1521-009X/47/2/67–70$35.00
Drug Metabolism and Disposition

https://doi.org/10.1124/dmd.118.084657
Drug Metab Dispos 47:67–70, February 2019

Short Communication

Identification of 19-(*S*/*R*)Hydroxyeicosatetraenoic Acid as the First Endogenous Noncompetitive Inhibitor of Cytochrome P450 1B1 with Enantioselective Activity[S]

Received September 24, 2018; accepted November 8, 2018

ABSTRACT

The overexpression of cytochrome P450 1B1 (CYP1B1) is a common characteristic of several diseases and conditions, such as inflammation, cancer, and cardiac hypertrophy. CYP1B1 is believed to contribute to pathogenesis of these diseases by mediating the formation of toxic compounds, either from exogenous or endogenous origin. We recently reported that an arachidonic acid metabolite, 19(*S*/*R*-)hydroxyeicosatetraenoic (HETE) acid, protects from cardiac hypertrophy by inhibiting the formation of toxic compounds, midchain HETEs, known to be formed by CYP1B1. This raised the question whether 19(*S*/*R*)-HETE can directly inhibit CYP1B1. In the current study, we report that 19(*S*/*R*)-HETE enantioselectively inhibits human recombinant CYP1B1 activity measured by 7-ethoxyresorufin O-deethylation assay. 19(*S*)-HETE is more potent than the *R* enantiomer (K_i = 37.3 and 89.1 nM, respectively). Noncompetitive inhibition was identified as the mechanism of CYP1B1 inhibition, which underlines the potentially important physiologic role of 19(*S*/*R*)-HETE as an endogenous CYP1B1 inhibitor; to our knowledge, 19(*S*/*R*)-HETE is the first inhibitor of its kind to be reported.

Introduction

Cytochrome P450 1B1 (CYP1B1) is a member of the heme-thiolate mono-oxygenase superfamily, a group of enzymes that are known to have the capacity to metabolize a wide range of endobiotics and xenobiotics (Faiq et al., 2014). Arachidonic acid (AA) and 17β-estradiol are examples of endogenous substances that are metabolized by human CYP1B1, and polycyclic aromatic hydrocarbons and dioxins represent some of its xenobiotic substrates (Shimada et al., 1997; Tsuchiya et al., 2004). Several CYP1B1-dependent metabolic transformations result in toxic and/or carcinogenic metabolites. CYP1B1 mediates the formation of dihydroxy-epoxy-polycyclic aromatic hydrocarbons, which are very potent electrophiles that attack DNA to form adducts leading to mutation and neoplastic transformation. Also, CYP1B1 mediates the formation of midchain hydroxylated arachidonic acid, namely 5-, 8-, 9-, 11-, 12-, and 15-hydroxyeicosatetraenoic (HETE) acids (Choudhary et al., 2004). Accumulating studies report the cardiotoxic effects of midchain HETEs and their involvement in the pathogenesis of hypertension, cardiac hypertrophy, and heart failure (Dołegowska et al., 2009; Kayama et al., 2009; El-Sherbeni and El-Kadi, 2014a). CYP1B1 is selectively expressed at a low level in extrahepatic tissues and not in normal human liver. The expression of CYP1B1 only achieves high levels in abnormal tissues; CYP1B1 overexpression is actually regarded as a marker of malignancy phenotype; it was also reported in cardiovascular diseases (Jennings et al., 2010; El-Sherbeni and El-Kadi, 2014; Maayah et al., 2017). Therefore, considerable interest has been expressed in CYP1B1 as a novel therapeutic target for cancer (Li et al., 2015; Chang et al., 2017; D'Uva et al., 2018).

Another group of AA-associated metabolites that are formed by cardiac cytochrome P450s is subterminal HETEs, exemplified by 16-, 17-, 18-, and 19-HETE. Recently, we demonstrated that both enantiomers of 19-HETE (Fig. 1) protect against angiotensin II (Ang II)-induced cardiac hypertrophy with preferential effect of 19(*S*)-HETE. One important finding of our study was that both enantiomers of 19-HETE inhibited the formation of midchain HETEs and the catalytic activity of CYP1B1 in two different cell lines, human ventricular cardiomyocytes RL-14 cells and rat cardiomyoblasts H9c2 cells (Shoieb and El-Kadi, 2018). To obtain a comprehensive understanding of the protective role of *R*- and *S*-enantiomers of 19-HETE in the case of cardiac hypertrophy, we undertook the current study to examine, using 7-ethoxyresorufin O-deethylation (EROD) assay, the inhibitory effect of both enantiomers on human recombinant CYP1B1 enzyme.

Materials and Methods

Materials. 19(*R*)- and 19(*S*)-HETEs were purchased from Cayman Chemical (Ann Arbor, MI). 7-Ethoxyresorufin (7-ER) and nicotinamide adenine dinucleotide phosphate (NADPH) tetrasodium salt were purchased from MilliporeSigma (St. Louis, MO). Human recombinant CYP1B1 microsomes supplemented with NADPH–cytochrome P450-oxidoreductase (Corning-Supersomes) were obtained from Fisher Scientific (Hampton, NH); all other chemicals were purchased from Thermo Fisher Scientific (Toronto, ON).

Determination of CYP1B1-Inhibition Kinetics by 19-HETE Enantiomers. The O-dealkylation rate of 7-ER by recombinant human CYP1B1 was measured in the absence and presence of 19(*R*)-HETE or 19(*S*)-HETE. Briefly, 96-well solid black polystyrene microplates were used to carry out the fluorescent

This work was supported by a grant from the Canadian Institutes of Health Research [Grant 106665] to A.O.S.E.-K. S.M.S. is the recipient of Antoine Noujaim Graduate Scholarship in Pharmaceutical Sciences.

https://doi.org/10.1124/dmd.118.084657.

[S]This article has supplemental material available at dmd.aspetjournals.org.

ABBREVIATIONS: AA, arachidonic acid; Ang II, angiotensin II; 7-ER, 7-ethoxyresorufin; EROD, 7-ethoxyresorufin O-deethylation; HETE, hydroxyeicosatetraenoic acid; NADPH, nicotinamide adenine dinucleotide phosphate; S002-333, 2-(4-methoxy-benzenesulfonyl)-2,3,4,9-tetrahydro-1H-b-carboxylic acid amide.

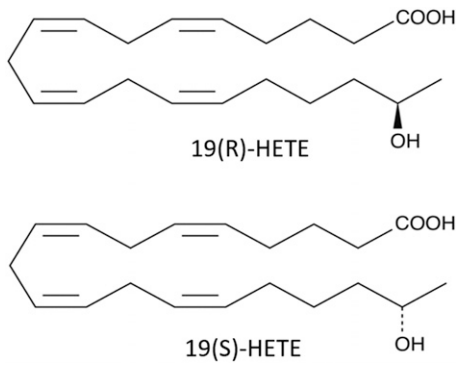

Fig. 1. *R*- and *S*-enantiomers of 19-HETE. Both enantiomers represent an example of stereoisomerism of P450-mediated arachidonic acid metabolites. Incorporation of an hydroxyl group in the carbon skeleton of arachidonic acid generates a chiral center, hence there are two enantiomers for each HETE metabolite.

assay. The reaction mixture containing 100 mM potassium phosphate (pH 7.4) buffer supplemented with 5 mM magnesium chloride hexahydrate and 1 pmol of human CYP1B1 was incubated with 10–100 nM 7-ER. In addition, 0, 5, 10, 20, or 40 nM 19(*R*)-HETE or 19(*S*)-HETE was added to the reaction mixture. The volume of the reaction mixture was 100 μl. The reaction was initiated by the addition of 100 μl of 2 mM NADPH; the fluorescent signal related to the formation of resorufin was measured every minute (excitation and emission wavelengths of 550 and 585 nm, respectively) for 30 minutes at 37°C using a BioTek Synergy H1 Hybrid Reader (BioTek Instruments, Inc., Winooski, VT). The quantity of formed resorufin was measured by the construction of a standard curve of 0–200 nM resorufin dissolved in the same incubation buffer. The formation rate of resorufin in each well was determined over the first 9-minute period using linear regression. The formation rates of resorufin at different 7-ER concentrations were fitted to Michaelis-Menten equation, while 19(*R*)-HETE-CYP1B1 and 19(*S*)-HETE-CYP1B1 inhibition rates were fitted to competitive, noncompetitive, uncompetitive, and mixed models of enzyme inhibition. The most probable enzyme inhibition model was selected according to Akaike information criteria (AIC). The fitting was carried out using GraphPad Prism (version 5.01; GraphPad Software, Inc., La Jolla, CA).

Determination of 19(*R*)-HETE and 19(*S*)-HETE In Vitro Stability. To assess the stability of 19-HETE enantiomers in solution under the same experimental conditions used for the kinetic study, the stability experiment was performed using 7-ER concentration corresponding to the of the reaction (86 nM). 19(*R*)-HETE and 19(*S*)-HETE were added to the reaction mixture at their K_i concentrations of 89.1 and 37.3 nM, respectively. After initiation of the reaction with 2 mM NADPH, 200 μl was taken from the mixture at the start of the reaction (T0), after 10, 20, and 30 minutes and at 37°C. The reaction was stopped using 400 μl ice-cold acetonitrile and the extraction of 19-HETE enantiomers was performed using ethyl acetate and dried using speed vacuum (Savant, Farmingdale, NY). The analysis of 19-HETE enantiomers was performed using liquid chromatography–electrospray ionization mass spectrometry (LC-ESI-MS) as previously described (Shoieb and El-Kadi, 2018).

Results and Discussion

Inhibition of CYP1B1 is proposed as one of the strategies in the treatment of cardiovascular diseases and cancer chemoprevention (Mikstacka et al., 2007). In this regard, we tested the capacity of both enantiomers of 19-HETE to inhibit the catalytic activity of human recombinant CYP1B1, on the basis of our previous finding that 19(*R*)- and 19(*S*)-HETE protected against Ang II-induced cellular hypertrophy via decreasing the level of cardiotoxic midchain HETEs, the major metabolites of CYP1B1 (Shoieb and El-Kadi, 2018). For that purpose, the inhibition kinetics of both enantiomers of 19-HETE on CYP1B1 activity were carried out using CYP1B1 substrate 7-ER, a compound that is metabolized via oxidative reaction by this enzyme (Shimada et al., 1997). The experimental conditions, such as the concentrations of 7-ER, concentration of the recombinant enzyme, in addition to the incubation time, were all optimized before the addition of either 19(*R*)-HETE or 19(*S*)-HETE.

Inhibition of EROD is a well reported assay carried out predominantly to examine the inhibitory potencies of CYP1B1 inhibitors (Androutsopoulos et al., 2011). Inhibitory activity of 19(*R*)-HETE (Fig. 2A) and 19(*S*)-HETE (Fig. 2B) on CYP1B1 was studied. Foremost experiments involved the assessment of 7-ER-O-deethylase kinetics by determination of the rate of resorufin formation over time. On the basis of the nonlinear regression analysis and fitting to the Michaelis-Menten model, the maximal EROD activity (V_{max}) in the control containing 0.1% dimethyl sulfoxide was 16.7 pmol resorufin/pmol P450 per minute ($R^2 = 0.9888$) (Table 1). The Michaelis-Menten constant (K_m) value for the current EROD reaction is 86.6 nM (Table 1). During the coincubation of either 19-HETE enantiomers at 10 nM concentration with different concentrations of 7-ER, we observed a significant inhibition of CYP1B1 enzymatic activity. This finding prompted us to test increasing concentrations (0–40 nM) of either 19(*R*)-HETE or 19(*S*)-HETE with different concentrations (0–100 nM) of 7-ER to characterize the mode of inhibition by 19-HETE enantiomers.

Nonlinear regression analysis and comparisons showed that the mode of inhibition for 19(*R*)-HETE and 19(*S*)-HETE is noncompetitive inhibition of CYP1B1 enzyme. Dixon plots showed that 19(*R*)-HETE (Fig. 3A) and 19(*S*)-HETE (Fig. 3B) have K_i values of 89.1 and 37.3 nM, respectively (Table 1). The K_i values of both enantiomers showed that the *S*-enantiomer is more potent than the *R*-enantiomer by approximately 2.4-fold. This comes in agreement with our previous work demonstrating that the *S*-enantiomer of 19-HETE has a preferential effect over the *R*-enantiomer in inhibiting the formation of midchain HETEs (Shoieb and El-Kadi, 2018). In accordance with being noncompetitive inhibitors, the in vitro stability study showed that both enantiomers of 19-HETE are metabolically stable in solution during the 30-minute time course of the kinetic experiment as there was no significant difference in the level of both enantiomers in the duration of the experiment (Supplemental Figs. 1 and 2; Table 2). Discovery of selective and potent inhibitors of CYP1B1 enables the design of CYP1B1-targeted cardiovascular treatment and prevention in addition to cancer chemotherapy (Pingili et al., 2017; Ware, 2017).

To date, more than 50 synthetic and natural compounds have been designed or recognized as inhibitors of CYP1B1 (Li et al., 2017). Various CYP1B1 inhibitors with varying mode of inhibition have been identified. For instance, *trans*-resveratrol inhibited CYP1B1 enzymatic activity via mixed-type inhibitory mechanism, and the K_i was 0.75 μM (Chang et al., 2000). Also, myricetin and quercetin with K_i 230 and 120 nM, respectively, exhibited mixed-type inhibition, whereas apigenin with K_i 60 nM acted as a competitive inhibitor of CYP1B1 (Chaudhary and Willett, 2006). Moreover, tetramethoxystilbene (TMS) noncompetitively inhibited EROD activity of CYP1B1 with an IC_{50} value of 2 nM, when 2 μM 7-ER was used as a substrate (Chun et al., 2009). In the present study, both enantiomers of 19-HETE inhibited CYP1B1 enzymatic activity in the nanomolar range and more potently than some of the previously mentioned CYP1B1 inhibitors, which recommends their introduction as therapeutic and preventive modalities in CYP1B1-targeting therapies.

In the current study, there is a significant difference in the inhibitory activity between *R*- and *S*-enantiomers of 19-HETE. To the best of our knowledge, the current study is the first to characterize the enantioselective pattern of inhibition of CYP1B1 by enantiomers of 19-HETE. Enantioselective inhibition of cytochrome P450 enzymes has been previously documented. S002-333, a novel and potent antithrombotic agent, inhibited CYP2B6-mediated bupropion 6-hydroxylation in a stereoselective manner. Although the *S*-enantiomer showed strong inhibition with IC_{50} value of 5.28 μM, the *R*-enantiomer showed

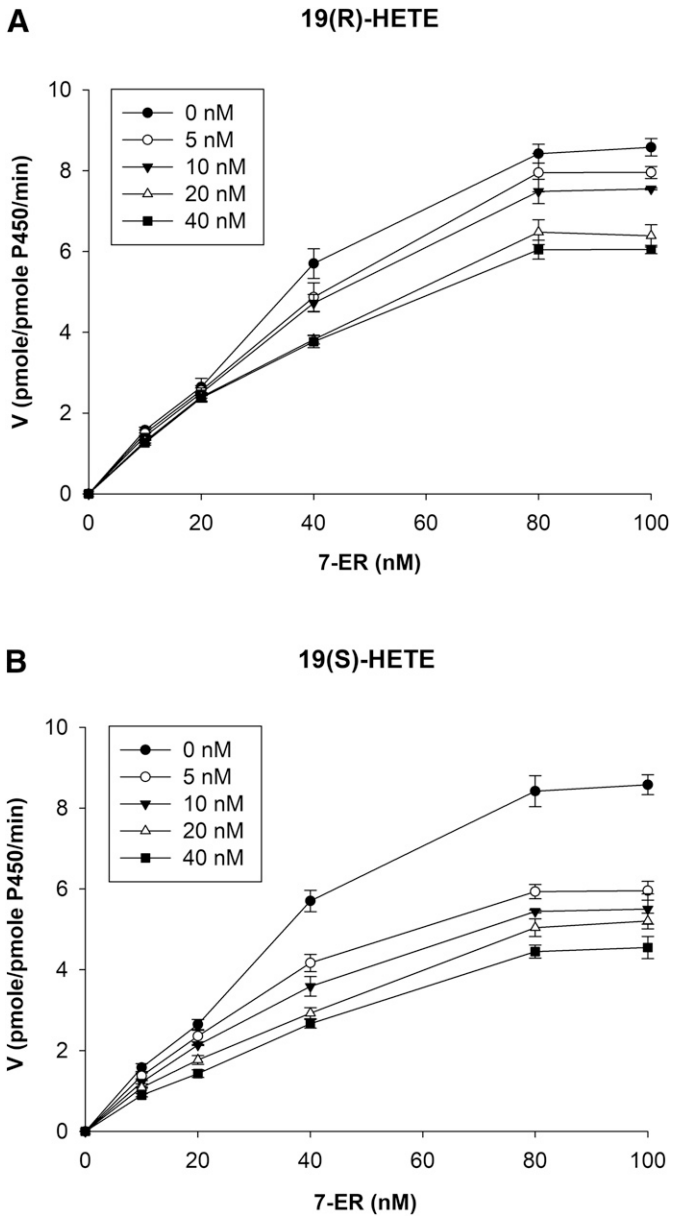

Fig. 2. Inhibitory effect of 19(*R*)-HETE and 19(*S*)-HETE on EROD activity mediated by human recombinant CYP1B1. In 96-well solid black polystyrene plates, the reaction mixture containing 100 mM potassium phosphate (pH 7.4) buffer supplemented with 5 mM magnesium chloride hexahydrate and 1 pmol of human CYP1B1 was incubated with 10–100 nM 7-ER. In addition, either 19(*R*)-HETE (A) or 19(*S*)-HETE (B) at 0, 5, 10, 20, or 40 nM concentration was added to the reaction. The reaction was initiated by the addition of 100 μl of 2 mM NADPH, the fluorescent signal related to the formation of resorufin was measured every minute (excitation and emission wavelengths of 550 and 585 nm, respectively) for 30 minutes at 37°C using a BioTek Synergy H1 Hybrid. The quantity of formed resorufin was measured by the construction of standard curve of 0–200 nM resorufin dissolved in the same incubation buffer. Each point represents the mean of six independent experiments ± S.E.

much less inhibition on CYP2B6 catalytic activity with IC_{50} value of more than 50 μM (Bhateria et al., 2016).

It is noteworthy that CYP1B1 regulates several metabolic pathways. For example, AA is oxidized by CYP1B1 to produce the cardiotoxic midchain HETE metabolites. In rat neonatal cardiomyocytes, 15-HETE has been reported to be capable of elevating isoproterenol-induced β-adrenergic response sensitivity. Moreover, 5-, 12-, and 15-HETE were capable of inducing cellular hypertrophy in RL-14 cells, human

TABLE 1

The mean values and S.E. of kinetic parameters of resorufin formation by human recombinant CYP1B1 in the absence and presence of 19(*R*)-HETE or 19(*S*)-HETE

Results are presented as mean and S.E., of at least three individual experiments. K_m, V_{max} and K_i mean ± S.E. were determined by the Enzyme Kinetics module of GraphPad Prism, version 5.01.

K_m	V_{max}	K_i	
		19(*R*)-HETE	19(*S*)-HETE
nM	*pmol/pmol P450 per minute*	*nM*	*nM*
86.6 ± 10.4	16.7 ± 1.1	89.1 ± 11.5	37.3 ± 5.5

ventricular cardiomyocytes, via mitogen-activated protein kinase- and nuclear factor κB-dependent pathways (Zhang et al., 2014; Maayah and El-Kadi, 2016). Furthermore, CYP1B1 is responsible for some vital physiologic processes in blood vessels important to the development and progression of hypertension. In Ang II-induced hypertension, CYP1B1 mediates cell migration, induces proliferation, and increases protein

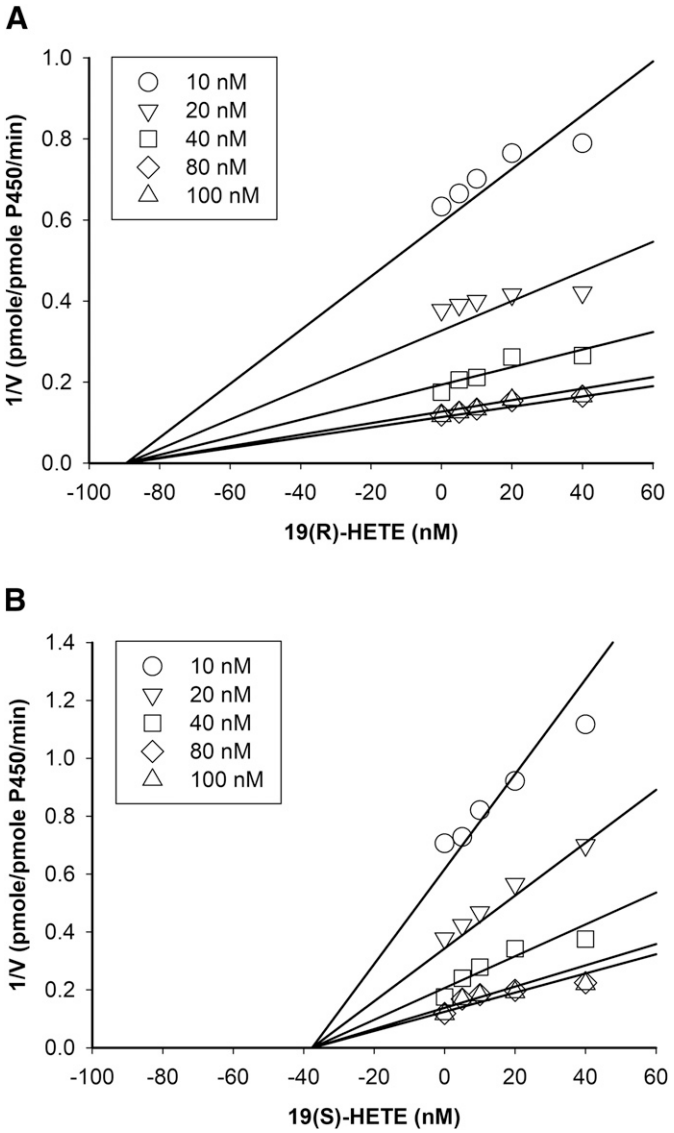

Fig. 3. Dixon plots representing the inhibitory effect of 19(*R*)-HETE and 19(*S*)-HETE on EROD activity mediated by human recombinant CYP1B1. The *y*-axis represents the reciprocal of EROD activity expressed in picomoles of resorufin/picomoles CYP1B1/minute and the *x*-axis represents 19(*R*)-HETE (A) or 19(*S*)-HETE (B) concentrations in nanomolar concentrations in the presence of increasing concentration of the substrate 7-ER (10–100 nM). Each point represents the mean of six independent experiments ± S.E.

TABLE 2

The mean values and S.E. of 19(*R*)-HETE or 19(*S*)-HETE levels calculated as a percentage of the metabolite level at the beginning of the experiment (T_0)

Data are represented as mean ± S.E. where n = 3.

	19(R/S)-HETE levels as a percentage of their levels at zero time (%)			
Time interval (T_{minute})	T_0	T_{10}	T_{20}	T_{30}
19(*R*)-HETE	100 ± 17.1	98.6 ± 7.8	96.3 ± 1.3	98.5 ± 8.9
19(*S*)-HETE	100 ± 5.9	99.7 ± 1.3	100.1 ± 0.9	98.9 ± 4.6

synthesis in vascular smooth muscle cells through regulation of the metabolism of AA (Yaghini et al., 2010). The fact that CYP1B1 expression is vastly enhanced in several tumors, such as prostate, breast, and colon cancers, suggests that CYP1B1 inhibitors could be considered an accepted therapeutic strategy in treatment and prevention of cancer (Gibson et al., 2003; Tokizane et al., 2005).

In summary, CYP1B1 is considered one of the important cytochrome P450s. It is involved in the metabolism of many endobiotics, xenobiotics, and in the activation pathways of various procarcinogens. The findings of the current study show that both enantiomers of 19-HETE noncompetitively inhibited CYP1B1 enzymatic activity with the *S*-enantiomer having a higher potency. The current study suggests that 19(*R*)-HETE and 19(*S*)-HETE could be considered a novel therapeutic modality in the treatment of hypertension, cardiac hypertrophy, and cancer. Moreover, given that a noncompetitive inhibitor might bind to the enzyme regulatory region, 19(*R*)-HETE and 19(*S*)-HETE could be the focus for unraveling the obscure mechanisms of CYP1B1 enzymatic reaction.

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Authorship Contributions

Participated in research design: Shoieb, El-Kadi.

Conducted experiments: Shoieb.

Performed data analysis: Shoieb, El-Sherbeni, El-Kadi.

Wrote or contributed to the writing of the manuscript: Shoieb, El-Sherbeni, El-Kadi.

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