Supporting Information

An Exceptionally Potent Inhibitor of Human CD73

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I. MATERIALS AND METHODS

1. Protein Expression and Purification

The sequence of human CD73 (Uniport ID: P21589, aa 27-549) was modified for protein expression and incorporation into the expression vector. In brief, the native leader sequence was replaced with the mouse immunoglobulin heavy-chain leader sequence, MEWSWVFLFFLSVTTGVHS, to allow for protein secretion, Asn was mutated to Asp in the following instances N53D, N311D, N333D, N403D, to remove N-glycosylation sites, and a C-terminal 6 × His-tag was added.

Plasmid generation and expression was performed as a contract service at Lake Pharma. The modified gene was inserted into Lake Pharma's proprietary high expression mammalian vector and expressed in HEK293 cells using their Tuna293^{TM} Process. One liter was harvested and delivered to Arcus Biosciences for purification.

At Arcus Biosciences, five tablets of Complete™, EDTA-free Protease Inhibitor Cocktail were added to the harvested supernatant. Purification was performed using an AKTA Pure FPLC system (GE Healthcare Life Sciences). All procedures were carried out at 4 °C. Supernatant was applied to a 5 mL HisTrap Excel column (GE Healthcare Life Sciences) and washed with eight column volumes of wash buffer (25 mM Tris, pH 8.0, 400 mM NaCl, 2 mM imidazole). In a single step elution, the protein was eluted with 25 mM Tris, pH 8.0, 300 mM NaCl, and 400 mM imidazole. Thirty micromolar $ZnCl_2$ and 5 mM $CaCl_2$ were added to the eluate. The eluted protein was further purified by gel filtration chromatography using a HiLoad 16/60 Superdex 200 pg column (GE Healthcare Life Sciences) in 50 mM Tris, pH 8.0, 100 mM NaCl. The protein was

concentrated to 5 mg/ml with an Amicon Ultra-15 spin tube (Millipore Sigma), and the protein concentration was initially measured using absorbance at 280 nm with $\varepsilon = 0.8969$ $(mg/ml)^{-1}$ cm^{-1}. Confirmation of hCD73 protein expression and purity was evaluated by reducing SDS–PAGE electrophoresis and western blot.

2. hCD73 Concentration Measurement by Active-site Titration

The concentration of hCD73 is critical for the design of the reversibility assay, and for the analysis of the tight-binding data and progress curves for K_i determination. Therefore, the enzyme concentration was determined by active-site titration (Figure S1) for these applications and is reported as the concentration determined by active-site titration except as noted. Active-site titration was conducted by adding hCD73 to a Nunc 96-well polypropylene plate to a final concentration of 20 nM (determined from A_{280} measurements) in assay buffer. AB680 was added at varying concentrations with a final DMSO concentration of 2%. AB680 and hCD73 were incubated at room temperature at 300 rpm for 1 h. After the incubation, the reaction was initiated by adding AMP to a final concentration of 30 µM. The reaction was stopped after 30 s by transferring 80 µL of the reaction mixture to 20 µL of P_iColorLock Gold Phosphate Detection System (malachite green based detection reagent, Innova Biosciences), which was sufficiently acidic to quench the reaction. The detection plate was incubated shaking at room temperature for 30 min, then read on an Envision multimode reader at 620 nm. A condition containing no enzyme was used for 0% activity and a condition using DMSO instead of AB680 was set to 100% enzyme activity. From the absorbance data, the ratio of the reaction velocity with AB680 to the velocity without AB680, v/v_o, was calculated and plotted as a function of AB680 concentration. The *x*-intercept extrapolated from a linear regression of the linear portion of the curve yielded the

concentration of active sites capable of binding the inhibitor. A division factor of 2.5 was applied to protein concentration determined by the absorbance at 280 nm to generate the concentration determined by active-site titration.

3. Measurement of k_{cat} and K_M

The k_{cat} and K_M of hCD73 with AMP were determined from a substrate saturation curve using the AMP-Glo assay (Promega), monitoring AMP depletion (Figure S2). Recombinant human CD73 (hCD73) was added to the assay plate to a final assay concentration of 0.2 nM. The assay buffer, used at 2X, was composed of 50 mM HEPES pH 7.4, 100 mM NaCl, and 0.1% PEG-8000. To measure k_{cat} and K_M as close as possible to experimental conditions in experiments containing AB680, 2% DMSO was added and the hCD73 + 2% DMSO mixture was incubated for 1 h at room temperature. Varying concentrations of AMP (0, 1.6, 3.1, 6.3, 12.5, 25, 50, and 100 µM) were used to initiate the enzymatic reaction, and 10 µM AB680 was used to quench the reaction at the following time points: 0, 3, 6, 9, 12, 15, 20, 25, 30, 45, 60, and 90 min. While the reactions were running the plate was sealed and shaking at room temperature. After the reactions were quenched, 25 µL of the reaction mixture from the plate were transferred to a white, opaque, 96-well plate containing 25 µL of the R1 reagent. The plate was mixed at 150 rpm for 1 h, then the R2 solution was prepared by adding 80 µL R2 to 8 mL Kinase Glo. Fifty microliters of the resulting solution were added to the development plate, and it was again incubated shaking at 150 rpm at room temperature for 1 h. After 1 h, luminescence was read on an Envision multimode reader. Using a standard curve, luminescence values were converted to AMP concentrations. The slopes from the linear portion of the reaction time courses were replotted to generate a substrate saturation curve which was fitted to the Michaelis-Menten equation: $v = (V_{max} \times S) / (K_M + S)$

where v is the initial velocity, V_{max} is the maximum enzyme velocity, S is the substrate concentration, and K_M is the Michaelis constant. Only substrate concentrations less than 10-fold K_M were used in the fit. To obtain k_{cat}, the V_{max} was divided by the concentration of enzyme.

4. Discovery and Synthesis of AB680

The discovery and synthesis of AB680 was presented at the 2019 American Chemical Society Meeting in Orlando, FL[1] and in U.S. Patent 10,239,912[2]. A separate publication on the discovery and synthesis of AB680 is in preparation.

5. Tight-binding of AB680

To test for tight-binding of AB680, dose-response curves of hCD73 were determined at different enzyme concentrations (Figure S3). The highest AB680 concentration was 200 nM and each curve consisted of 10 points of successive 4-fold dilutions. Enzyme was added to the plate at the following concentrations: 2, 1.6, 1.2, 1.0, 0.8, 0.4, 0.2, and 0.1 nM. DMSO at 2% was present in each well. Inhibitor and enzyme were incubated at room temperature at 300 rpm for 1 h, at which point 30 μM AMP were added to initiate the reaction. After 02:24, 03:00, 04:00, 04:48, 06:00, 12:00, 24:00, 48:00 (mm:ss) corresponding with then enzyme concentrations above, the reaction was stopped by transferring the reaction to a development plate containing PiColorLock Gold, incubated for 30 min at room temperature, shaking, and read at 620 nm on an Envision multimode reader. For the experiment shown in Figure S3E, the enzyme concentrations were shifted lower, so that the highest concentration tested was 0.4 nM and the lowest was 0.05 nM. Reaction times were adjusted accordingly. The AB680 dose-response curve was also shifted lower

so that it was a 10-point, 3-fold dilution with the highest concentration of AB680 being 10 nM. IC_{50}s were plotted as a function of enzyme concentration and fit with linear regression. The slope and y-intercept (K_{iapp}) were determined from the fit.

6. Jump-dilution Experiment

Enzyme and AB680 or DMSO were mixed such that the final concentrations of enzyme and compound or vehicle were equal to 20 nM, and incubated, shaking, for 1 h. The enzyme/AB680 or DMSO mixture was diluted 200-fold into a 96-well Nunc polypropylene microwell plate containing 100 μM AMP. At several times postdilution, 80 μL from the assay plate were transferred to a 96-well spectra plate containing 20 μL of PiColorLock Gold. In the analysis, time points were corrected for the exact time the assay mixture was transferred to the PiColorLock Gold plate. The 96-well spectra plate was then placed on a shaker for 30 min. Subsequently absorbance was read using an Envision multimode reader at 620 nm. The data from the AB680 points was fit to the equation $P = (v \times t) - (v/k_{obs}) \times (1 - e^{-k_{obs}t})$ where P is inorganic phosphate (P_i) concentration, t is time postdilution, v is the steady-state rate and k_{obs} is the observed first-order rate constant for dissociation of the EI complex (Figure S4).

7. Slow-binding of AB680

To test for slow-binding of AB680, dose-response curves of AB680 were determined at different incubation times between enzyme and inhibitor (Figure S5). Enzyme concentration was 100 pM. The highest AB680 concentration was 10 nM and each curve consisted of 10 points of successive 3-fold dilutions. Control wells contained no added AB680. Reactions were started by

addition of 30 μM AMP. After 45 min, the reaction was stopped by transferring the reaction to a development plate containing PiColorLock Gold. The development plate was incubated for 30 min at room temperature on a plate shaker and then read at 620 nm on an Envision multimode reader. The half-maximal inhibition concentrations (IC_{50}) of AB680 were plotted as a function of incubation time.

8. K_i Determination

Progress curves of hCD73 inhibition by AB680 were measured in a Nunc 96-well polypropylene microwell plate in 30 μM AMP, AB680 concentrations of 0, 400, 500, 600, 1000, 1500, 2000, and 3400 pM in 2% DMSO at room temperature (Figure 3A). The plate containing AB680 and AMP was placed on the shaker at 300 rpm for 10 min to mix. A separate 96-well plate contained 200 pM hCD73 in 2X assay buffer (final assay concentration of hCD73 = 100 pM). The reaction was initiated by adding an equal volume of enzyme to the substrate/AB680 mixture. After 0, 3, 6, 9, 12, 15, 20, 30, 45, 60, 75, and 90 min of reaction, 80 μL from the assay plate were transferred to a spectra plate containing 20 μL of PiColorLock Gold. Time points were corrected by 30 s for the exact timing of addition of PiColorLock Gold. The development plate was placed on the shaker for 30 min. Subsequently, absorbance was read using an Envision multimode reader at 620 nm. Outlier data points at 2.5 min and 1000 and 2000 pM AB680 were excluded from the fit. Progress curves were fitted by numerical integration as implemented in KinTek Explorer[3], and parameter boundaries were determined by FitSpace analysis[4] (Figure 3B-D). The χ^2 threshold for FitSpace analysis was 0.8333.

II. RESULTS

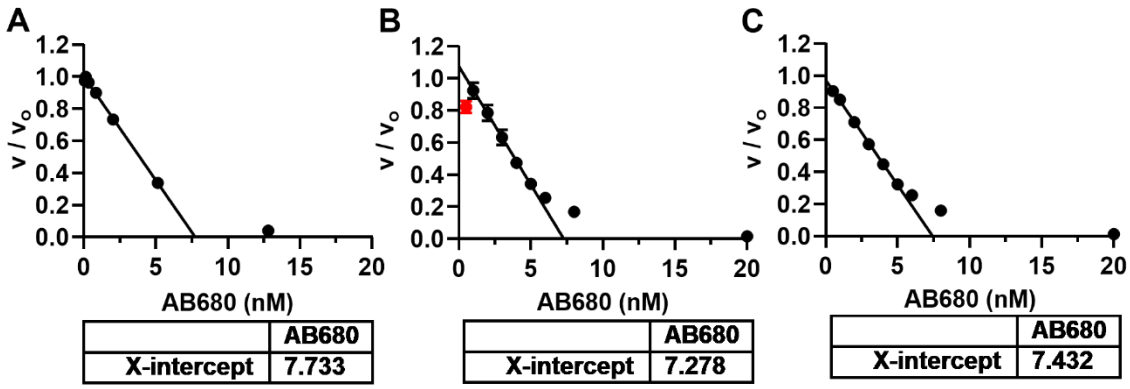


Figure S1. Active-site titration of hCD73. The red point in panel B was deemed to be an outlier and was excluded from the fit. The data points in panel A are the average of technical duplicates and the data points in panels B and C are the average of technical triplicates.

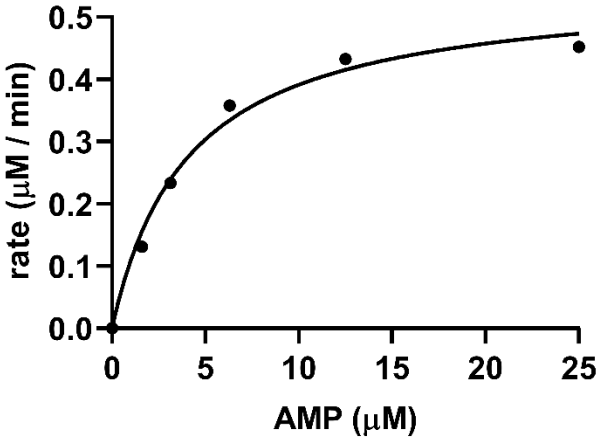


Figure S2. Substrate saturation curve for hCD73 with AMP. A k_{cat} = 46 ± 3 s^{-1} and a K_M = 4.1 ± 0.7 μM were obtained, resulting in a k_{cat}/K_M = (11 ± 2) x 10^6 s^{-1} M^{-1}.

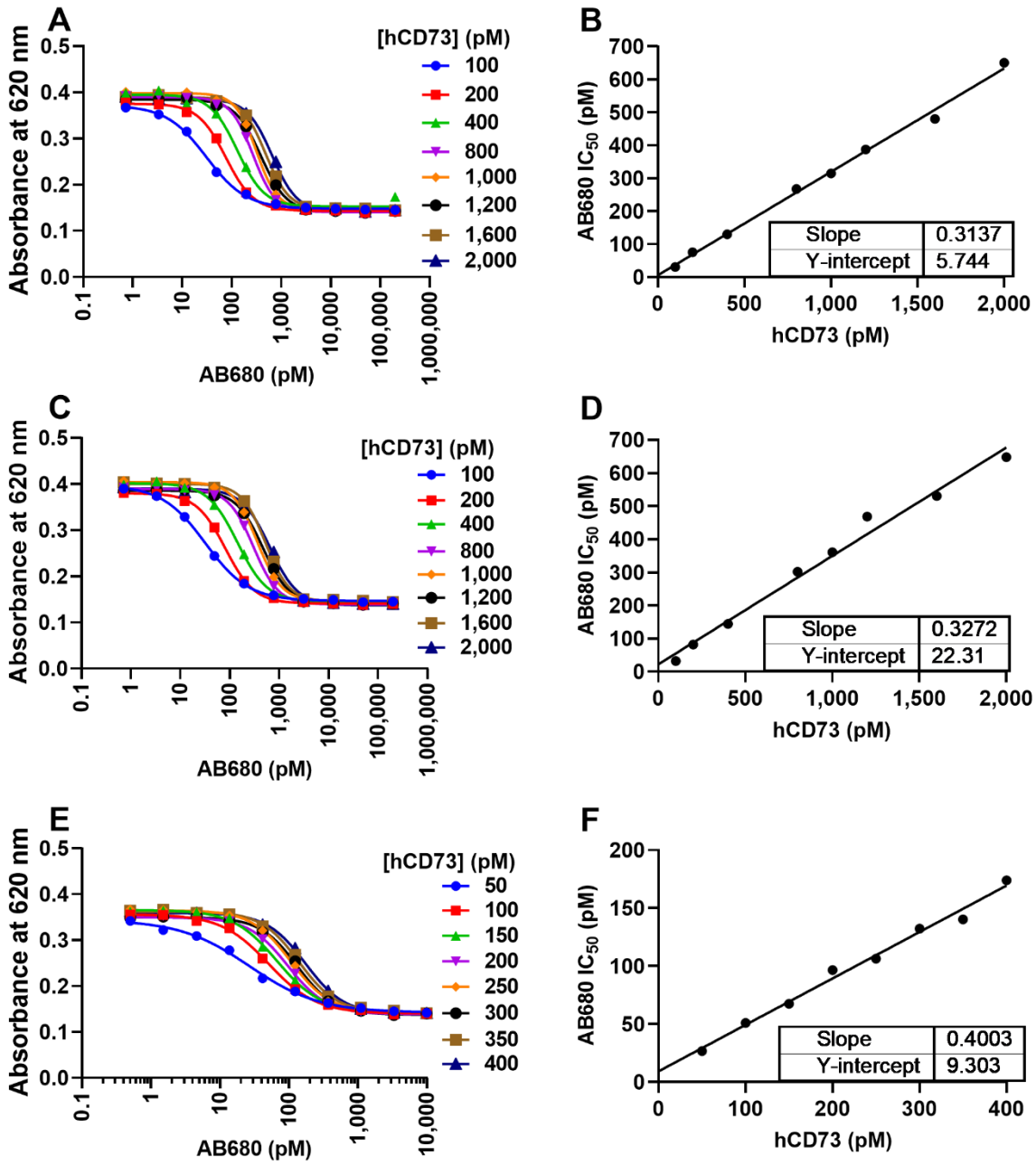


Figure S3. Tight-binding of AB680 to hCD73. Panels A/B, C/D, and E/F are three independent experiments. Panels A and B are also shown in the main text.

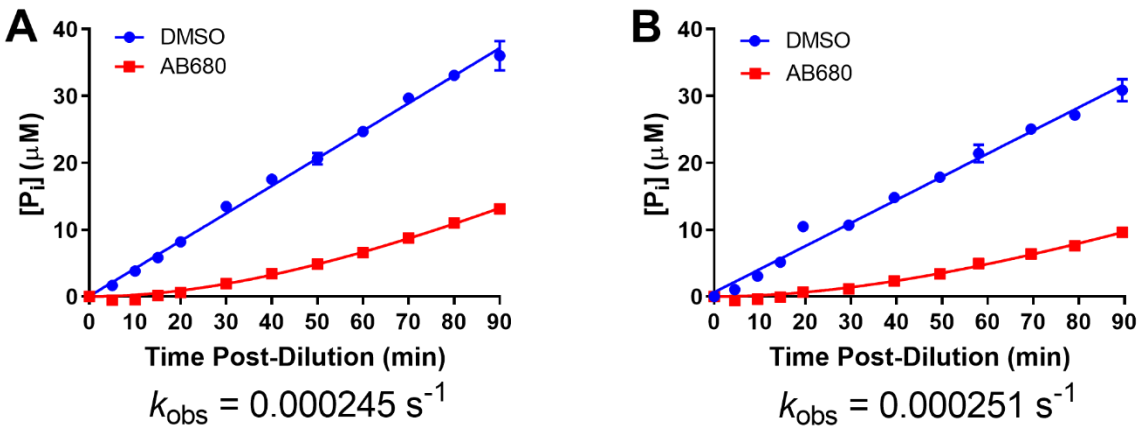


Figure S4. Reversibility of AB680 binding to hCD73. Panels A and B are two independent experiments, panel A is also shown in the main text.

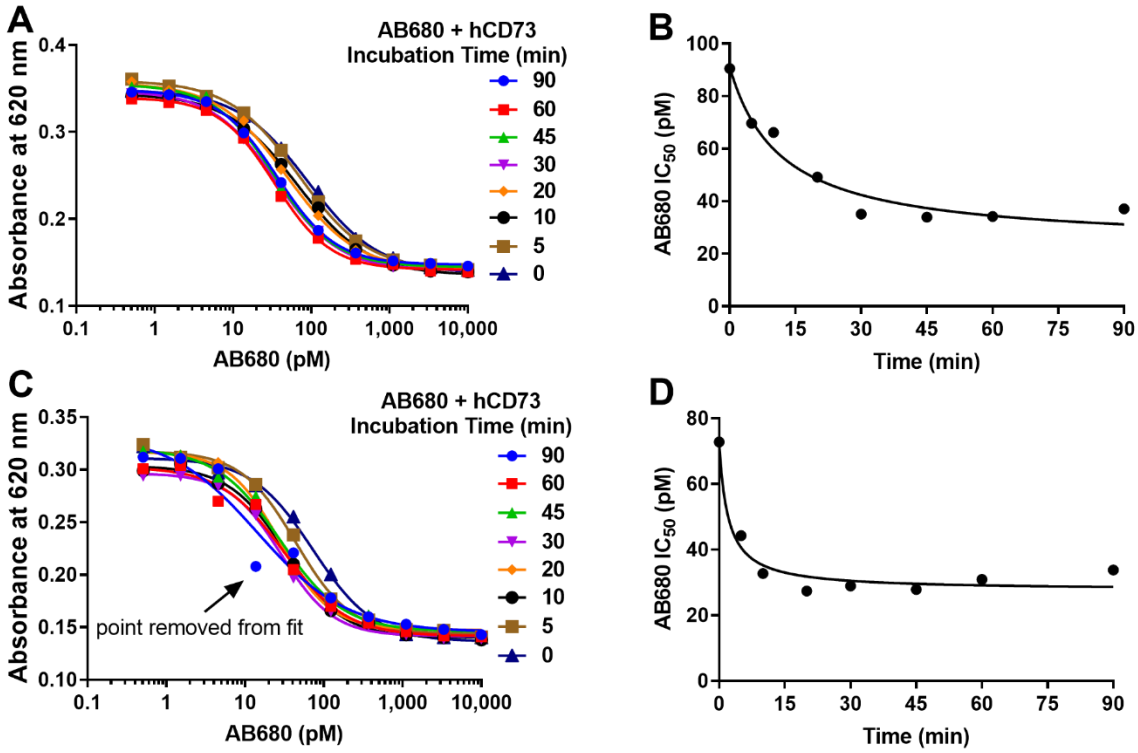
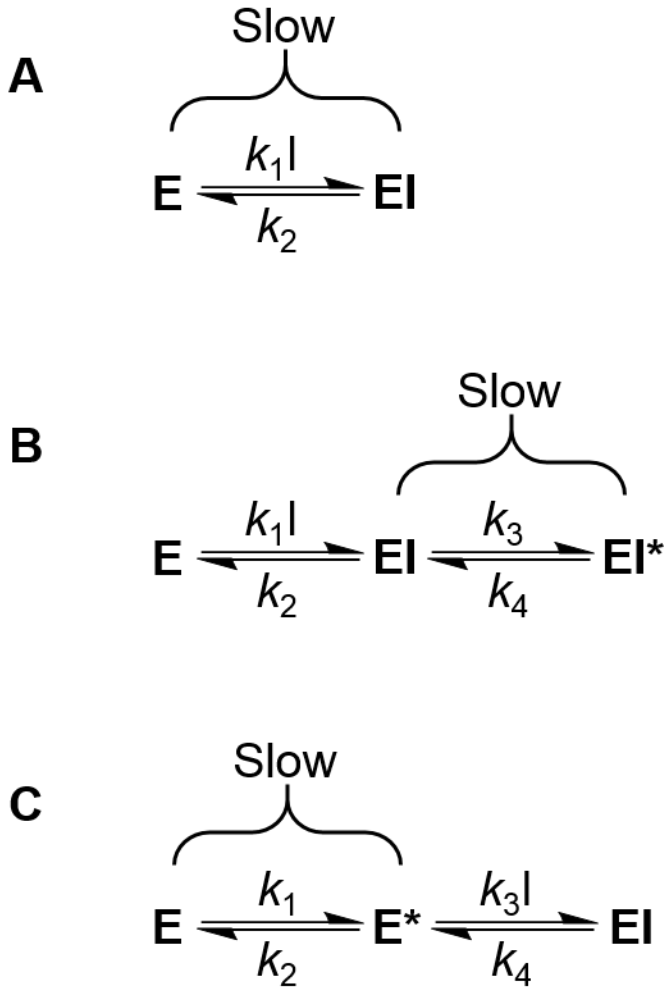


Figure S5. Slow-binding of AB680 to hCD73. Panels A/B and C/D are two independent experiments, and panel A/B are also shown in the main text. AB680 binds to hCD73 slowly, leading to an increase in potency over time until a plateau is reached.

III. SCHEME

Scheme S1: Mechanisms for slow-onset non-covalent inhibition.



IV. REFERENCES

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