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Carbonic anhydrase inhibitors: Inhibition of the β-class enzymes from the fungal pathogens *Candida albicans* and *Cryptococcus neoformans* with simple anions

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ABSTRACT

The catalytic activity and inhibition of the β-carbonic anhydrases (CAs, EC 4.2.1.1) from the pathogenic fungi *Candida albicans* (Nce103) and *Cryptococcus neoformans* (Can2) with inorganic anions such as halogenides, pseudohalogenides, bicarbonate, carbonate, nitrate, nitrite, hydrogen sulfide, bisulfite, perchlorate, sulfate were investigated. The two enzymes showed appreciable CO_2 hydrase activity (k_{cat} in the range of (3.9–8.0) × 10^5 s^{-1}, and k_{cat}/K_m in the range of (4.3–9.7) × 10^7 M^{-1} s^{-1}). Can2 was weakly inhibited by cyanide and sulfamic acid (K_Is of 8.22–13.56 mM), while all other anions displayed more potent inhibition. Nce103 was strongly inhibited by cyanide and carbonate (K_Is of 10–11 μM), and weakly inhibited by sulfate, phenylboronic, and phenyl arsonic acid (K_Is of 14.15–30.85 mM). These data demonstrate that pathogenic, fungal β-CAs may be targets for the development of antifungals that have a novel mechanism of action.

Carbonic anhydrases (CAs, EC 4.2.1.1) are ubiquitous Zn(II)-, Cd(II)- or Fe(II)-containing metalloenzymes present in prokaryotes and eukaryotes, being encoded by at least five distinct, evolutionarily unrelated gene families: the α-CAs (in prokaryotes from the *Bacteria* domain, algae, cytoplasm of green plants and vertebrates—with 15 isozymes presently known in humans), the β-CAs (predominantly in *Bacteria*, *Fungi*, algae and chloroplasts of both mono- and dicotyledons), the γ-CAs (mainly in *Archaea* and some *Bacteria*), the δ-CAs (found so far only in marine diatoms) and the recently described ζ-CAs, which have a cadmium metal centre (present again in marine diatoms), respectively.[1–9] The β-class enzymes predominate in most bacterial and fungal species, for which the complete genome has been sequenced, but they are poorly investigated.[1,2,8,9] These enzymes catalyse the reversible hydration of carbon dioxide to bicarbonate and protons by means of a metal-hydroxide mechanism, but at least the α-CAs possess other catalytic activities (esterase, phosphatase, cyanate/cyanamide hydrase, etc.).[3]

CAs play an important role in many physiological processes of eukaryotes including respiration, CO_2 transport, electrolyte secretion and photosynthesis among others.[1–3] Although ubiquitous in organisms from the *Eukarya* domain (mainly vertebrates) in which they have been thoroughly investigated, these enzymes have received scant attention in prokaryotes from the *Bacteria* and *Archaea* domains as well as microscopic eukaryotes, such as pathogenic fungi.[1,2,8,9] Recently, we and others characterized two β-CAs (Nce103 and Can2) from the fungal pathogens *Candida albicans* and *Cryptococcus neoformans*.[8–10] These enzymes were denominated Nce103 in *C. albicans* and Can2 in *C. neoformans*.[8–10] CO_2 sensing plays an important role in fungal pathogenesis. In fact physiological concentrations of CO_2/HCO_3^- induce prominent virulence attributes in *C. albicans* (filamentation) or *C. neoformans* (capsule biosynthesis) through direct activation of the fungal adenylyl cyclise.[8–10] CO_2/HCO_3^- equilibration by fungal β-CAs equally plays a critical part in fungal CO_2 sensing and pathogenesis. For example, Nce103 is essential for pathogenesis of *C. albicans* in niches where the available CO_2 is limited (e.g., the skin), or essential for the growth of *C. neoformans* in its natural environment.[8–10] Thus the link between cAMP signalling and CO_2/HCO_3^- sensing is conserved in fungi and revealed CO_2 sensing to be an important mediator of

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fungal metabolism and pathogenesis. No inhibitors of these new β-CAs have been described up to now, but it has been hypothesized,[1,8–10] that novel therapeutic agents could target this unexplored pathway at several levels, in order to control fungal infections as a different class of antifungals. Indeed, these pathogens cause potentially life-threatening disease, and drug resistance to the currently used antifungals is increasing worldwide.[11,12]

Similarly to all metalloenzymes, CAs are inhibited by inorganic, metal-complexing anions (acting as non-competitive inhibitors with CO_2 as substrate)[1,13,14] but they also possess a class of specific inhibitors, the sulfonamides and their bioisosteres (sulfamates, sulfamides, etc.), which coordinate to the metal ion within the enzyme cavity as anionic species.[1,3,6,7,13–15] Sulfonamides and sulfamates are clinically used inhibitors of the mammalian α-CAs, mainly as diuretics, antiglaucoma, anticonvulsant, antiobesity or antitumor drugs/diagnostic tools.[1,3] However, very few anion/sulfonamide inhibition studies are available for non-α-CAs. The archaeal β-CA from *Methanobacterium thermoautotrophicum* (Cab) and the prototypical γ-CA from *Methanosarcina thermophila* (Cam) were investigated by our and Ferry's groups for their interaction with both inorganic anion and sulfonamide inhibitors,[14] whereas Nishimori et al.[15] reported a detailed inhibition study of the β-CA from the pathogenic bacterium *Helicobacter pylori* with sulfonamides and sulfamates. Here, we present the first detailed inhibition study of the fungal β-CAs from the pathogenic species *C. neoformans* (Can2) and *C. albicans* (Nce103) with a series of simple, mostly inorganic anions. This study is useful both for understanding the catalytic/inhibition mechanism of these poorly investigated β-CAs and for detecting potential zinc-binding groups (ZBGs) for the design of potent inhibitors targeting these enzymes, which might have pharmacologic applications.

Although Can2 and Nce103 have been cloned, purified and characterized earlier,[8,9] their kinetic parameters for the catalyzed physiological reaction, that is, CO_2 hydration to bicarbonate and a proton, are not available in the literature.[16,17] Therefore, we performed a kinetic investigation of purified Can2 and Nce103, comparing their kinetic parameters (k_{cat} and k_{cat}/K_m) with those of thoroughly investigated CAs, such as the cytosolic, ubiquitous human isozymes hCA I and II (α-class CAs) as well as Cab, the best characterized β-CA from the archaeon *M. thermoautotrophicum*,[18,19] (Table 1). Data of Table 1 show that similarly to other CAs belonging to the α- or β-class, Can2 and Nce103 possess appreciable CO_2 hydrase activity, with a k_{cat} in the range of $(3.9–8.0) \times 10^5$ s^{-1}, and k_{cat}/K_m in the range of $(4.3–9.7) \times 10^7$ M^{-1} s^{-1}. Data of Table 1 also show that these enzymes (except Cab)[14] are inhibited appreciably by the clinically used sulfonamide acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide), with inhibition constants in the range of 10.5–250 nM. Thus, our data prove the following.

(i) β-CAs from the fungal pathogens Can2 and Nce103 are effective catalysts for the interconversion between carbon dioxide and bicarbonate. Indeed, these enzymes show a moderate–high catalytic activity for the physiological reaction catalyzed by CAs. Can2, the less active enzyme of the two β-CAs investigated here, possesses a k_{cat}/K_m value in the same range as the ubiquitous, house-keeping human isoform hCA I[1] (Can2 has 86% of the catalytic activity of hCA I, considering the turnover number, k_{cat}/K_m). However, the fungal enzyme is almost two times (1.95-fold) faster than the human one, as observed by comparing their k_{cat} values. Nce103, on the other hand, displays 64.66% of the catalytic activity of hCA II for the CO_2 hydration reaction, with CA II being one of the best catalysts known in nature[1] (again considering the k_{cat}/K_m values). Nce103 is considerably more effective as a catalyst for the physiological reaction as compared to Cab (53.88 times), Can2 (2.25 times) or hCA I (1.94 times). Taken together this is an excellent example of convergent evolution of highly efficient catalytic activity (for CO_2 hydration) in genetically unrelated enzymes. It should be also mentioned that unlike mammalian α-CAs (including hCA I and II),[3d] the investigated β-CAs Can2 and Nce103 do not possess esterase activity with 4-nitrophenyl acetate as substrate (data not shown).

(ii) Similarly to α- or β-class CAs investigated earlier,[14,15] Can2 and Nce103 are susceptible to inhibition by the clinically used sulfonamide inhibitor acetazolamide, with inhibition constants in the range of 10.5–132 nM. The human α-CAs hCA I and II, are inhibited with K_I values in the range of 12–250 nM, very similar to those of Can2 and Nce103. Cab on the other hand was poorly inhibited by this sulfonamide (K_I of 12.1 μM),[14] being, however, susceptible to inhibition by other types of sulfonamides (e.g., ethoxzolamide), as previously shown.[14]

Table 2 shows the Can2 and Nce103 inhibition data with anionic, physiological species (such as chloride, bicarbonate and sulfate) as well as other non-physiologic anions. Here we also include inhibition data for hCA I and II as well as Cab (reported earlier).[13,14] This helps to compare the newly generated data with those of the better investigated CAs belonging to the α- and β-CA families. The following should be noted regarding the fungal β-CA inhibition data of Table 2:

(i) Can 2 was not inhibited by perchlorate, similarly to all other α- and β-CAs investigated up to now, and it was weakly inhibited by cyanide and sulfamic acid, which showed inhibition constants of 8.22–13.56 mM. The weak inhibitory activity of cyanide against Can2 is quite surprising considering the fact that this metal-complexing anion is a submicromolar inhibitor of hCA I, a micromolar inhibitor of Nce 103 (see later in the text), and also appreciably inhibits hCA II (Table 2). Thus from this point of view Can2 is similar to Cab, which is also weakly inhibited by this anion (K_I of 27.8 mM). The remaining investigated anions had a very compact behaviour of modest inhibitors, with inhibition constants in the range of 0.60–1.11 mM. The best Can2 anion inhibitor was hydrogen sulfide, and the worst one iodide, in this subgroup of discussed anions. Since the X-ray crystal structure of these fungal enzymes is not known it is currently difficult to fully interpret these results.

Table 1
Kinetic parameters for the CO_2 hydration reaction catalysed by the human cytosolic isozymes hCA I and II (α-class CAs) at 20 °C and pH 7.5 in 10 mM HEPES buffer and 20 mM Na_2SO_4, and the β-CAs Cab (from *M. thermoautotrophicum*) and Can2 and Nce103 (from *C. neoformans* and *C. albicans*, respectively), measured at 20 °C, pH 8.3 in 20 mM Tris buffer and 20 mM $NaClO_4$[17]

Isozyme	Activity level	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	K_I (acetazolamide) (nM)
hCA I[a]	Moderate	2.0×10^5	5.0×10^7	250
hCA II[a]	Very high	1.4×10^6	1.5×10^8	12
Cab[a]	Low	3.1×10^4	1.8×10^6	12,100
Can2[b]	Moderate	3.9×10^5	4.3×10^7	10.5
Nce103 [b]	High	8.0×10^5	9.7×10^7	132

Inhibition data with the clinically used sulfonamide acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide) are also provided.
[a] Data from Ref. 1.
[b] This work.

Table 2
Inhibition constants of anionic inhibitors against isozymes hCA I, and II (α-CA class), and β-isozymes Cab (from the archaeon *Methanobacterium thermoautotrophicum*) as well as Can2 (from *Cryptococcus neoformans*) and Nce103 (from *Candida albicans*), for the CO_2 hydration reaction, at 20 °C[17]

Inhibitor	K_I (mM)[a]				
	hCA I[c]	hCA II[c]	Cab[c]	Can2[d]	Nce103[d]
F^-	>300	>300	>1000	0.86	0.69
Cl^-	6	200	152	0.92	0.85
Br^-	4	63	42.1	1.00	0.94
I^-	0.3	26	13.2	1.11	1.40
CNO^-	0.0007	0.03	11.2	1.01	1.18
SCN^-	0.2	1.60	0.52	0.94	0.65
CN^-	0.0005	0.02	27.8	13.56	0.011
N_3^-	0.0012	1.51	55.7	0.73	0.52
HCO_3^-	12	85	44.9	0.75	0.62
CO_3^-	15	73	9.6	0.60	0.010
NO_3^-	7	35	7.8	0.92	0.69
NO_2^-	8.4	63	44.8	0.96	0.53
HS^-	0.0006	0.04	0.70	0.60	0.37
HSO_3^-	18	89	45.1	0.71	0.54
SO_4^-	63	>200	>200	0.86	14.15
ClO_4^-	>200	>200	>200	>200	>200
$H_2NSO_2NH_2$	0.31	1.13	103	0.99	0.30
H_2NSO_3H[b]	0.021	0.39	44.0	8.22	0.70
Ph-$B(OH)_2$	58.6	23.1	0.20	0.81	30.85
Ph-AsO_3H_2[b]	31.7	49.2	0.33	0.87	30.84

[a] Errors were in the range of 3–5% of the reported values, from three different assays.
[b] As sodium salt.
[c] From Ref. 14.
[d] This work.

However, it can be stated that Can2 is much better inhibited by most of these anions as compared to Cab, an archaeal β-CA for which the X-ray crystal structure has been reported by Ferry's group.[18] Most of these anions inhibit Cab in the range of 7.8–103 mM, except for hydrogen sulfide, thiocyanate as well as phenyl boronic/phenyl arsonic acids which were the only submillimolar Cab inhibitors (Table 2). On the other hand, Can2 was generally less susceptible to inhibition with anions as compared to the other investigated fungal enzyme, Nce103 (see discussion later). However, as shown in Table 1, Can2 has a 12.5 times higher affinity for the sulfonamide inhibitor acetazolamide compared to Nce103 which was moderately inhibited by this clinically used compound. Notably, the inhibition profile of Can2 is also very different from those of the α-class enzymes hCA I and II (Table 2). This is predictable considering the different metal coordination spheres in these enzymes as well as the fact that the overall charge can be considered as +2 for the α-CAs (three neutral His residues as metal(II) ion ligands). By contrast, this charge is formally 0 for the open active site β-CAs, with the two cysteinate metal ion ligands 'neutralizing' the +2 charge of the metal ion. These different distributions of the charge at the bottom of the enzyme active site cavity in the α- and β-CAs is clearly reflected in their quite distinct inhibition profiles with small anions as those investigated here, but obviously this is just one of the factors controlling inhibitory activity. The various hydrophobic contacts that these inhibitors, possessing various ionic radii, with amino acid residues within the enzyme active site can be evidenced only by resolving high-resolution X-ray crystal structures of such enzyme–anion adducts, which for the moment are not available.[20]

(ii) When compared to Can2, Nce103 shows a distinct inhibition profile. Thus, except for perchlorate, which was not an inhibitor in concentrations up to 200 mM, the other weak Nce103 inhibitors were sulfate, and the phenyl boronic/phenyl arsonic acids (K_Is in the range of 14.15–30.85 mM). Again most of the remaining investigated anions showed rather similar inhibition constants, in the range of 0.30–1.40 mM, except for two anions which behave as quite potent inhibitors: cyanide and carbonate (K_Is of 10–11 μM). Several comments must be made regarding these very interesting data. Thus, there are many anions, such as cyanide, carbonate, sulfate, sulfamic acid (sulfamate), as well as the neutral inhibitor phenyl boronic acid or the anionic one phenyl arsonate, which show very different inhibition profiles for Can2 and Nce103. For example, sulfate is 16.8 times better inhibitor of Can2 than Nce103, whereas the isoelectronic and isostructural sulfamate is 11.7 times better at inhibiting Nce103 than Can2. These data provide strong hints for the design of more potent inhibitors targeting these enzymes, with organic sulfonates as leads for obtaining Can2 inhibitors, whereas organic sulfamates as leads for strong Nce103 inhibitors. Work is in progress in our laboratories to detect such inhibitors.

The affinity of the two enzymes for cyanide is also very different and difficult to explain with the data available at this time. It may be observed that some β-CAs such as Cab and Can2 show a weak inhibition with this complexing, metal–poison anion, whereas Nce103 or the α-CAs hCA I and II are much better inhibited by it. Again an X-ray structure of these adducts would help us understand this very different behaviour. However, maybe the most interesting data regard the carbonate inhibition of these fungal enzymes. Indeed, this anion is a 60 times more potent Nce103 than Can2 inhibitor (Table 2). Since carbonate is in equilibrium with one of the enzyme substrate, i.e., bicarbonate (a much weaker inhibitor of both Can2 and Nce103), our data raise several important questions. Is this potent carbonate inhibition of Nce103 an evolutionary adaptation which silences this enzyme once the pH, at which *C. albicans* grows, reaches above 7.5? How do these processes affect the growth and pathogenicity of this ascomycete? And why is this effect not observed for the orthologue of Nce103 in the basidiomycete *C. neoformans*, Can2, which does not show this susceptibility to inhibition by carbonate?

In order to try to rationalize the kinetic and inhibition data reported here, an alignment of the amino acid sequences of Cab, Nce103 and Can2 is shown in Figure 1.[21] We chose this archaeal β-CA for comparison since it is one of the best characterized β-class enzymes both from the point of view of the catalytic and inhibition mechanism,[14,18,19] is the smallest such enzyme known up to now, and its high-resolution X-ray crystal structure is reported.[18] Data from Figure 1 show that the putative zinc ligands of the fungal β-CAs Can2 and Nce103 are conserved, corresponding to residues Cys37, His92 and Cys94 in the Cab sequence. A second pair of conserved amino acid residues in all sequenced β-CAs, known to date, is constituted by the dyad Asp39 - Arg41. These amino acids are close[18] to the zinc-bound water molecule, which is the fourth zinc ligand in this type of open active site β-CAs, making a network of hydrogen bonds with it, which probably assists its deprotonation and formation of the nucleophilic, zinc hydroxide species of the enzyme. Indeed, in β-CAs, unlike the α-class enzymes, as mentioned above, the formal zinc charge is zero, and as a consequence the activation of the zinc-coordinated water molecule needs the assistance of additional amino acids. The pair Asp39-Arg41 probably has this function, as it is conserved in all β-CAs. As a consequence, the catalytic water molecule is activated both by the metal ion (as in metalloproteases[22] and α-CAs[1,23]), but also by an aspartic acid residue, as in aspartic proteases.[24] This particular mechanism makes the β-CAs very different as compared to all other known enzyme classes involved in hydrolytic or hydration processes.

In conclusion, we investigated the catalytic activity and inhibition of the β-CAs from the pathogenic fungi *C. albicans* (Nce103) and *C. neoformans* (Can2) with simple inorganic anions such as halogenides, pseudohalogenides, bicarbonate, carbonate, nitrate, nitrite, hydrogen sulfide, bisulfite, perchlorate, sulfate and some of its isosteric species. The two enzymes showed appreciable CO_2 hydrase activity, with a k_{cat} in the range of $(3.9–8.0) \times 10^5\ s^{-1}$,

```
CAB             ------------------------------------------------------------
NCE103          MGRENILKYQLEHDHESDLVTEKDQSLLLDNNNNLNGMNNTIKTHPVRVSSGNHNNFPFT 60
CAN2            ------------------------------------MPFHAEPLKPSDEIDMDLGHS 21


CAB             -----------MRFVSMIIKDILRENQDFRFRDLSDLKHSPKLCIITCMDSRLIDLLERA 49
NCE103          LSSEST--LQDFLNNNKFFVDSIKHNHGNQIFDLNGQGQSPHTLWIGCSDSRAG---DQC 115
CAN2            VAAQKFKEIREVLEGNRYWARKVTS-EEPEFMAEQVKGQAPNFLWIGCADSRVP--EVTI 78
                           .   .      :   .  .:   .   ::*:   * Z ***

CAB             LGIGRGDAKVIKNAGNIVDDGVIRS---AAVAIYALGVNEIIIVGHTDCG--MARLDEDL 104
NCE103          LATLPGEIFVHRNIANIVNANDISSQGVIQFAIDVLKVKKIIVCGHTDCGGIWASLSKKK 175
CAN2            MARKPGDVFVQRNVANQFKPEDDSSQALLNYAIMNVGVTHVMVVGHTGCGGCIAAFDQPL 138
                :.   *:  * :* .* ..     *      **  : *.:::: *Z*.Z*   * :.:

CAB             IVSRMRELGVEEEVIENFS-IDVLNPVGDE---------EENVIEGVKRLKSSP-----L 149
NCE103          IGGVLDLWLNPVRHIRAAN-LKLLEEYNQDPKLKAKKLAELNVISSVTALKRHPSASVAL 234
CAN2            PTEENPGGTPLVRYLEPIIRLKHSLPEGSDVN----DLIKENVKMAVKNVVNSPTIQGAW 194
                            . :.    :.     ..:         : **  .*. :   *

CAB             IPES------IGVHGLIIDINTGRLKPLYLDED-------------------- 176
NCE103          KKNE------IEVWGMLYDVATGYLSQVEIPQDEFEDLFHVHDEHDEEEYNPH 281
CAN2            EQARKGEFREVFVHGWLYDLSTGNIVDLNVTQGPHP---FVDDRVPRA----- 239
                          : * * : *: ** :   : : :.
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Figure 1. Alignment of Cab, Nce103 and Can2 amino acid sequences. The three zinc ligands (Cys37, His92 and Cys94, Cab numbering)[19] are evidenced in blue (and the "Z" sign) whereas the other conserved amino acid residues between the three β-CAs are evidenced by an asterisk. The two conserved residues Asp39, Arg41, thought to be involved in the β-CA catalytic cycle[18] are shown in red. Amino acids where conserved substitutions were identified are represented with the ":" sign, while semi-conserved substitutions are represented by the "." symbol.[21]

and k_{cat}/K_m in the range of $(4.3–9.7) \times 10^7\ M^{-1}\ s^{-1}$. Can2 was weakly inhibited by cyanide and sulfamic acid (K_Is of 8.22–13.56 mM) whereas all other anions showed a compact behaviour of stronger inhibition (K_Is of 0.60–1.11 mM). Notably, Nce103 was strongly inhibited by cyanide and carbonate (K_Is of 10–11 μM), weakly inhibited by sulfate, phenylboronic and phenyl arsonic acid (K_Is of 14.15–30.85 mM), whereas other anions where medium potency inhibitors (K_Is of 0.30–1.40 mM). Perchlorate was not an inhibitor of these enzymes. Can2 and Nce103 show distinct inhibition profiles compared to the β-CA from the archaeon *M. thermoautotrophicum* (Cab) or the cytosolic, human α-CA isozymes I and II. These data demonstrate that β-CAs from fungal pathogens of humans may function as attractive drug targets for developing a class of therapeutic agents with a novel mechanism of action.

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16. Buffers and metal salts (sodium or potassium fluoride, chloride, bromide, iodide, cyanate, thiocyanate, cyanide, azide, bicarbonate, carbonate, nitrate, nitrite, hydrogen sulfide, hydrogen sulfite, sulfate and perchlorate) were of highest purity available, and were used without further purification. Sulfamide, sulfamic acid, phenylboronic acid and phenylarsonic acid were from Sigma–Aldrich. Can2 and Nce103 were recombinant, purified enzymes obtained as described by Mühlschlegel's group.[8,9]
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catalysed CO_2 hydration reaction for a period of 10–100 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (100 mM) were prepared in distilled–deionized water and dilutions up to 0.01 μM were done thereafter with distilled–deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E–I complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3, whereas the kinetic parameters for the uninhibited enzymes from Lineweaver–Burk plots, as reported earlier, Refs. 14, 15 and represent the mean from at least three different determinations..

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