Activation of $\alpha$, $\beta$, $\gamma$, $\delta$, $\zeta$, and $\eta$-class of carbonic anhydrases with amines and amino acids: a review

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**ABSTRACT**

Eight genetically distinct carbonic anhydrase (EC 4.2.1.1) enzyme families ($\alpha$, $\beta$, $\gamma$, $\delta$, $\zeta$, $\eta$, $\Theta$- and $\iota$-CAs) were described to date. On the other hand, 16 mammalian $\alpha$-CA isoforms are known to be involved in many diseases such as glaucoma, edema, epilepsy, obesity, hypoxic tumors, neuropathic pain, arthritis, neurodegeneration, etc. Although CA inhibitors were investigated for the management of a variety of such disorders, the activators just started to be investigated in detail for their in vivo effects. This review summarizes the activation profiles of $\alpha$, $\beta$, $\gamma$, $\delta$, $\zeta$, and $\eta$-CAs from various organisms (animals, fungi, protozoan, bacteria and archaea) with the most investigated classes of activators, the amines and the amino acids.

**1. Introduction**

Carbonic anhydrases (CAs; EC 4.2.1.1) are metalloproteins present virtually in all living organisms. CA enzymatic activity was first observed in the early 1930s, when experiments performed with hemolyzed blood samples have demonstrated that the rate of carbon dioxide release from the hemolyzed blood was higher than expected, indicating that blood could contain a catalyst for the dehydration of bicarbonate, which allows the formation of CO$_2$ [1]. This catalyst, named carbonic anhydrase, was thereafter extracted from erythrocytes in 1933 by Meldrum and Roughton [2]. Upon the discovery in 1940 that zinc ions are an intrinsic cofactor of the protein, CA became the first recognized metalloenzyme. This enzyme efficiently catalyzes the reversible hydration of carbon dioxide (CO$_2$) to yield bicarbonate (HCO$_3^-$) and protons (H$^+$) [2,3].

$$\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{HCO}_3^- + \text{H}^+$$

It has been known since the 1940s that CA is ubiquitous in plants [4], where it performs an essential role in CO$_2$ fixation [5]. CAs, under the form of many enzyme families and isoforms, are virtually found in all living organisms, from the unicellular ones to higher vertebrates including humans. Their structure is encoded virtually in all living organisms, from the unicellular ones to CAs, under the form of many enzyme families and isoforms, are virtually found in all living organisms, from the unicellular ones to higher vertebrates including humans. Their structure is encoded virtually in all living organisms, from the unicellular ones to

- $\alpha$-CAs are Zn$^{2+}$ metalloproteins expressed in animals, vertebrates, prokaryotes, fungi, algae, protozoa and plants [9].
- $\beta$-CAs are Zn$^{2+}$ metalloproteins present in bacteria, plants, fungi, chloroplasts of mono-/dicotyledons [6].
- $\gamma$-CAs are Zn$^{2+}$ or Fe, Co metalloproteins present in some plants, fungi, bacteria and archaea [6].
- $\delta$-CAs are Co metalloproteins present in marine diatoms [7,10].
- $\zeta$-CAs are Cd or Zn metalloproteins identified only in some marine diatoms [11].
- $\eta$-CA are Zn metalloproteins identified in Plasmodium spp. [12].
- $\Theta$-CA are Zn metalloproteins identified in Marine diatoms [11].
- $\iota$-CAs were only recently reported to be present in diatoms and bacteria and seem to be Mn(II) proteins [13].

CA inhibitors (CAIs) targeting mammalian CAs, are in clinical use as diuretics, antiglaucoma, antiepileptic or antiobesity agents for decades [3,6,14–18]. These diverse applications are due to the fact that at least 15 different $\alpha$-CA isoforms are present in humans, being involved in critical physiological and pathological processes [14–18].

In the current review, we focused our attention on recent activation studies on $\alpha$, $\beta$, $\gamma$, $\delta$, $\zeta$, and $\eta$-CA classes which were explored with at least two classes of modulators of activity, amines and amino acids. The catalytic mechanism of these enzymes is in fact well understood [3]. A metal hydroxide species present in the active site of these enzymes as the fourth ligand (Figure 1(A,B)) acts as a strong nucleophile (at physiologic pH) converting the CO$_2$ to bicarbonate, bis-coordinated to Zn(II), in a trigonal bipyramidal geometry (Figure 1(C)). This adduct is not very stable and reaction with a water molecule leads to liberation of bicarbonate in solution and generation of an acidic form of the enzyme incorporating a M$^{2+}$(OH)$_2$ species at the metal center, which is catalytically ineffective for the hydration of CO$_2$ (Figure 1(D)). In order to generate the nucleophile, M$^{2+}$(OH$_2^-$) species, a proton transfer reaction occurs, which is rate determining for the catalytic cycle in many of these quite rapid enzymes. CA enzymes typically use a metal ion (Zn$^{2+}$ in $\alpha$, $\beta$- and $\gamma$-CAs, Fe$^{2+}$/Co$^{2+}$/Zn$^{2+}$) which favors in the reduction pK$_a$ of H$_2$O from...
Human CAs use a Zn$^{2+}$ ion to decrease the pKa of H$_2$O bound with Zn$^{2+}$ ion which also binds to histidine residues (His94, His96 and His119). For many α-CAs this step is assisted by a proton shuttle residue, which is His64 in most mammalian isoforms. Possessing a flexible orientation, inwards (the in conformation) or outwards (the out conformation) the zinc ion center, the imidazole moiety of this histidine, with a pKa of 6.0–7.5 is an appropriate proton shuttling residue and crucially important for the entire catalytic cycle. The process can be also assisted by endogenous molecules, which bind within the enzyme active site (as proven by X-ray crystallography and other techniques) which have been termed CA activators (CAAs) [19]. They facilitate the proton transfer reactions between the metal ion center and the external medium. It was understood that CA activators act by speeding up the deprotonation of zinc-bound water (the rate-determining step, Equation (2) in the catalytic mechanism) [19–21], with the generation of the active form of the enzyme [22]

\[
\begin{align*}
\text{E} + \text{Zn}^{2+} + \text{OH}^- + \text{CO}_2 &\rightarrow \text{EZn}^{2+} - \text{H}_2\text{O} + \text{HCO}_3^- \quad (1) \\
\text{E} + \text{Zn}^{2+} + \text{OH}^- + \text{H}^+ &\rightarrow \text{EZn}^{2+} + \text{OH}^- + \text{H}^+ \quad \text{- rate determining step} \quad (2)
\end{align*}
\]

In the presence of an activator ‘A’, Equation (2) becomes (3):

\[
\begin{align*}
\text{E} + \text{Zn}^{2+} + \text{OH}^- + \text{A} &\rightarrow [\text{EZn}^{2+} - \text{OH}^- - \text{A}] \\
[\text{EZn}^{2+} - \text{OH}^- - \text{A}] &\rightarrow \text{EZn}^{2+} - \text{OH}^- + \text{A}^+ \quad \text{enzyme - activator complexes} \quad (3)
\end{align*}
\]

CAAs may have pharmacologic applications, activation of the mammalian enzymes was shown to enhance cognition and memory in experimental animals [23], likewise its inhibition has an opposite effect [24].

In order to better understand the catalytic mechanism of CAs belonging to the β-, δ-, γ-, ζ-, η-CAs and Θ-CAs classes, it is of crucial importance to see if these enzymes act, similar to the α-CAs, which can be activated by compounds that shuttle protons between the active site and the environment. The activation of CAs from pathogenic bacteria may be relevant for understanding the factors governing virulence and colonization of the host, because pH in the tissues surrounding the pathogens likely plays a key role in such processes and many compounds that are CAAs (biogenic amines and amino acid derivatives) are abundant in such tissues. In this review, we have carefully analyzed the activation potential of different natural, non natural, aromatic/heterocyclic amino acids and amines (compounds 1–19) across 6 different families of CAs that were investigated based on the existing literature (Chart 1) [19–24]. These compounds have functional groups similar to their endogenous proton shuttlers, and can participate in proton transfer processes during the catalytic cycle. This study is relevant as no X-ray crystal structures of enzyme activator complexes have been reported so far for β-, γ-, δ-, ζ-, η-CAs and Θ-CAs.

2. Activation of α-CAs with amino acids and amines

Activation of the twelve catalytically active human (h) or murine (m) CA isoforms, hCA I, hCA II, hCA III, hCA IV, hCA VA, hCA VB, hCA VII, hCA IX, hCA XII, mCA XIII, hCA XIV and mCA XV with amino acids and amines (1–19) has been investigated by stopped
This bioassay is in excellent agreement with results from native mass spectrometry [30]. The following structure-activity relationship (SAR) can be summarized from data presented in Table 1 based on the activation profile of these derivatives.

Compounds 1–19 generally activated, these CA isoforms in a very different manner based on their structures. Nanomolar potencies were observed for several isozymes. For example, hCA I was activated by compounds 1 (L-His), 2 (D-His), 3 (L-Phe), 4 (D-Phe), 9 (L-Tyr), 10 (D-Tyr), and 19 (L-adrenaline) with K_{A} ranging from 20 to 90 nM. The best activation profile was observed against one of the most abundant cytosolic isofrom hCA II with K_{A} ranging from 125 μM to 11 nM. Specifically, compounds 3 (L-His), 9 (L-Tyr), and 10 (D-Tyr) showed good activation potency with K_{A} of 13–11 nM, respectively. Other cytosolic isoforms hCA III and hCA VII were weakly activated, in general, by these series of amines and amino acids 1–19. The remaining cytosolic isoform mCA XIII was moderately activated by most of the compounds with K_{A} ranging from 0.24 to 48.3 μM. Among the mitochondrial isoforms hCA VB was slightly better activated than hCA VA by these amines and amino acids. Interestingly, compound 5 (L-DOPA) showed nanomolar potency against both isozymes, hCA VA and VB, with K_{A} of

### Table 1. In vitro hCA I [25], hCA II [25], hCA III [26], hCA IV [26], hCA VA [27], hCA VB [27], hCA VII [28], hCA IX [29], hCA XII [29], mCA XIII [25], hCA XIV [28] and mCA XV [30] activation data with amines and amino acids (1–19) by a stopped-flow CO₂ hydrase assay.

| No | Compound       | hCA I (μM) | hCA II (μM) | hCA III (μM) | hCA IV (μM) | hCA VA (μM) | hCA VB (μM) | hCA VII (μM) | hCA IX (μM) | hCA XII (μM) | mCA XIII (μM) | hCA XIV (μM) | mCA XV (μM) |
|----|----------------|------------|-------------|--------------|-------------|-------------|-------------|--------------|-------------|--------------|---------------|--------------|-------------|
| 1  | L-His          | 0.03       | 10.9        | 35.9         | 7.30        | 1.34        | 0.97        | 0.92         | 9.71        | 37.5         | 0.13          | 0.90         | 32.1        |
| 2  | D-His          | 0.09       | 43.7        | 1.13         | 12.3        | 0.12        | 4.38        | 0.71         | 12.5        | 24.7         | 0.090         | 2.37         | 14.1        |
| 3  | L-Phe          | 0.07       | 0.013       | 34.7         | 36.3        | 9.81        | 10.45       | 10.93        | 16.3        | 1.38         | 1.02          | 0.24         | 33.4        |
| 4  | D-Phe          | 86         | 0.035       | 15.4         | 49.3        | 4.63        | 0.072       | 9.74         | 9.30        | 0.37         | 0.051         | 7.21         | 9.5         |
| 5  | L-DOPA         | 3.1        | 11.4        | 13.5         | 15.3        | 0.036       | 0.063       | 58.3         | 51.3        | 1.67         | 1.67          | 12.1         | 6.5         |
| 6  | D-DOPA         | 4.9        | 7.8         | 28.7         | 34.7        | 4.59        | 3.71        | 34.7         | 54.7        | 0.89         | 0.73          | 36.8         | 4.0         |
| 7  | L-Trp          | 44         | 27          | 20.5         | 37.1        | 1.13        | 0.89        | 57.5         | 37.5        | 26.0         | 16            | 16.5         | 13.5        |
| 8  | D-Trp          | 41         | 12          | 19.0         | 39.6        | 1.24        | 1.35        | 39.6         | 43.6        | 28.1         | 0.81          | 18.0         | 8.7         |
| 9  | L-Tyr          | 0.02       | 0.011       | 34.1         | 25.1        | 2.45        | 0.044       | 20.3         | 25.3        | 25.8         | --            | 21.8         | 8.9         |
| 10 | D-Tyr          | 0.04       | 0.013       | --           | --          | --          | --          | --           | --          | --           | --            | --           | --         |
| 11 | 4-H₂N-L-Phe    | 0.24       | 0.15        | 43.2         | 0.079       | 2.76        | 2.17        | 18.7         | 48.7        | 1.09         | --            | 2.90         | 16.3        |
| 12 | Histamine      | 2.1        | 125         | 36.9         | 25.3        | 0.010       | 3.52        | 37.5         | 35.1        | 27.9         | 4.6           | 0.010        | 18.5        |
| 13 | Dopamine       | 13.5       | 9.2         | 33.2         | 30.9        | 0.13        | 7.85        | 0.89         | 0.92        | 0.67         | 27            | 14.6         | 7.1         |
| 14 | Serotonin      | 45         | 50          | 0.78         | 3.14        | 6.33        | 0.11        | 0.93         | 33.1        | 0.30         | 0.51          | 6.5          | 7.5         |
| 15 | 2-Pyridyl-methylamine | 26 | 34 | 1.03 | 5.19 | 23.56 | 0.24 | 43.7 | 1.07 | 41.5 | 3.8 | 21.7 | 11.6 |
| 16 | 1–(2–Aminoethyl)pyridine | 13 | 15 | 1.10 | 7.13 | 7.62 | 0.094 | 27.8 | 0.013 | 69.6 | 46 | 6.9 | 11.9 |
| 17 | 1–(2–Aminoethyl)piperazine | 7.4 | 2.3 | 0.32 | 24.9 | 6.04 | 0.91 | 32.5 | 0.009 | 48.3 | 54 | 18.3 | 10.4 |
| 18 | 4–(2–Aminoethyl)morpholine | 0.14 | 0.19 | 0.091 | 1.30 | 0.089 | 1.15 | 64.3 | 0.43 | 0.24 | 0.013 | 5.4 | 9.3 |
| 19 | L-Adrenaline   | 0.09       | 96          | 36.4         | 45.0        | --          | --          | --           | 60          | 0.87         | --            | 36.1         | 6.9         |

*aMean from 3 different determinations (errors in the range of 5–10% of the reported values.

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flow CO₂ hydrase assay method and are shown in Table 1 [25–29]. This bioassay is in excellent agreement with results from native mass spectrometry [30]. The following structure-activity relationship (SAR) can be summarized from data presented in Table 1 based on the activation profile of these derivatives.
36 and 63 nM, respectively. Only one compound 11 (4-H$_2$N-L-Phe) had nanomolar activity against membrane-bound isofrom hCA IV with a $K_A$ of 79 nM. On the other hand, another transmembrane-bound tumor overexpressed isofrom hCA IX was moderately activated by most of the tested compounds, except the compound 16 and 17 which showed one of the best activation profile from the Table 1 with $K_A$s of 13 and 9 nM, respectively. The CA activating effects of amines and amino acids 1–19 on the remaining membrane-bound isofroms hCA XII, hCA XIV and mCA XV were moderate to weak and most of the results were very close to the others (Table 1).

3. Activation of β-CAs with amino acids and amines

In literature, there are many β-CAs which were investigated in details, among which Cab (from Methanobacterium thermoautotrophicum), scCA (from Saccharomyces cerevisiae), CgCA (from Candida glabrata), MgCA (from Malassezia globosa), VchCAβ (from Vibrio cholerae, mtCA 3 (from Mycobacterium tuberculosis), BsuCA1 (from Brucella suis), FtuCA (from Francisella tularensis), LdcCA (from Leishmania donovani chagasi), and EhiCA (from Entameba histolytica). Furthermore, VchCAβ and BsuCA1 was also activated efficiently, with $K_A$s of 0.18–20.3 and 0.70–43.1 µM, by amino acids and amines, respectively. Specifically, VchCAβ was activated slightly more effectively by amines ($K_A$s: 0.18–12.8 µM) than by amino acid derivatives ($K_A$s: 0.94–20.3 µM). For BsuCA1 activities of most compounds are close to each other, except the compounds 2, 8, and 17 with $K_A$s of 12.3, 13.7 and 43.1 µM, respectively, which are the least effective CAAs. In the case of FtuCA, most of the amines and amino acid derivatives (compounds 5, 9–14, 16, 18 and 19) investigated so far showed weak activation effects, with activation constants $>$100 µM. The remaining activators were also moderately active against FtuCA, with $K_A$s ranging between 30.5 to 78.3 µM. Other β-CAs (Cab, CgCA, MgCA, mtCA 3 and EhiCA) were activated in a different manner, as seen from Table 2, with most of the activation constants in a limited range of values.

4. Activation of γ-, δ-, ζ-, and η-CAs with amino acids and amines

Activation studies were also performed recently against γ-CAs, such as Zn-Cam and Co-Cam (from the Archaeon Methanosarcina thermophila), Bps;CA (from the pathogenic bacterium Burkholderia pseudomallei), PhaCA (from the cyanobacterium Pseudoalteromonas haloplanktis), and CpsCA (from another cyanobacterium, Colwellia psychrerythraea), as well as δ-CAs, such as TweCA$\delta$ (from the diatom Thalassiosira weissflogii), ζ-CAs, such as ZnTweCA$\zeta$ (from the same diatom, Thalassiosira weissflogii), and η-CAs, such as PfaCA (from Plasmodium falciparum) [31–44]. Among them, an interesting activation profile was observed for some of the γ- class CAs, such as Bps;CA. Most of the tested compounds showed nanomolar potency against this enzyme. Specifically, Bps;CA was efficiently activated by compounds 2, 5, 8, 11, 13, and 16–19 with activation constants ranging between 9 to 86 nM. Interestingly, the ζ- class CA, ZnTweCA$\zeta$ was activated slightly more efficiently by amines ($K_A$s of 92 nM to 10.1 µM) than by amino acids ($K_A$s of 0.62 to 15.4 µM), which is just the opposite in the case of the η- class CA PfaCA, for which $K_A$s ranging from 0.12 to 8.55 µM were obtained for amino acid derivatives and between 0.71 and 9.97 µM for amines (Table 3). A wide range of activities of the various activators for the remaining CAs was observed, such as for γ- class CAs, Co-Cam and PhaCA, which were moderately activated by amino acid derivatives and amines with $K_A$s of 0.72–135 µM (Table 3). Other γ-CAs, such as Zn-Cam and CpsCA were less prone to be activated, as compared to other γ-CAs investigated so far, with activation constants ranging between 4.79 to $>$100 µM. The unique δ- class CA investigated in details at this moment, TweCA$\delta$, was efficiently activated by most

| No | Compound | Cab | scCA | CgCA | MgCA | VchCAβ | mtCA 3 | BsuCA1 | FtuCA | LdcCA | EhiCA |
|----|----------|-----|------|------|------|--------|--------|--------|-------|-------|-------|
| 1  | L-His    | 69  | 82   | 37   | 29.3 | 20.3   | 18.2   | 1.76   | 40.7  | 8.21  | 78.7  |
| 2  | D-His    | 57  | 85   | 21.2 | 18.1 | 18.0   | 32.5   | 12.3   | 78.3  | 4.13  | 9.83  |
| 3  | L-Phe    | 70  | 86   | 24.1 | 34.1 | 15.4   | 30.6   | 1.16   | 69.1  | 9.16  | 16.5  |
| 4  | D-Phe    | 10.3| 86   | 15.7 | 10.7 | 5.12   | 441    | 1.21   | 75.0  | 9.97  | 10.1  |
| 5  | L-DOPA   | 11.4| 90   | 23.3 | 8.31 | 8.26   | 30.0   | 2.07   | $>$100| 1.64  | 16.6  |
| 6  | D-DOPA   | 15.6| 89   | 15.1 | 13.7 | 6.27   | 9.74   | 2.34   | 44.8  | 5.47  | 4.05  |
| 7  | L-Trp    | 16.9| 91   | 22.8 | 10.1 | 4.18   | 8.98   | 1.25   | 34.1  | 4.02  | 5.24  |
| 8  | D-Trp    | 41  | 90   | 12.1 | 12.5 | 5.89   | 43.7   | 13.7   | 30.5  | 6.18  | 4.95  |
| 9  | L-Tyr    | 10.5| 85   | 9.5  | 15.7 | 6.15   | 89.8   | 1.38   | $>$100| 8.05  | 4.52  |
| 10 | D-Tyr    | 19.2| 84   | 7.1  | 25.1 | 0.94   | 17.6   | 0.95   | $>$100| 1.27  | 1.07  |
| 11 | 4-H$_2$N-L-Phe | 89  | 21.3 | 31.6 | 13.4 | 7.21   | 40.5   | 1.18   | $>$100| 15.9  | 8.12  |
| 12 | Histamine| 76  | 20.4 | 27.4 | 10.9 | 9.50   | 34.2   | 3.71   | $>$100| 0.74  | 7.38  |
| 13 | Dopamine | 51  | 13.1 | 27.6 | 9.43 | 1.24   | 12.1   | 1.54   | $>$100| 0.81  | 30.8  |
| 14 | Serotonin| 62  | 15.0 | 16.7 | 14.2 | 1.37   | 10.3   | 4.26   | $>$100| 0.62  | 4.94  |
| 15 | 2-Pyridyl-methylamine | 18.7| 16.2| 15.0 | 6.12 | 0.18   | 43.3   | 1.62   | 46.3  | 0.23  | $>$100|
| 16 | 2-[(2-Aminoethyl)pyridine | 40  | 11.2| 16.3 | 7.30 | 1.00   | 45.9   | 5.20   | $>$100| 0.012 | $>$100|
| 17 | 1-[(2-Aminoethyl)piperazine | 13.8| 9.3  | 14.9 | 0.81 | 0.24   | 50.3   | 43.1   | 51.8  | 0.009 | 43.8  |
| 18 | 4-[(2-Aminoethyl)morpholine | 18.5| 10.2| 10.1 | 5.82 | 12.8   | 52.0   | 9.56   | $>$100| 0.94  | $>$100|
| 19 | L-Adrenaline | 11.5| 0.95| 10.8 | 0.72 | 8.73   | 52.2   | 0.70   | 4.89  | 25.6  |       |

Table 2. In vitro β-CA (Cab [31], scCA [31–34], CgCA [34], MgCA [32], VchCAβ [35], mtCA 3 [36], BsuCA1 [37], FtuCA [37], LdcCA [38, 39], and EhiCA [38]) activation data with amines and amino acids (1–19).

$K_A$ (µM)$^a$

$^a$Mean from 3 different determinations (errors in the range of 5–10% of the reported values, data not shown).
of the amino acid derivatives and amines 1–19, with $K_A$s ranging between 51 nM and 18.9 μM.

5. Conclusions and future perspective

To our knowledge, this is the first article that summarizes the activation profile of all classes of CAs (the $\alpha$, $\beta$, $\gamma$, $\delta$, $\zeta$, and $\eta$-CA) with a small library of amines and amino acid derivatives. This panel of investigated amino acids and amines showed considerable activating properties, with a well-defined structure–activity relationship, but without net differences between the various CA families. Even if the available activators are not isoform-selective (for the many $\alpha$-CAs of human or other origins), as already mentioned above, in the last period, their possible use as pharmacological agents for memory therapy or for artificial tissue engineering started to be explored [23,24], with very promising results being obtained. There is however a stringent need for having more effective, isoform-selective and possibly non-autacoid or amino acid derived compounds, which may possess a rather complicated polypharmacology [3]. Furthermore, the investigations of the activating effects of non-human CAs are still in their infancy, with very few in vitro studies being available on the non-$\alpha$-CA activators. Indeed, only in the few several years the first activation studies of $\beta_1$, $\gamma_1$, $\delta_1$, $\zeta_1$, and $\eta_1$-CAs from various organisms have been reported, which allowed the identification of compounds active in the nanomolar to micromolar range. However, no drug design studies of CAs targeting these enzymes were performed so far, which is one of the future objectives of research in this area. In addition, almost nothing is known regarding the in vivo effects of CAs in organisms other than the vertebrates (human and rodents). As briefly mentioned, many pathogenic bacteria, fungi or protozoans live in various niches which are potentially rich in endogenous activators of the amine and amino acid type. A deep understanding of the role that these modulators of activity may play in the interaction between the host and the pathogen, may lead to relevant biomedical discoveries, but this is an entire new field to be explored in the future.

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