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Report

Pharmacokinetic Screening of *o*-Naphthoquinone 5-Lipoxygenase Inhibitors

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The *o*-naphthoquinone derivative, CGS 8515 (I), is a potent inhibitor (IC_{50}, 0.1 μM) of 5-lipoxygenase, but its therapeutic potential is compromised by a short plasma half-life (22 min) and extremely poor oral bioavailability (<2%). Poor biopharmaceutical properties of CGS 8515 were attributed to poor aqueous solubility and rapid *in vivo* hydrolysis of its methyl ester function to an inactive metabolite (IC_{50}, 100 μM). An active amide analogue (II) was synthesized to prevent rapid hydrolysis. While analogue II appeared to be stable *in vivo*, its plasma half-life was also short (10 min), possibly because of rapid tissue distribution rather than metabolic elimination. Therefore, three potent analogues with increased aqueous solubilities were synthesized and compared with respect to their pharmacokinetic properties. The analogue with the highest aqueous solubility (V) demonstrated a plasma concentration vs time profile with the largest area under the curve (AUC) and the smallest distribution (α) phase of all the analogues studied. The percentage AUC of the terminal phase (β) for three analogs paralleled their aqueous solubilities. The oral bioavailability of V was improved to 27%, compared to 2% for the parent compound, CGS 8515.

KEY WORDS: drug design; pharmacokinetic screening; 5-lipoxygenase inhibitor; *o*-quinone analogues.

INTRODUCTION

Receptor-targeted drug design (1) leads to highly potent drugs that, however, may not be optimally active *in vivo* with respect to duration or intensity of effect after oral administration. Unfavorable biopharmaceutical properties (poor aqueous solubility, instability in the gastrointestinal tract, poor gastrointestinal membrane permeability, rapid first-pass metabolism in the GI tract or liver) can result in poor oral bioavailability. The development of novel lead molecules as orally active drugs may, therefore, require biopharmaceutical tailoring through prodrug or analogue design during the drug discovery process (2–4).

Development of 5-lipoxygenase inhibitors (LOI) for potential therapeutic use in inflammatory diseases targets the obligatory step in the synthesis of leukotrienes from arachidonic acid (5). CGS 8515 (I) was selected as a lead LOI; its IC_{50} was approximately 0.1 μM in an *in vitro* assay of 5-lipoxygenase activity (6–8).

The high potency and selectivity of CGS 8515 in this assay encouraged the evaluation of its *in vivo* efficacy in an animal model. Suppression of calcium ionophore (A-23187) stimulated (*ex vivo*) production of leukotriene B_4 (LTB_4) in dog blood following drug administration served to evaluate this drug with respect to its bioavailability and pharmacological half-life. Intravenous (iv) administration of only 0.5 mg/kg caused 100% suppression of the *ex vivo* LTB_4 production in dog plasma. In spite of its high potency, the effect half-life was brief (12 min). The oral efficacy of this compound was also poor. An oral dose of as much as 100 mg/kg resulted in a maximum inhibition of LTB_4 production of only 26%, 3 hr after drug administration (unpublished data).

The large oral-to-iv dose ratio for comparable efficacy suggested a major biopharmaceutical problem with CGS 8515. In this study, the chemical structure of CGS 8515 was modified to improve its biopharmaceutical properties.

MATERIALS AND METHODS

Biopharmaceutical Evaluation of CGS 8515

CGS 8515 (methyl 2-[(3,4-dihydro-3,4-dioxo-1-naphthalenyl)amino]-benzoate) was administered initially by iv bolus to three male beagle dogs at a dose of 1 mg/kg. The chemical purity of this compound (>98%) was verified by HPLC,

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NMR spectroscopy, and elemental analyses. Because the drug was not soluble in water, the dosing vehicle consisted of a polyethylene glycol 400 and dimethylacetamide mixture (PEG 400:DMA, 80:20) with a final CGS 8515 concentration of 10 mg/ml. The dosing vehicle alone was shown to have no effect on *ex vivo* production of LTB_4. The same vehicle was used for all analogues. After a 2-week washout period, the same dogs also received an oral dose of 100 mg/kg CGS 8515 (1 ml/kg). Serial blood samples were collected at scheduled intervals up to 120 min postdose.

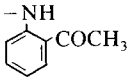
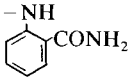
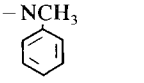
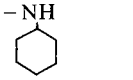
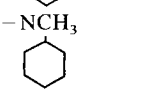
Blood Sampling

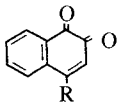
Blood samples were collected into polypropylene tubes containing 10 units/ml of heparin. Collection tubes for CGS 8515 samples also contained 0.5 mg/ml of eserine (Sigma Chemical Co., St. Louis, MO) to prevent artifactual ester hydrolysis *in vitro*. Blood samples were centrifuged immediately, and the plasma was harvested and stored frozen until analyzed. The *in vitro* stability of all analogues was tested by incubating the fresh heparinized blood samples supplemented with 1 μg/ml of the individual analogue at 37°C for 2 hr. With the exception of CGS 8515, all analogues were found to be stable under these conditions. CGS 8515 was also stable over this time period when the sample was supplemented with the eserine as described above.

Pharmacokinetic Evaluation of CGS 20111, the Amide Analogue

A potent analogue of CGS 8515, CGS 20111 (II; Table I), containing a metabolically stable amide, was selected for biopharmaceutical evaluation. The analogue was administered iv (1 mg/kg) to the same dogs after at least a 2-week washout period to compare its pharmacokinetic characteristics with those of CGS 8515.

Table I. Summary of Aqueous Solubilities of the Analogues of CGS 8515

Analogue No.	ID	Solubility (μg/ml)	Structure,[a] $R=$
I	CGS 8515	<1.0	
II	CGS 20111	1.0	
III		30.0	
IV		20.0	
V	CGS 19213	50.0	

[a] Structure of basic *o*-naphthoquinone moiety: 

Evaluation of Solubility of Different *o*-Quinone Analogues

The aqueous solubility of several analogues with good *in vitro* potency (IC_{50}, <1 μ*M*) was determined by stirring 250 mg of the drug powder in 250 ml of distilled water at room temperature for about 6 hr, followed by filtration using a 0.45-μm filter (Millipore Corp., Bedford, MA). The filtrate was then analyzed by HPLC.

Pharmacokinetic Screening of Three Analogues of CGS 8515 [III, IV, and CGS 19213 (V)]

Three other analogues of CGS 8515 [III, IV, and CGS 19213 (V)] were selected for pharmacokinetic screening. These three analogues were administered by iv bolus to each of the three male beagle dogs on three separate occaasions in a random crossover design with a 2-week washout period between doses. The analogues were administered at a dose of 1 mg/kg. Serial blood samples were taken at scheduled intervals over 3 hr postdose to obtain the plasma concentration vs time profile.

Determination of the Oral Bioavailability of CGS 19213 (V)

Based on the results of the iv screen, the analogue CGS 19213 (V) was selected for evaluation of its oral absorption. This analogue was administered via oral intubation as a solution, to the same three dogs used for the iv study, at a dose of 10 mg/kg. The tube was then flushed with 20 ml of PEG 400. Blood samples were collected for 6 hr at scheduled intervals.

Determination of First-Pass Metabolism in a Porta-Caval Transposed Dog

The porta-caval preparation was performed in a male beagle dog with surgically transposed inferior vena cava and portal veins, such that the hindleg infusion of a drug causes its direct administration to the liver before reaching the systemic circulation (9). Analog V was administered in the porta-caval dog model on three separate occasions by the iv route via the foreleg (systemic) and the hindleg (intraportal) at a dose of 1 mg/kg and by the oral route at a dose of 10 mg/kg. Blood samples were collected at scheduled intervals over 6 hr postdose.

Determination of the Plasma Concentration–Effect Relationship for CGS 8515 and Analogue V

To determine the relationship between the plasma drug concentrations and the biological effects of these compounds, blood samples were drawn for simultaneous determination of the plasma drug concentration and A-23187 stimulated production of LTB_4 in samples collected after iv administration of CGS 8515 and analogue V (1 mg/kg) to normal male beagle dogs. Four-milliliter blood samples were collected 60 min before drug administration and at various times postdose. The sample was then divided into four aliquots of 1 ml each. One milliliter was used as the unstimulated control, while three other aliquots were used to determine the stimulated LTB_4 production. The reaction was started by the addition of A-23187 (100 μ*M*) and 10 μ*M* *N*-formyl-met-leu-phe (fMLP; Sigma Chemical, St. Louis, MO). After 15

min at 37°C, the reaction was terminated by placing the samples at 4°C. Plasma was separated, and LTB_4 was quantitated by radioimmunoassay (Amersham, Arlington Heights, IL), according to the manufacturer's procedure, either immediately or within 1 day of storage at −80°C. Inhibition of LTB_4 formation was calculated as the mean change in LTB_4 levels at each time point compared to the mean of the values obtained at −60 min.

Analytical

Plasma concentrations of each of the analogues studied were determined by an HPLC method. The method consisted of acetonitrile precipitation of plasma proteins followed by direct injection of the supernatant on a C_{18} column. Analogue III was used as an internal standard for all of the other analogues, and analogue V was used as the internal standard for analogue III. Electrochemical detection (BAS, Indianapolis, IN) was employed in the reductive mode using a glassy carbon electrode set at −0.15 V. The reference electrode was Ag/AgCl. The mobile phase consisted of a methanol and sodium acetate buffer (pH 4.5, 0.1 *M*) mixture (63:37) and was run at 1 ml/min. The hydrolyzed acid metabolite of CGS 8515 was synthesized. The metabolite of the analogue CGS 20111 (II) was not identified but was quantitated as equivalents of parent compound using the parent's standard curve. The sensitivity was comparable among the analogues studied. The detection limit of the assay was about 25 ng/ml, and the standard curve was linear to at least 10 μg/ml. The between-day variability of the standard curve (<10%) was determined for each individual analogue. The precision and accuracy of the method were tested for each compound from blind analyses in plasma and ensured to be within the acceptable range (CV <10%). Standard curves were constructed daily and linearity of the HPLC assay was excellent (multi-r^2 >0.99).

Data Analysis

Plasma concentration vs time data were analzyed by noncompartmental methods as well as by nonlinear regression (FIT FUNCTION, RS/1 User's Guide, BBN Software Products Corporation, Cambridge, MA) using a biexponential function (10).

RESULTS

Biopharmaceutical Evaluation of CGS 8515

The mean (n = 3) plasma concentration vs time profiles for CGS 8515 following iv (1 mg/kg) and oral (100 mg/kg) administrations are depicted in Fig. 1. The plasma concentration declined rapidly after iv administration, with a terminal half-life of 22 ± 9 min. An acidic metabolite (CGS 19984) was apparent in the plasma at the first sampling time (2 min), and its concentration remained much higher than that of CGS 8515 at all times. This result suggested rapid deesterification of CGS 8515 as the primary route of its elimination. The pharmacokinetic parameters estimated after iv drug administration are presented in Table II. Plasma concentrations of CGS 8515 following oral drug administration were very low (Fig. 1). The peak plasma concentrations were only

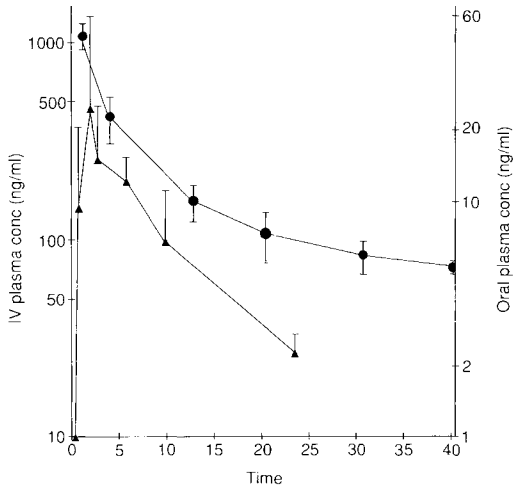


Fig. 1. Mean (±SD) plasma concentration vs time profiles for CGS 8515 following 1-mg/kg iv and 100-mg/kg oral drug administrations. Time is in minutes for iv administration and in hours for oral administration. iv, ●; po, ▲. N = 3.

10–100 ng/ml at 1–2 hr postdose. Comparison of the AUCs between the iv and the oral studies suggested an oral bioavailability of only 1.4 ± 1.1%.

Pharmacokinetic Evaluation of the Amide Analogue

In spite of the amide analogue's (CGS 20111, II) stability toward enzymatic hydrolysis, plasma concentrations of II still declined rapidly (half-life, 10 min; Fig. 2). One metabolite peak was detected in the HPLC chromatogram, but the size of the peak was small relative to that of the parent compound. Because of the undesirably short plasma half-life of the analogue, no attempt was made to characterize this metabolite. In the absence of any evidence of other metabolites, the rapid disappearance of II from plasma was assumed to reflect extensive distribution rather than rapid metabolic elimination.

Evaluation of the Aqueous Solubility and Pharmacokinetics of *o*-Quinone Analogues of CGS 8515

Based on the assumption that the high lipophilicity of the amide analogue may have contributed to its rapid disappearance from the plasma, three analogues of CGS 8515 containing the active *o*-naphthoquinone moiety (III to V) were selected based on their aqueous solubilities (Table I). Analogues III, IV, and V had considerably improved aqueous solubility over that of CGS 8515 and its amide analogue (II). These compounds had an aromatic or an alicyclic ring with or without additional methyl substitution on the nitrogen attached to the naphthoquinone ring (Table I). The plasma concentration vs time profiles of these three analogues in one dog are compared in Fig. 3. Analogue V consistently showed the highest plasma levels in all three animals. The mean area under the plasma concentration–time curve (AUC) after administration of V was five times larger than the AUCs for III and IV and 12 times larger than the AUC for CGS 8515. The calculated pharmacokinetic parameters are presented in Table II. The mean (±SD) clearances were 10 (±0.9), 48.8 (±7.6), 54.7 (±8.0), and 135 (±23) ml/min/kg

Table II. Mean (±SD) Pharmacokinetic Parameters of Four CGS 8515 Analogues Following Their Intravenous Administration in Dogs[a]

Parameter	Analogue I (CGS 8515)	Analogue III	Analogue IV	Analogue V (CGS 19213)
Compartment independent				
AUC (mg · min/L)	7.6	20.8	18.5	100
	(1.4)	(3.5)	(2.7)	(8.3)
Clearance (ml/min/kg)	135	48.8	54.7	10.0
	(23)	(7.6)	(8.0)	(0.9)
Vd_{ss} (L/kg)	1.6	2.64	1.62	1.07
	(0.43)	(1.11)	(0.65)	(0.37)
MRT (min)	12	53	30	107
	(3)	(17)	(13)	(41)
Compartment dependent				
A (ng/ml)	—	2410	1395	1185
		(1850)	(429)	(1000)
B (ng/ml)	—	184	254	941
		(104)	(193)	(278)
α (min^{-1})	—	0.243	0.177	0.178
		(0.091)	(0.151)	(0.166)
β (min^{-1})	—	0.014	0.025	0.010
		(0.006)	(0.012)	(0.003)
AUC_β (%)	—	58	45	93
		(9)	(25)	(3)

[a] Dose = 1 mg/kg.

for analogues V, III, IV, and CGS 8515, respectively. There were only small differences in the volumes of distribution (Vd_{ss}) among the four analogues.

Determination of the Oral Bioavailability of Analogue V in the Dog

The oral absorption of analogue V was relatively rapid, with the peak plasma concentration occurring at about 1–2 hr; the plasma concentration vs time data from a representative dog is shown in Fig. 4. The pharmacokinetic parameters are presented in Table III. The mean (±SD) oral bioavailability at 10 mg/kg was estimated to be 28 ±16% compared to the iv dose in the same dogs.

Determination of the First-Pass Metabolism of Analogue V in a Porta-Caval Transposed Dog

The plasma concentration vs time profiles for analogue V after three routes of administration are compared in Fig. 4. Comparison of the dose-corrected AUCs demonstrated almost complete oral absorption for V when the liver was bypassed in the transposed dog. The first-pass liver bioavailability after iv hindlimb intraportal administration was only

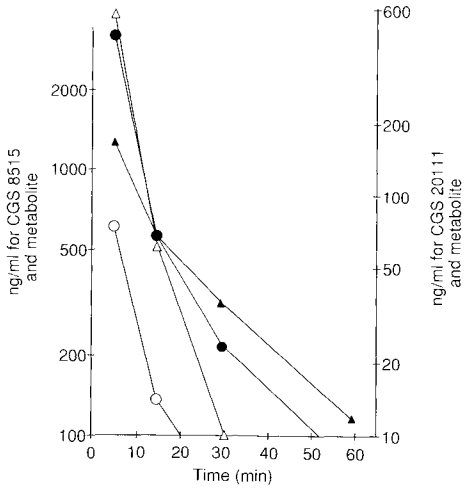


Fig. 2. Plasma concentration vs time profiles for CGS 8515 and its amide analogue (II) and their individual metabolites after 1 mg/kg iv drug administration in one dog. CGS 8515, ○; CGS 8515 metabolite, ●; analogue II, △; analogue II metabolite, ▲.

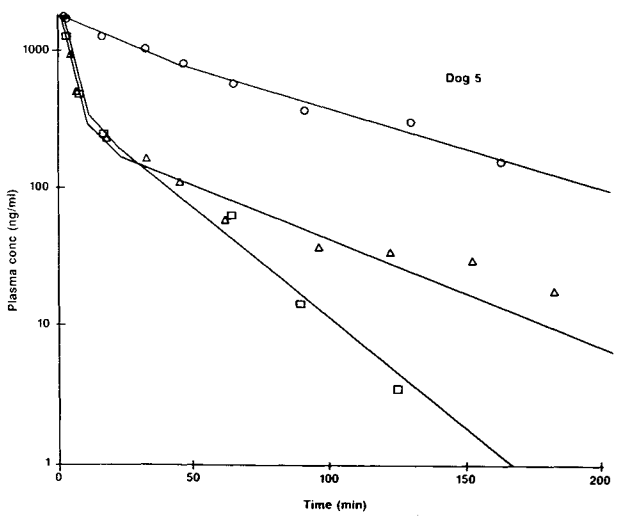


Fig. 3. Biexponential functions fitted to plasma concentration vs time profiles of three analogues following iv administrations at 1 mg/kg in one dog. Analogue V, ○; analogue III, △; analogue IV, □.

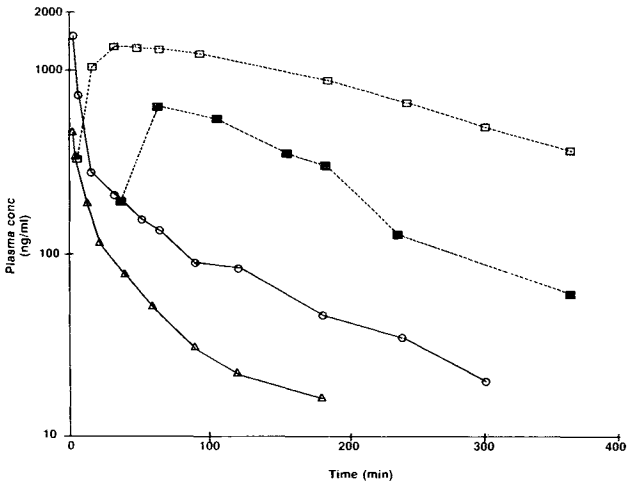


Fig. 4. Plasma concentration vs time profiles for analogue V in a portacaval dog and a normal dog. Portacaval dog: intravenous 1 mg/kg, ○; intraportal 1 mg/kg, △; oral 10 mg/kg, □. Normal dog: oral 10 mg/kg, ■.

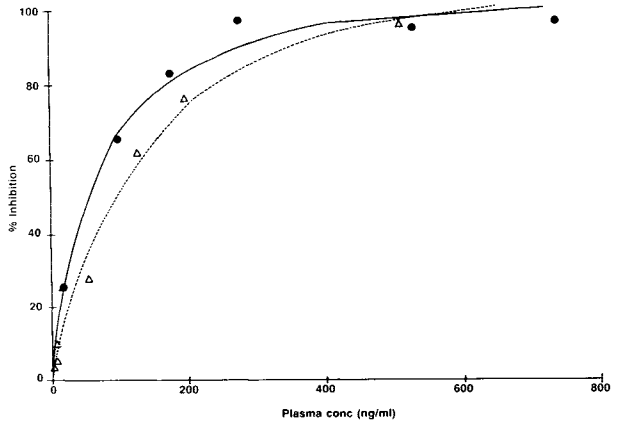


Fig. 5. Relationship of percentage inhibition (%I) of LTB_4 *ex vivo* to plasma concentration of CGS 8515 and analogue V fitted by a hyperbolic function. CGS 8515: △, %I = 122 * Cp/(124 + Cp). analogue V: ●, %I = 112 * Cp/(69 + Cp).

37%. This result suggests that the observed mean oral bioavailability of 28% in the normal dogs was the result primarily of a first-pass liver inactivation of the drug rather than low gastrointestinal absorption.

Plasma Concentration–Effect Relationship for CGS 8515 and Analogue V

There was a direct relationship between the plasma concentration of CGS 8515 and analogue V (Fig. 5) after iv administration and the pharmacological effect measured as the percentage inhibition of LTB_4 production, *ex vivo*. Intravenous administration of V (1 mg/kg) caused complete (>90%) inhibition at 5 min, the earliest time point of sample collection. The inhibition declined to 30% at 3 hr after drug administration.

DISCUSSION

Several biopharmaceutical problems were identified with the lead LOI candidate CGS 8515, including rapid inactivation to the less active free acid metabolite (IC_{50}, 100 μ*M*) and large hepatic first-pass effect after oral drug administration. Poor aqueous solubility (<1 μg/ml) may also have contributed to the poor oral bioavailability. As a consequence, the oral bioavailability of CGS 8515 was only 2% or less. A pharmacokinetic study demonstrated that an amide analogue (II) of CGS 8515 was metabolically more stable; however, poor aqueous solubility (<1 μg/ml) still remained a problem. The rapid disappearance of the amide from plasma was assumed to reflect extensive distribution rather than rapid elimination.

Three analogues (III, IV, V) with good *in vitro* potency (IC_{50}, <1 μ*M*) but increased aqueous solubility were then selected for biopharmaceutical evaluations. The methyl substitution on the nitrogen and the replacement of the aromatic ring with a nonaromatic ring increased the basicity of the nitrogen and the aqueous solubility of the analogues. All three analogues demonstrated longer mean residence times (MRT) compared to CGS 8515 and II. The rank order of MRT values for these analogues (V > III > IV) was the same as the rank order of their aqueous solubilities. The contribution of the early distribution phase (α phase) decreased dramatically for V, as calculated by its contribution to the total AUC. Percentage AUC_α was 7% for V, compared to

Table III. Compartment Independent Pharmacokinetic Parameters of CGS 8515 and Analog V following Oral and Intravenous Administrations

Analogue	AUC (mg · min/L)	Route	Dose (mg/kg)	Clearance (ml/min/kg)	Bioavailability (%)
I (CGS 8515)					
Normal dogs (n = 3)					
Mean	9.7	Oral	100	16,200	1.4
SD	7.1			13,400	1.1
V (CGS 19213)					
Normal dogs (n = 3)					
Mean	268	Oral	10	45	28
SD	137			25	16
Porta-caval dog	31	i.v. (forelimb)	1	32	—
	11.3	Intraportal (hindlimb)	1	88	37
	302	Oral (liver bypass)	10	33	98

42 and 55% for III and IV, respectively. Further, the removal of the ester function from CGS 8515 reduced the plasma clearance of these compounds. The mean clearance of CGS 8515 was 135 ml/min/kg, while clearance for V was only 10 ml/min/kg.

The decreased clearance of V resulted in the expected increase in the oral bioavailability (27%, as compared to 2% for CGS 8515). The study in the porta-caval dog model demonstrated that the magnitude of the oral bioavailability was due primarily to first-pass liver metabolism, while intestinal absorption was essentially complete.

The improved plasma concentration vs time profile for V correlated with a similar improvement in the biological profile for plasma LTB_4 inhibition as compared to CGS 8515. The IC_{50} measured *ex vivo* in plasma was comparable to that measured from *in vitro* studies, suggesting the measured activity was due primarily to V.

In conclusion, CGS 19213, an analogue of the LOI lead drug CGS 8515, was designed to improve specifically biopharmaceutical properties, especially with regard to clearance and aqueous solubility. Systematic improvement in physicochemical properties significantly changed the biopharmaceutical properties of this series of novel compounds.

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