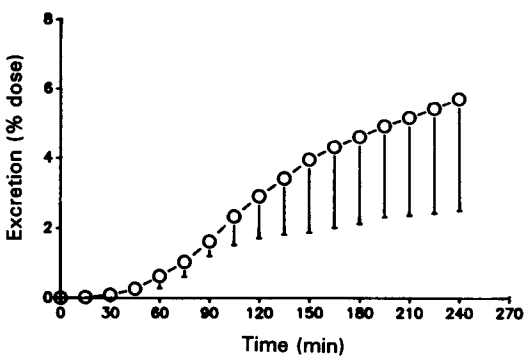


FIG. 4. Cumulative excretion of [^{3}H]GAZT-derived radioactivity in bile after intraduodenal administration of [^{3}H]GAZT (1 mg/rat, 15 μCi/rat) in-vivo in rats with indwelling bile duct cannulae. Each point represents the mean ± s.d. of four determinations.

[^{3}H]GAZT for kinetic study was prepared by biosynthesis in rat perfused liver.

Kinetics of excretion of radioactivity in bile after intraduodenal administration of [^{3}H]GAZT to rats

To elucidate whether GAZT might undergo enterohepatic recirculation in-vivo, four rats were dosed intraduodenally with [^{3}H]GAZT. The time course of cumulative excretion of [^{3}H]GAZT-derived radioactivity into the bile is depicted in Fig. 4. The total amount accounted for 5·8 ± 3·3% (mean ± s.d.) of administered GAZT excreted into the bile within 4 h post dosing.

Discussion

The presence of the GAZT and AMT, two main catabolites of AZT, as determined by TLC in rat recirculating liver perfusate samples is in accord with the finding of Cretton et al (1991a). On incubating AZT with rat isolated hepatocytes, those workers found a high GAZT/AMT ratio in both the extracellular and intracellular compartment. In our study, however, a high GAZT/AMT ratio was established in bile only, whereas in the perfusate a high AMT/GAZT ratio was found. The discrepancy between the rat perfused liver data and the isolated hepatocytes data is accounted for by preservation of the liver architecture in the former model and its disruption in the latter one. Yet each study may provide unique information contributing to our understanding of the disposition of a given drug in the living system.

After perfusion, the amount of GAZT in the perfusate of control was low (< 1% of AZT dose; see Table 1), while excretion in bile was relatively high (> 10% of AZT dose; see Table 1). The molecular weight of GAZT (443 Da) is a prerequisite for its preferential biliary excretion (Millburn et al 1967; Hirom et al 1972). Recently Mays et al (1991) showed that the rat was a suitable model for the study of AZT pharmacokinetics and the results of their study may be extrapolated to man. In the present study we found a very similar disposition pattern of GAZT as did Mays et al (1991). Although administration of AZT into the reservoir used for perfusion of the liver simulated an intravenous bolus, the non-existence of AZT renal elimination in the system of rat perfused liver, which enables clearance of AZT from blood, must be taken into consideration. In spite of this, the perfused liver with recirculating perfusate appears to be a good model for mimicking AZT disposition in-vivo with regard to GAZT formation and to its selective elimination into bile. Therefore, the effects of influencing GAZT formation in isolated perfused liver by drugs might be relevant to the rat in-vivo and consequently extrapolatable to man.

Failure to prepare GAZT by incubation of AZT with bovine UDPGT and UDPGA may be explained by the species differences in glucuronidation patterns. de Miranda et al (1990) were also unable to synthesize GAZT using commercially available UDPGT from bovine and rabbit livers.

The most polar catabolite in the perfusate of control and phenobarbitone-induced livers was unidentified material (R_f = 0·05 in 80% acetonitrile). On the basis of its high polarity, it could be anticipated that this catabolite might be a sulphate or phosphoric acid derivative of AZT (Liebes et al

1990). The plateau of this material in the perfusate of phenobarbitone-induced livers after 5-min perfusion suggests the presence of a strong diffusional barrier for this catabolite between the perfusate and liver cells.

Phenobarbitone, a well established microsomal inducer, increased both the rate and extent of catabolites in the perfusate and in the bile. Approximately 2-fold stimulation of glucuronidation of AZT by phenobarbitone has already been reported when rat liver microsomes were used as a source of UDPGT (Resetar & Spector 1989). The induction of GAZT formation by phenobarbitone was most probably mediated by an increase of liver content of UDPGA and of the activity of UDPGT (Watkins & Klaassen 1983). While glucuronidation of AZT is thought to be a detoxification process, the stimulation of AMT formation is a bioactivation process, as the AMT catabolite has been found to be more toxic to human bone marrow cells than the parent compound (Cretton et al 1991a). The reduction of the azide moiety of AZT was NADPH-dependent (Cretton et al 1991a) and it may involve cytochrome P450 reductase, as suggested for some azido-containing drugs by Nicholls et al (1991), as well as nonenzymatic reduction (Kamali et al 1988).

AZT is known to penetrate erythrocyte and lymphocyte membranes (Zimmerman et al 1987) as well as the placental barrier (Liebes et al 1990) by nonfacilitated diffusion. Uptake experiments with isolated hepatocytes were conducted to gain information on how AZT may cross the hepatocyte membrane. Its uptake by isolated hepatocytes was found to be independent of temperature, metabolic inhibitors, and thymidine, and it showed no evidence of saturation at the concentrations studied (Fig. 3). These results are indicative of simple diffusion of AZT through the hepatocellular membrane.

GAZT administered to rats was almost completely excreted in urine as AMT, since the azido group underwent reduction followed by cleavage of the β-linkage between the pyranose and furanose rings of GAZT (de Miranda et al 1990). This aspect of GAZT reabsorption and its subsequent elimination by bile, however, has formerly not been taken into consideration. On the basis of excretion of [^{3}H]GAZT-derived radioactivity by exteriorized bile duct (Fig. 4), GAZT may be expected to undergo enterohepatic recirculation in the rat.

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