Intrapulmonary Pharmacokinetics of Azithromycin in Healthy Volunteers Given Five Oral Doses

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The intrapulmonary pharmacokinetics of oral azithromycin were studied in 25 healthy volunteers, each of whom received an initial dose of 500 mg and then 250 mg once daily for four additional doses. Bronchoscopy, bronchoalveolar lavage, and venipuncture were performed 4, 28, 76, 124, 172, 244, 340, and 508 h after the first dose was administered. Azithromycin concentrations in epithelial lining fluid (ELF), alveolar macrophages, peripheral blood monocytes, and serum were measured by high-performance liquid chromatography. Azithromycin was extensively concentrated in cells and ELF. Drug concentrations in AMs (peak mean ± standard deviation, 464 ± 65 μg/ml) exceeded 80 μg/ml up to 508 h (21 days) following the first dose, while concentrations in PBMs (peak, 124 ± 28 μg/ml) exceeded 20 μg/ml up to 340 h (14 days). Azithromycin concentrations in ELF peaked at 124 h (3.12 ± 0.93 μg/ml) and were detectable up to 172 h (7 days), when they were 20 times the concurrent serum concentrations. Although the clinical significance of antibiotic concentrations in these compartments is unclear, the sustained lung tissue penetration and extensive phagocytic accumulation demonstrated in this study support the proven efficacy of azithromycin administered on a 5-day dosage schedule in the treatment of extracellular or intracellular pulmonary infections.

Azithromycin is an azalide antibiotic that contains a nitrogen atom in the macrolide aglycone ring (10). It is active in vitro against *Streptococcus pneumoniae*, group A streptococci, *Streptococcus agalactiae*, *Staphylococcus aureus*, *Haemophilus influenzae*, *Moraxella catarrhalis*, and intracellular organisms such as *Chlamydia*, *Mycoplasma*, and *Legionella* species (8, 12, 18). Azithromycin retains the gram-positive activity of erythromycin as the result of a common mechanism of action but provides enhanced activity against gram-negative organisms (12, 18). To a greater extent than other macrolides, azithromycin is concentrated in phagocytic cells (15) and has been shown in vitro to reduce the viability of intracellular bacteria (23).

The pharmacokinetics of azithromycin are characterized by rapid and extensive concentration within the intracellular and interstitial compartments of tissues (20). High and sustained tissue antibiotic concentrations are accompanied by relatively low concentrations in serum (10). In vivo activity in models of respiratory tract infection correlates with pulmonary tissue antibiotic concentrations, even though serum drug concentrations remain below the MIC for the infecting organism (19). For clinical infections, azithromycin is administered in a 5-day oral dosage regimen (500 mg on the first day, followed by 250 mg once daily on four additional days), providing sustained drug levels at sites of infection for up to 10 days (9).

Baldwin et al. (4) evaluated the pulmonary pharmacokinetics of azithromycin in 22 patients undergoing diagnostic bronchoscopy 12 to 96 h after a single 500-mg oral dose. Mean peak concentrations of azithromycin were high in epithelial lining fluid (ELF; 2.18 μg/ml), alveolar macrophages (AMs; 23.0 μg/ml), bronchial mucosa (3.89 μg/ml), and sputum (1.56 μg/ml) as observed 48 h after dosage. Serum concentrations were low at 12 h (mean, 0.13 μg/ml) but were still detectable at 96 h

(mean, 0.01 μg/ml). In contrast, pulmonary concentrations—particularly intracellular concentrations—remained elevated through 96 h and exceeded MICs for common respiratory tract pathogens, including intracellular organisms.

To further define the pharmacokinetics of azithromycin in pulmonary sites, the study reported here estimated antibiotic concentrations in serum, peripheral blood monocytes (PBMs), AMs, and ELF during and after the 5-day, once-daily oral regimen administered to healthy volunteers. Bronchoscopy and bronchoalveolar lavage (BAL) were performed up to 504 h (21 days) after the start of azithromycin administration.

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MATERIALS AND METHODS

Study design and subjects. Healthy volunteers 19 years of age or older were eligible for this open-label study. Women with childbearing potential had to demonstrate a negative serum gonadotropin pregnancy test prior to entry and were required to practice contraception (e.g., barrier method or abstinence) both during and up to 3 months after the end of the study. A history of cigarette smoking of less than five pack-years (the number of packs smoked per day times the number of years smoked) and abstinence from smoking within the previous 6 months were required.

Any of the following conditions were grounds for exclusion: known hypersensitivity or intolerance to azithromycin, macrolide antibiotics, or benzodiazepine or lidocaine-related anesthetics; use of ergotamine, terfenadine, or digitalis glycoside; treatment with another investigational drug within 4 weeks prior to enrollment; evidence or history of significant disease (hematologic, renal, cardiovascular, or hepatic); malabsorption or other conditions affecting drug absorption; lactation; drug or alcohol dependence; or a severe infection or underlying medical condition likely to result in death within 90 days.

After providing written informed consent, each volunteer gave a medical history and underwent a physical examination and baseline laboratory testing (urinalysis, complete blood count and platelet count, blood urea nitrogen, and serum levels of alanine and aspartate aminotransferases, alkaline phosphatase, total bilirubin, total protein and albumin, lactate dehydrogenase, creatinine, calcium, phosphorus, glucose, and electrolytes).

Azithromycin (Zithromax; Pfizer, Inc., New York, N.Y.) was administered orally as two 250-mg capsules on the first study day, followed by one capsule once daily on each of the next 4 days. The initial dose was taken under direct supervision in the study center. Subjects were instructed to take azithromycin at 0900

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and at least 1 h before or 2 h after meals. Each subject was examined approximately 4 h after the last dose, at which time blood and urine samples were obtained for laboratory testing.

Bronchoscopy and BAL. Standardized fiber-optic bronchoscopy (Olympus BF P-10 bronchoscope; Olympus Inc., Tokyo, Japan) and BAL were performed with the subjects receiving mild sedation with intravenous midazolam (Versed; Hoffmann-La Roche, Nutley, N.J.). In the upper airway (from the oropharynx to the true vocal cords), approximately 6 ml (range, 4 to 10 ml; given in 2- to 3-ml boluses) of 4% lidocaine (Roxane Labs, Columbus, Ohio) was used as a local anesthetic. In the lower airway (distal to the true vocal cords), approximately 12 ml (range, 6 to 18 ml; given in 3-ml boluses) of 1% lidocaine (Abbott Laboratories, Chicago, Ill.) was used as a local anesthetic.

All subjects underwent two bronchoscopies approximately 1 week apart, except for subjects at the 24-h time point, for whom only one bronchoscopy was completed. Bronchoscopies were performed at 1300 hours, or 4 h after drug dosing. BAL was performed 4, 28, 76, 124, 172, 244, 340, and 508 h after the start of drug administration, with five to six subjects studied at each time point.

BAL was carried out by infusion of 10 20-ml aliquots of sterile 0.9% saline into the medial segment of the right middle lobe and direct aspiration of fluid. The first aspirate, representing the bronchial wash, was discarded. Aspirated samples were collected and pooled in a plastic trap at 4°C, and the total volume was recorded. An aliquot of BAL fluid was retained for determination of cell counts and differential as well as albumin and urea concentrations. The samples were subsequently centrifuged at $2,500 \times g$ and 4°C. The supernatant and cell fraction were separated and frozen at −80°C until assayed.

Leukocytes from BAL samples and plasma were quantified in an identical manner. Total leukocyte counts in BAL and plasma were performed by diluting plasma with a pipette (Unipet; Becton Dickinson, Rutherford, N.J.) with a preset 1:20 dilution with acetic acid to lyse the erythrocytes. Cells were then counted with a hemocytometer with Neubauer ruling (Spencer, McGraw Park, Ill.). To obtain the total number of cells per microliter, the following formula was used: (number of cells) × (dilution)/(number of squares counted) × (volume of each square) = total leukocytes per microliter.

Manual differentials were performed on Cytospin samples. Each sample was prepared with a Cytospin 2 (Shandon, Pittsburgh, Pa.) with samples spun for 3 min at 100 rpm. The supernatant was then Wright stained in an Ames Hema-Tek (Miles Diagnostic Division, Elkhart, Ind.) slide stainer with a Hema-Tek stain pack (Miles Diagnostic Division). The leukocyte differential was obtained by microscopically scanning the stained slide at magnifications of ×50 and ×100 with oil objective lenses and counting up to 100 cells. A Schilling differential counter (Fisher Scientific Laboratories, Burr Ridge, Ill.) was used to tally the different cell types. From a 50-ml blood sample obtained prior to bronchoscopy, mononuclear cells were isolated by differential centrifugation through Histopaque (Sigma Diagnostics, St. Louis, Mo.). This method utilizes differential migration during slow-spin centrifugation through a low-viscosity solution that enables layering of separate populations of cells in a single tube. Following cell isolation, absolute cell counts for each sample were performed with an automated cell counter. Samples were then diluted up to 1 ml with phosphate-buffered saline (PBS) to yield final suspension concentrations of about 3.0×10^6 cells per ml. Cells were frozen at −80°C until assayed. A second 10-ml blood sample was obtained and centrifuged at $2,500 \times g$. The serum was separated and frozen until assayed.

Azithromycin assay. Azithromycin was measured in serum, PBMs, BAL fluid, and AMs by high-pressure liquid chromatography with an aluminum oxide column and a liquid chromatograph equipped with a dual electrochemical detector (BAS 200 liquid chromatograph; Bioanalytical Systems, Inc., West Lafayette, Ind.) (21). With the detector set in the series mode with dual glassy carbon electrodes at 600 and 850 mV versus Ag-AgCl, an aliquot was injected onto the column following extraction from a sample. Separation was achieved on a reverse-phase Chromegabond alkylphenyl analytical column (50 by 4.6 mm, 5-μm-diameter particle size) (E.S. Industries, Marlton, N.J.). The mobile phase was a 0.02 M sodium phosphate-acetonitrile mixture (25:75 [vol/vol]) at pH 10 with a flow rate of 1.5 ml/min at room temperature. A microsampler (CMA, Stockholm, Sweden) was used, and data were acquired on a PC-based system (InjectR software; Bioanalytical Systems).

For the serum assay, samples were run against a standard curve in serum (10 ng/ml to 1 μg/ml). In each batch, six quality control samples (duplicates of three concentrations) were tested. Linear regression of standard solutions gave a correlation coefficient of 0.9997 with intraday and interday coefficients of variation of 4.1 and 6.5%, respectively. Prior to analysis, PBM and BAL pellets were exposed to several freeze-thaw cycles to fracture cell walls and release the antibiotic content. For the PBM assay, the cell pellet in 1 ml of saline was further diluted with 1 ml of serum and saline to a volume of 5 ml. The standard curve and quality control samples each contained 1 ml of spiked serum plus 4 ml of saline. Samples for the standard curve each contained 1 μg to 31 ng of antibiotic in five steps, while those for quality control had 50, 100, or 200 ng per tube with intraday and interday assay coefficients of variation of 6.9 and 8.85%, respectively. For assays of BAL supernatants and cell pellets, a standard addition method was used with three samples containing 0, 12.5, or 25 ng of azithromycin per ml. To ensure lysis of cell pellets, the samples were diluted with water to a volume of 50 ml, frozen, and thawed. Peak height ratios were regressed against the added concentration. The intra- and interday percent coefficients of variation for all

BAL assays were 7.9 and 9.7%, respectively, with standard curve regression lines greater than 0.990. The lower limit of detection was 2 ng/ml for BAL fluid and leukocytes and 10 ng/ml for serum.

Albumin and urea determinations. Albumin concentrations were determined by an enzyme-linked immunosorbent assay. Each well of Immulon II 96-well flat-bottomed polystyrene microtiter plates (Dynatech Labs, Inc., Chantilly, Va.) was coated with 200 μl of human serum albumin (Sigma Chemical Co., St. Louis, Mo.) at 1.0 μg/ml in Voller's buffer (12 mM sodium carbonate, 35 mM sodium bicarbonate, 3 mM sodium azide [all from Fisher Scientific, Plano, Tex.]; pH adjusted to 9.6 with sodium hydroxide) and incubated overnight at 4°C in the dark.

Titertek U-shaped 96-well polyvinyl chloride microtitration plates (ICN Biomedical, Inc., Irvine, Calif.) were loaded with 100-μl samples and standard human serum albumin (10 μg/ml) diluted 1:3 in PBS-Tween (136 mM sodium chloride, 1.5 mM potassium phosphate, 10.8 mM sodium phosphate heptahydrate, and 10 mM potassium chloride with 750 μl of Tween 20; all from Fisher). One hundred ten microliters of primary antibody (anti-human albumin [goat; ICN]) diluted 1:7,500 in PBS-Tween was added to all wells of the polyvinyl chloride plates, which were then incubated for 2 h at 4°C.

Coated flat-bottomed plates were washed three times with PBS-Tween, and the contents of the U-shaped polyvinyl chloride plates were transferred to the flat-bottomed ones (200 μl per well) and incubated for 30 min at room temperature (the U-shaped plates were discarded). The plates were washed three times with PBS-Tween, and 200 μl of secondary antibody (horseradish peroxidase-conjugated anti-goat IgG [rabbit; Sigma] diluted 1:1,000 in PBS-Tween) was added per well. Plates were incubated for 90 min at room temperature and again washed three times with PBS-Tween. Two hundred microliters of substrate (ortho-phenylenediamine [100 μg/ml; Sigma] in methanol, diluted to 10 μg/ml in distilled water and 0.1 mM hydrogen peroxide) was added to each well and allowed to incubate for 20 min at room temperature. The enzymatic reaction was terminated by addition of 27.5 μl of 8 M sulfuric acid to each well. The plates were read on a Bio-Rad model 2550 enzyme immunoassay reader at a wavelength of 492 nm.

BUN determination. BUN assays were performed with a commercially available kit (BUN-20 Endpoint; Sigma Diagnostics). BAL fluid samples were assayed undiluted, and serum samples were diluted 1:10 in PBS in accordance with the commercial protocol.

Calculation of azithromycin concentration in ELF. The concentration of azithromycin in ELF was determined from the volume of ELF contained in each BAL sample by means of the urea dilution method. The hypothesis behind this technique is that there is equal diffusion of urea or albumin throughout the body. Thus, the urea concentrations in serum and ELF are the same, and ELF volume can be calculated by simple dilution techniques. With this technique, the quantity of urea in serum and lavage fluid is determined, and the volume of ELF (V_{ELF}) can then be calculated by using the equation V_{ELF} = volume of BAL × [urea] in BAL/[urea] in plasma, where [urea] is the concentration of urea. Once the volume of ELF in BAL is known, concentrations of noncellular contents can be determined. We confirmed the ELF concentration of V_{ELF} by determining the albumin in serum and BAL and then substituting the albumin value for [urea] in the equation above. The results obtained by the two techniques were within 5% and were in agreement with the V_{ELF} determined by Rennard and colleagues (17). Azithromycin concentrations in V_{ELF} are determined from the total drug present in BAL with the ratio calculation.

Calculation of macrophage and monocyte azithromycin concentrations. Macrophages and monocytes were isolated and counted as described above. Macrophage cell volume was determined by velocity gradient centrifugation (13). The mean cell volume was 2.45 μl × 10^6 cells and is in agreement with other determinations (4, 7). Therefore, the absolute macrophage count divided by the cell volume yielded a cell volume. The mass concentration (in micrograms) divided by the cell volume (in milliliters) yielded a concentration of drug in micrograms per milliliter of cell volume.

Statistical analysis. All data are presented as means ± standard deviations. Azithromycin concentrations in serum, mononuclear cells, ELF, and AMs were grouped by the sample matrix and plotted against time.

RESULTS

Twenty-five volunteers (14 men and 11 women) with a mean age of 32.1 ± 8.6 years and a mean weight of 77.4 ± 20.9 kg participated in the study. Table 1 summarizes the mean concentrations of azithromycin in serum, PBMs, ELF, and AMs. With the employed sampling schedule, the serum azithromycin concentration was highest 4 h after the initial dose was administered and declined thereafter, although the drug was still detectable on day 10 (Fig. 1). At all time points, concentrations in ELF were greater than those in serum (Fig. 2); the mean peak concentration in ELF on day 5 was 58 times the concurrent serum azithromycin level (Table 1). ELF demonstrated detectable azithromycin concentrations up to 172 h (7 days)

TABLE 1. Azithromycin concentrations in cells, epithelial lining fluid, and serum[a]

Sample	Azithromycin concn (µg/ml, mean ± SD) at hour (day)[a]:							
	4 (0)	28 (1)	76 (3)	124 (5)	172 (7)	244 (10)	340 (14)	508 (21)
AMs[b]	74 ± 26	180 ± 105	217 ± 64	464 ± 65	304 ± 71	112 ± 20	110 ± 40	82 ± 88
PBMs[b]	58 ± 13	120 ± 28	110 ± 31	107 ± 18	77 ± 22	47 ± 12	21 ± 14	0
ELF	0.45 ± 0.15	1.53 ± 0.31	2.67 ± 0.85	3.12 ± 0.93	0.61 ± 0.23	0	0	0
Serum	0.178 ± 0.05	0.122 ± 0.055	0.093 ± 0.036	0.054 ± 0.008	0.031 ± 0.055	0.015 ± 0.005	0	0

[a] The number of subjects was five at 28, 767, 124, and 508 h; at all other time points it was six.
[b] Drug concentrations are in micrograms per milliliter of monocyte/macrophage volume.

following the initial dose, at which time the mean concentration was 20 times that in serum (Fig. 2; Table 1). Azithromycin concentrations in PBMs and AMs were high 4 h after the first dose and persisted for 340 and 508 h, respectively (Fig. 1 and 2). At day 10, the mean drug concentrations in PBMs and AMs were 3,133 and 7,467 times greater, respectively, than the concurrent serum concentration (Table 1).

DISCUSSION

As reported previously for a single-dose study (4), azithromycin is extensively concentrated in lung ELF and AMs. We found that elevated drug concentrations in AMs (>80 µg/ml) persisted up to 21 days after the start of the 5-day oral dosage regimen. High concentrations in ELF were sustained for 7 days following initiation of azithromycin administration. Drug concentrations in PBMs exceeded 20 µg/ml up to day 14 despite declining serum drug concentrations measured 1 day following the 500-mg loading dose. Peak drug concentrations occurred on day 1 for PBMs and on day 5 for AMs. Because the majority of monocytes undergo margination into tissues—including the lung—after 48 h, migration of azithromycin-loaded monocytes from the blood could contribute to the marked increase in AM concentrations by day 5 (3, 4).

The sustained azithromycin concentrations observed in PBMs and macrophages are consistent with the observations of others. Thus, both mononuclear phagocytes and neutrophils have been shown to accumulate azithromycin in vitro (11, 16, 24). The peak intracellular concentration in human monocytes was about six times the extracellular concentration at an extracellular pH of 6.9 (16). Furthermore, the antibacterial activity of azithromycin against ingested *S. aureus* was enhanced compared with the effect in cell-free medium. Uptake of azithromycin by human AMs produced an intracellular/extracellular concentration ratio of 1:634 after 90 min (23). Gladue et al. (11) demonstrated extensive uptake by human and mouse neutrophils, mouse and rat AMs, and murine peritoneal macrophages.

Intracellular concentration of azithromycin has demonstrated no detrimental effect on phagocyte functions, including chemotaxis (15), oxidative metabolism (5), and intracellular killing (11). Release of azithromycin is slow, even in the absence of extracellular drug, although it is significantly enhanced by phagocytosis (11). Therefore, release of azithromycin by phagocytes is believed to provide locally high concentrations of active drug, which—together with normal phagocytic killing mechanisms—could help eradicate extracellular infections caused by sensitive organisms (11). Moreover, sustained intracellular concentrations could enhance antibacterial activity against intracellular organisms such as *S. aureus*, *Legionella pneumophila*, and *Chlamydia trachomatis* (14, 15). In a murine model of nonparenchymatous respiratory tract infection due to *H. influenzae*, Vallée et al. (22) demonstrated that the efficacy of

azithromycin coincided with the arrival of large numbers of azithromycin-containing neutrophils within bronchoalveolar spaces, suggesting that active extracellular concentrations were provided by drug released from these cells.

Other macrolides, including erythromycin and clarithromycin, are actively accumulated by phagocytes (1, 15). Recently, the intrapulmonary pharmacokinetics of clarithromycin were evaluated through BAL of healthy adults given 500 mg of the drug every 12 h for five doses (6). Clarithromycin was present in high concentrations in ELF and alveolar cells up to 48 h following the last dose of drug (ELF, 23.4 ± 19.2 µg/ml; alveolar cells, 17.0 ± 34.0 µg/ml). The alveolar cell/plasma concentration ratio at 48 h was 1:1,700. The clinical significance of antibiotic concentrations in alveolar cells and ELF remains to be determined (3).

ELF represents an important site of extracellular infection in pneumonia, and AMs are sites of infection by intracellular pathogens. The alveolar epithelial membrane is a significant barrier that separates ELF from blood. Thus, ELF constitutes an important protected microenvironment (2). Clinical efficacy, particularly with pathogens that are confined to sites separated from blood by significant barriers, may be more closely related to drug concentrations at actual sites of infection (3).

Although serum drug concentration in relation to MIC has traditionally predicted antibiotic efficacy, the pharmacokinetics of azithromycin suggest that tissue and intracellular concentrations may be more useful for assessing its antibacterial ac-
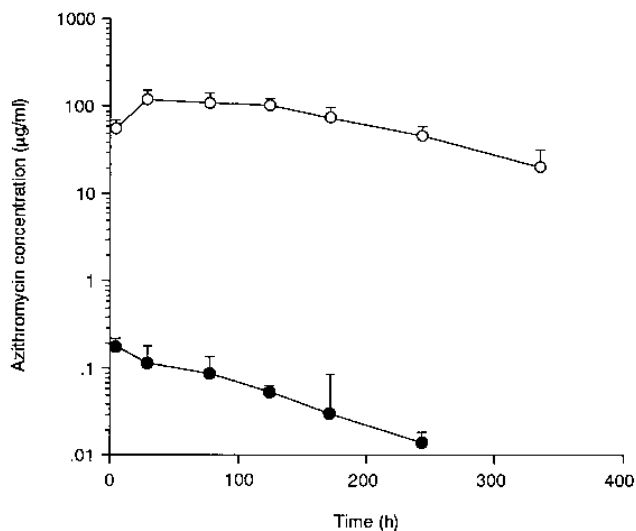

FIG. 1. Concentration of azithromycin in PBMs (○) and human serum (●) during and after a once-daily, 5-day dosage regimen. Each point represents the average ± the standard deviation.
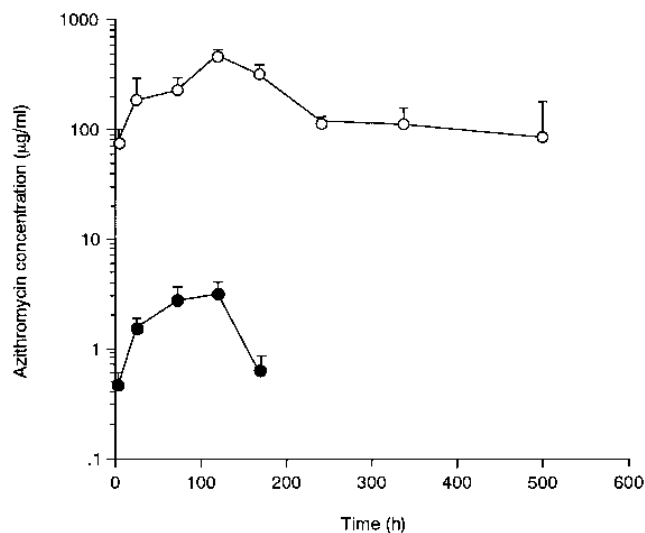
FIG. 2. Concentrations of azithromycin in ELF (●) and AMs (○) during and after a once-daily, 5-day dosage regimen. Each point represents the average ± the standard deviation.

tivity (14). The rapid decline in serum drug concentrations after absorption observed in this study is consistent with extensive redistribution into tissues. The average half-life in tissue after a single dose is estimated to be between 2 and 4 days (14). On the basis of pharmacokinetic data in this study and others, the sustained lung tissue penetration (10), extensive accumulation by phagocytes, and targeted drug delivery to sites of infection (11, 22) support the proven efficacy of azithromycin, in the face of relatively low serum drug concentrations, against both extracellular and intracellular respiratory infections, such as mild, community-acquired pneumonia or acute exacerbations of chronic bronchitis caused by sensitive pathogens.

ACKNOWLEDGMENT

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