

## Interstitial Methotrexate Kinetics in Primary Breast Cancer Lesions

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### Abstract

The transfer of cytotoxic agents across the tumor endothelium into the interstitial tumor space is considered a critical step in clinical resistance of solid tumors to antineoplastic chemotherapy. However, experimental data on drug transfer from the blood into the interstitium of solid tumors are scarce. Therefore, in this study, we used an innovative technique, *in vivo* microdialysis, for measuring interstitial tumor pharmacokinetics and plasma-to-tumor transfer rates of methotrexate (MTX) in breast cancer patients.

Microdialysis probes were inserted into the primary tumor and the periumbilical s.c. adipose layer of nine previously chemotherapy-naive breast cancer patients to monitor interstitial concentrations following i.v. administration of MTX (40 mg/m<sup>2</sup>) during a three-drug treatment regimen. Mean interstitial MTX load in breast tumors, expressed as area under curve (AUC), was 60 ± 20% (mean ± SE; coefficient of variation = 100%) of mean plasma MTX load. There was no correlation between plasma AUC and the AUC in the interstitial space of tumor tissue (*P* = 0.93). Not one of the parameters plasma, interstitial tumor load, and transfer rate of MTX to the interstitial space was associated with favorable clinical response.

In conclusion, plasma levels of MTX are not predictive of intratumor levels. There is a high interindividual variability in transendothelial MTX transfer. Under the present conditions, access of MTX to the interstitial space is not a rate-limiting step for clinical response to chemotherapy.

### Introduction

Many drugs initially raised enthusiasm concerning their therapeutic potential against solid tumors but failed to prove efficacious in clinical trials. No single factor is responsible for this discouraging phenomenon (1). Although tumor resistance at a molecular level is widely recognized as playing a role in therapeutic failure, several lines of evidence indicate that an impaired drug transfer across the tumor endothelium into the interstitial tumor space may also be a critical step in determining clinical response of tumors (2). Because the interstitial fluid represents the compartment of immediate vicinity of tumor cells, the concentration-time profile in the interstitial fluid may be regarded as the true *in vivo* dose intensity, comparable to *in vitro* dose intensity in cell culture.

Data on intratumoral concentrations of anticancer drugs are scarce and are only available from biopsy studies (3) and from nuclear magnetic resonance spectroscopy studies (4), which provide no information on interstitial space pharmacokinetics. Moreover, clinical studies on transendothelial drug transfer into the interstitium of solid tumors were hampered by the limited availability of appropriate methods. Interstitial fluid measurements using micropore diffusion chambers were described for animal studies (5) but cannot be used for

dynamic measurements and are not readily feasible in a clinical setting.

Recently, we have shown the feasibility and the potential utility of interstitial drug measurements in solid human tumors by using microdialysis, an innovative clinical technique for *in vivo* measurement of pharmacokinetics in the interstitial space. A pilot study in patients with metastasized malignant melanoma demonstrated that microdialysis allows for the measurement of the free, unbound fraction of cytotoxic drugs in the interstitial fluid (6) and, thus, enables direct assessment of tumor exposure to the pharmacologically active drug fraction. Moreover, a recent study in breast cancer patients provided preliminary evidence that measurement of interstitial pharmacokinetics by *in vivo* microdialysis may even predict response to chemotherapy (7).

The aim of this study was to test whether access of MTX<sup>2</sup> to the interstitial tumor space is a rate-limiting step for chemotherapeutic success. Therefore, intratumoral MTX pharmacokinetics and plasma-to-tumor transfer rates were measured by microdialysis in breast cancer patients receiving a preoperative chemotherapy with a regimen including MTX. On the basis of previous experiments (7), we hypothesized that interstitial dose intensity, *i.e.*, the product of MTX concentration and the time of cell exposure, would be more predictive of clinical tumor response than would plasma dose intensity.

### Patients and Methods

The study protocol was approved by the local ethics committee. All patients were given a detailed description of the study, and their written informed consent was obtained. The study was performed in accordance with the Declaration of Helsinki and the Good Clinical Practice Guideline of the European Commission.

### Patients

The study population included nine female patients with histologically confirmed diagnosis of primary breast cancer stage T<sub>3/4</sub>N<sub>x</sub>M<sub>0</sub> [age, 52 ± 4 years (mean ± SE); body surface area, 1.77 ± 0.03 m<sup>2</sup>; WHO performance status, Eastern Cooperative Oncology Group scale, 0, 1, or 2; life expectancy, at least 3 months], who were scheduled to receive preoperative chemotherapy according to the cyclophosphamide-MTX-fluorouracil regimen (cyclophosphamide, 600 mg/m<sup>2</sup>; MTX, 40 mg/m<sup>2</sup>; and fluorouracil, 600 mg/m<sup>2</sup>). Admission of patients to the study was limited to the first treatment cycle. However, patients were restudied whenever possible; data from these experiments were analyzed separately.

### Microdialysis

The principles of the microdialysis technique for clinical studies have been described in detail previously (8). Briefly, microdialysis is based on sampling of analytes from the interstitial space by means of a semipermeable membrane at the tip of a microdialysis probe. The probe is constantly perfused with a

Received 2/2/98; accepted 5/29/98.

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<sup>2</sup> The abbreviations used are: MTX, methotrexate; CV, coefficient of variation; PR, partial response; AUC, area under the curve; 5-FU, 5-fluorouracil.

physiological solution (perfusate) at a flow rate of 1.5  $\mu\text{l}/\text{min}$ . Once the probe is implanted into the tissue, substances present in the interstitial fluid at a certain concentration ( $C_{\text{tissue}}$ ) are filtered by diffusion out of the interstitial fluid into the probe, resulting in a concentration ( $C_{\text{dialysate}}$ ) in the perfusion medium. Samples are collected and analyzed. For most analytes, equilibrium between interstitial tissue fluid and the perfusion medium is incomplete; therefore,  $C_{\text{tissue}} > C_{\text{dialysate}}$ . The factor by which the concentrations are interrelated is termed recovery. To obtain absolute interstitial concentrations from dialysate concentrations, each microdialysis probe was calibrated in each experiment for *in vivo* recovery as described below.

#### Assessment of Microdialysis Probe Recovery

**In Vitro Experiments.** To characterize the influence of drug concentration in the surrounding medium on the transfer rate of the drug across the dialysis membrane, the dialysis probe was placed in glass beakers containing different concentrations of MTX (0.5–300  $\mu\text{M}$ ). The probe was perfused at a flow rate of 1.5  $\mu\text{l}/\text{min}$  at room temperature (20°C). Drug concentrations were measured in the dialysate and expressed as percentages of the concentration in the surrounding medium.

**In Vivo Experiments.** To obtain absolute interstitial concentrations from dialysate concentrations, microdialysis probes were calibrated for *in vivo* recovery rates according to the retrodialysis method (9, 10). The principle of this method relies on the assumption that the diffusion process is quantitatively equal in both directions across the semipermeable membrane. Therefore, the study drug is added to the perfusate, and the disappearance rate through the membrane is taken as the *in vivo* recovery. The *in vivo* recovery value is calculated as:

$$\% \text{ recovery} = 100 - (100 \cdot C_{\text{dialysate}} \cdot C_{\text{perfusate}}^{-1})$$

#### Study Protocol

On the morning of the study day, patients were admitted to the clinical research ward. The patients were in a supine position throughout the study period. A plastic cannula was inserted into an antecubital vein to monitor total MTX plasma concentrations at 15-min intervals. The skin at the site of microdialysis probe insertion was cleaned and disinfected. Commercially available microdialysis probes (CMA 10; CMA, Stockholm, Sweden; molecular cutoff of  $M_r$  20,000, outer diameter of 500  $\mu\text{m}$ , and membrane length of 16 mm) were inserted into the primary tumor and into periumbilical s.c. adipose tissue without local anesthesia by a previously described procedure (7, 10). Subsequently, the microdialysis system was connected and perfused with Ringer's solution at a flow rate of 1.5  $\mu\text{l}/\text{min}$  by a microinfusion pump (Precidor; Infors-AG, Basel, Switzerland). The position of each probe was established by two-dimensional ultrasound scanning, as described previously (7). After a 30-min baseline sampling period, *in vivo* probe calibration was performed by retrodialysis with MTX as described above (9, 10) for a period of 30 min, and two 15-min samples were taken (7). Prior to the administration of MTX, the perfusate was changed to Ringer's solution, and the system was flushed for 30 min. Thereafter, MTX was administered as described above, and sampling was continued at 30-min intervals. Dialysates were collected and stored at  $-20^\circ\text{C}$  until analysis.

#### Study Drugs

Patients received MTX (Methotrexat; Roche, Basel, Switzerland) as a single i.v. dose of 40  $\text{mg}/\text{m}^2$  over 5 min. Concomitant medication included 8 mg of ondansetron, 4 mg of dexamethasone, and 50 mg of ranitidine. Following the experimental observation period, *i.e.*, 3 h following MTX application, patients received 5-fluorouracil (600  $\text{mg}/\text{m}^2$ ) and cyclophosphamide (600  $\text{mg}/\text{m}^2$ ), according to the cyclophosphamide-MTX-fluorouracil regimen.

#### Chemical Analyses

MTX concentrations in plasma and dialysates were measured by use of a Fluorescence Polarization Immunoassay (FPIA) (Abbott, Abbott Park, IL). To be able to measure MTX at low concentrations in low volumes, the assay was

adapted for use on a COBAS Fara II analyzer (Roche). No extraction was required before analysis of the microdialysates. The inter- and intraassay CVs were  $<5\%$ . The detection limit was 0.01  $\mu\text{M}$ .

#### Assessment of Tumor Response

A PR was defined as a reduction in tumor size of 50% or more, as studied by mammography, mammosonography, or direct clinical measurement of the tumor diameters. Stable disease was defined as a change in tumor size of less than  $+25\%$  to  $-50\%$ , as compared to prestudy conditions. Response was assessed following three cycles of chemotherapy.

#### Calculations and Data Analysis

**Calculations for Microdialysis Experiments.** For the assessment of *in vitro* recovery and to show that microdialysis is concentration independent over a wide concentration range, linear regression was performed as described previously (10). A consistent relationship between the concentration in a surrounding solution and in the dialysate over a wide concentration range for a given study drug is indicated by linear regression ( $r > 0.95$ ; Fig. 1). Absolute interstitial fluid concentrations were calculated from dialysate concentrations by the following equation:

Interstitial fluid concentration

$$= 100 \cdot \text{sample concentration} \cdot \text{in vivo recovery value}^{-1}$$

**Pharmacokinetic Calculations.** The following pharmacokinetic parameters were determined by model independent analysis: AUC, time to maximum concentration ( $t_{\text{max}}$ ), and maximum concentration ( $C_{\text{max}}$ ). Penetration ratios for tissues were determined as:  $\text{AUC}_{\text{tumor}}/\text{AUC}_{\text{plasma}}$ . Due to the long half-life of MTX ( $>8$  h), the elimination phase was not reached in our experiments, and  $t_{1/2-\beta}$  was not calculated.  $\text{AUC}_{0-180}$  values (from 0 to 180 min) were calculated according to the trapezoidal rule.

**Statistical Calculations.** For correlations and comparisons between pharmacokinetic parameters of different compartments, Spearman rank order correlations ( $r_s$ ) and Mann-Whitney  $U$  tests, respectively, were used, as pharmacokinetic parameters were nonnormally distributed.  $P < 0.05$  was considered the level of significance. CVs were calculated as  $100 \cdot \text{SD} \cdot \text{mean}^{-1}$ .

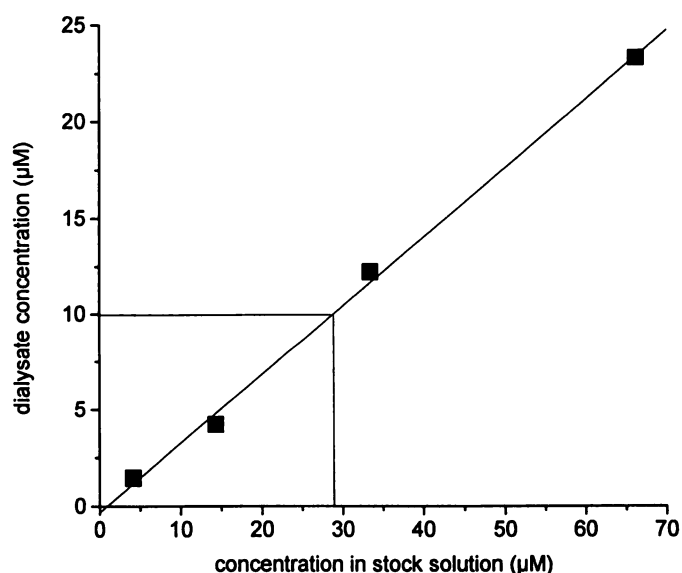


Fig. 1. *In vitro* calibration of the microdialysis probe for MTX. Analyte concentrations were measured in the dialysate and in the surrounding medium. The linear regression ( $r = 0.99$ ) indicates a consistent relationship between the concentration in a surrounding solution and in the dialysate over a wide concentration range. The vertical and horizontal lines illustrate the method for determining the *in vitro* recovery, which was 35% for MTX.

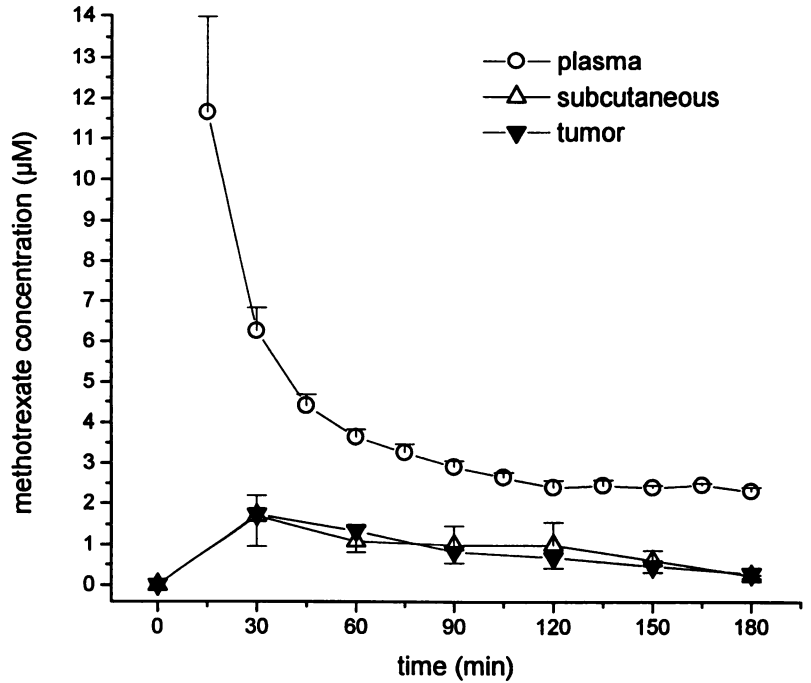


Fig. 2. Time versus plasma and interstitial fluid concentration curves for tumor and s.c. adipose tissue following administration of MTX (single i.v. dose of 40 mg/m<sup>2</sup> over 5 min; n = 9) in previously chemotherapy-naive breast cancer patients. Data points, means; bars, SE. 0–5 min, time of administration. Pharmacokinetic parameters are summarized in Table 1.

**Results**

**In Vitro Experiments.** Prior to performing the *in vivo* experiments, *in vitro* experiments were performed to characterize the transfer across the semipermeable membrane and to assure that the diffusion process of MTX across the microdialysis membrane is, indeed,

concentration independent over a wide concentration range. This is shown in Fig. 1.

**In Vivo Experiments.** The procedures were well tolerated by all patients. The results from experiments where probes were inserted simultaneously into the tumor and into the periumbilical s.c. adipose tissue (n = 9) are shown in Fig. 2. Pharmacokinetic parameters are summarized in Table 1. The mean AUC<sub>tumor</sub>/AUC<sub>plasma</sub> ratio was 0.60 ± 0.20 (mean ± SE), and the mean AUC<sub>subcutis</sub>/AUC<sub>plasma</sub> ratio was 0.49 ± 0.19. The absence of a correlation between total plasma and tumor AUCs is shown in Fig. 3 (r<sub>s</sub> = -0.03, P = 0.93).

Table 1 Pharmacokinetic parameters for tumor, subcutaneous adipose tissue, and plasma following administration of a single i.v. dose of 40 mg/m<sup>2</sup> MTX over 5 min (n = 9) in previously chemotherapy-naive breast cancer patients<sup>a</sup>

	C <sub>max</sub> (µM)	t <sub>max</sub> (min)	AUC <sub>0-180</sub> (µmol · min · liter <sup>-1</sup> )
Tumor	1.79 ± 0.76	43 ± 7	223.7 ± 76.8
Subcutis	1.75 ± 0.53	43 ± 10	210.3 ± 91.1
Plasma	11.7 ± 2.30	—	550.4 ± 46.4

<sup>a</sup> Results are presented as means ± SE. C<sub>max</sub>, peak concentration; t<sub>max</sub>, time to reach peak concentration; AUC<sub>0-180</sub>, area under the time versus concentration curve from 0 to 180 min.

4 patients were studied on two occasions. For these patients, the ratio AUC<sub>cycle1</sub>/AUC<sub>cycle2</sub> was 0.87 ± 0.07 (CV = 16%) for plasma values and 0.98 ± 0.17 (CV = 35%) for tumor values.

Of nine patients, five responded partially to chemotherapy and four had stable disease. Mean AUC<sub>tumor</sub> was 107.2 ± 46.7 µmol · min · liter<sup>-1</sup> for patients with PR and 34.9 ± 5.5 µmol · min · liter<sup>-1</sup>

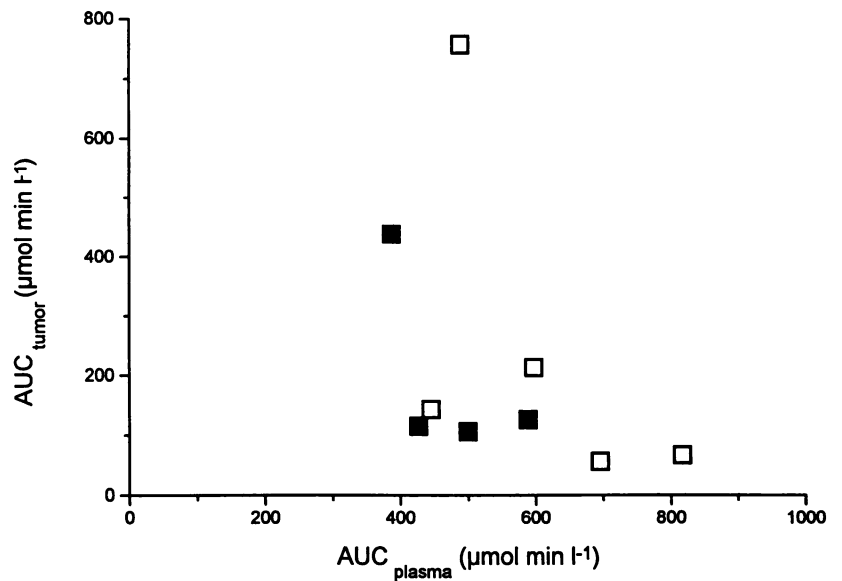


Fig. 3. Relationship between the AUC in the interstitial space of breast tumors and in plasma (r<sub>s</sub> = -0.03, P = 0.93). □, patients with PR; ■, patients with stable disease.

for patients with stable disease (not significant). Mean  $AUC_{\text{plasma}}$  was  $609.6 \pm 67.7 \mu\text{mol} \cdot \text{min} \cdot \text{liter}^{-1}$  for patients with PR and  $476.5 \pm 44.2 \mu\text{mol} \cdot \text{min} \cdot \text{liter}^{-1}$  for patients with stable disease (not significant).

## Discussion

Several lines of evidence support the notion that an impaired transfer of cytotoxic agents into the tumor interstitium might be critical for *in vivo* tumor response to chemotherapy (2, 7). These considerations notwithstanding, direct measurements of drug concentrations in the interstitial fluid in human tumors were hampered by a lack of appropriate methodology.

This study, therefore, aimed at using *in vivo* microdialysis for the measurement of interstitial MTX kinetics in a well-defined and relatively homogeneous group of breast cancer patients. Interstitial AUC of free intratumoral MTX was defined as the main outcome variable because the interstitial dose intensity of the free, non-protein-bound drug within the tumor compartment is a direct measure of the pharmacologically active drug concentration at the anatomical target site. Mean s.c. and tumor concentration time courses were not substantially different (Fig. 2 and Table 1) and the mean transfer rate constant from the central (plasma) to the peripheral (tumor and s.c.) compartments ( $k_{12}$ ) indicated a rapid mean equilibration between the plasma concentration and free interstitial concentrations for both tumor and s.c. tissue. Interstitial concentrations in both peripheral compartments were ~50% of corresponding plasma values (Fig. 2). Given a protein binding rate of 50% (11) these results are in line with previous findings in rats (12), reporting free interstitial levels in normal tissues close to unbound plasma levels.

Although MTX was administered at an individual dose based on body surface, interindividual variability in AUC values during the first cycle was relatively high, *i.e.*, CV was 41% for the plasma compartment and 103% for the tumor compartment. In contrast, intraindividual variability of tumor exposure, expressed as the  $AUC_{\text{cycle1}}/AUC_{\text{cycle2}}$  ratio, was considerably lower, *i.e.*, 16% for the plasma compartment and, more important, 30% for the tumor compartment. Under the present conditions, this provides indirect evidence for a relatively low variability of the penetration into different sites within one single tumor lesion and shows that permeability is a consistent characteristic of an individual tumor lesion.

The plasma-to-tumor transfer, expressed as  $AUC_{\text{plasma}}/AUC_{\text{tumor}}$  ratio, however, displayed a high interindividual variability, and for most pharmacokinetic parameters, the variability of tumor values was higher than the variability of corresponding plasma values. These observations indicate that the transfer from the plasma compartment into the interstitial space is not a fixed constant but rather a highly individual and variable determinant for each individual patient. The present experiments corroborate previous findings on intratumoral kinetics of other cytotoxic drugs, notably carboplatin (6) and 5-FU (7), where no association could be found between total plasma and interstitial tumor concentrations. Thus, the assumption of a linear relation between MTX plasma levels and intratumoral levels and, thereby, most probably, of a desired therapeutic effect could not be confirmed in this study. This lack in association may be explained by typical characteristics of tumor biology and vascular architecture. It was reported that, as a tumor grows, several pathophysiological and histological changes in interstitial structure and function take place, which, over time, lead to the development of functional and anatomical barriers to drug accumulation in some solid tumors (2). MTX monitoring of total MTX concentrations in plasma, which is acknowledged as a suitable technique for predicting adverse effects on tissues and for quantifying a rescue therapy with leukovorin, thus, appears to

have no additional predictive value of free intratumoral levels (tumor exposure) and, most likely, of therapeutic effect.

Previous clinical studies, particularly on intratumoral 5-FU kinetics in solid human tumors (4, 7), provided evidence that an increased intratumor dose intensity is associated with a favorable tumor response. These observations, which highlighted the importance of drug transfer from the plasma into the interstitial tumor compartment, also helped explain why plasma measurements do not provide surrogate markers for clinical outcome. Although the small sample size of this study precludes a detailed statistical evaluation, neither increased plasma nor tumor dose intensity were associated with a favorable tumor response. How could these observations be explained? First, the patients in this study received a chemotherapy regimen including three different antineoplastic drugs. Thus, against the background of previous studies showing a positive association between *e.g.*, intratumoral 5-FU kinetics and clinical response, it remains to be shown whether the success of a polychemotherapy is determined by intratumoral access of a "response-limiting" drug, *e.g.*, 5-FU or cyclophosphamide, as in the present therapeutic regimen. Second, clinical response to MTX may be determined by events beyond interstitial fluid kinetics, such as an impairment of the active transport process across the cell membrane (12). Cancer cell susceptibility to MTX therapy may further be determined by dihydrofolate reductase affinity to MTX (12), by adoptively increased intracellular dihydrofolate reductase levels (12) or altered levels of MTX polyglutamation (12). Thus, a simple concept of a threshold MTX level, comparable to a MIC level for antimicrobial agents (13), which is to be attained in the compartment surrounding the tumor cells, may not be appropriate.

In conclusion, plasma levels of MTX are not predictive of intratumor levels. There is a high interindividual variability in transendothelial MTX transfer. Under the conditions of this study, access of MTX to the interstitial space is not a rate-limiting step for clinical response to chemotherapy.

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*Cancer Res* 1998;58:2982-2985.

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