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Superoxide Dismutase Mimetics

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SUMMARY: In this review we describe the potential role(s) of superoxide in inflammatory disorders.

KEY WORDS: superoxide, superoxide dismutase, inflammation.

INTRODUCTION

Under normal circumstances, the levels of superoxide anion (O_2^-*, produced by the one electron reduction of molecular oxygen) are kept under tight control by endogenous superoxide dismutase (SOD) enzymes, the enzymatic activity of which was discovered in 1969 by McCord and Fridovich.[1] There are two forms of SOD: the Mn enzyme in mitochondria (SOD2) and Cu/Zn enzyme present in the cytosol (SOD1) or extracellular surfaces (SOD3). The importance of SOD2 is highlighted by the findings that in contrast to SOD1[2] and SOD3,[3] the SOD2 knockout is lethal to mice.[4–6] Superoxide anion is formed via a large number of pathways, including normal cellular respiration, inflammatory cells, endothelial cells and in the metabolism of arachidonic acid. In acute and chronic inflammation, the production of superoxide anion is increased at a rate that overwhelms the capacity of the endogenous SOD enzyme defence system to remove it. The consequence of this imbalance results in superoxide anion mediated damage (Fig. 1). Some important pro-inflammatory roles for superoxide anion include: endothelial cell damage and increased microvascular permeability,[7–9] formation of chemotactic factors such as leukotriene B_4,[10] recruitment of neutrophils at sites of inflammation,[11,12,13] auto-catalytic destruction of neurotransmitters and hormones such as norepinephrine and epinephrine respectively,[14] lipid peroxidation and oxidation, DNA single-strand damage[15] and activation of poly-ADP-ribose polymerase, formation of peroxynitrite, a potent cytotoxic and proinflammatory molecule[13,16–19] that also nitrates and deactivates superoxide dismutase[20,21] and causes the inactivation of nitric oxide.[22]

The list of patho-physiological conditions associated with the production of superoxide anion expands everyday. The most exciting realization is that there appears to be a commonality to the tissue injury observed in various disease states; namely, superoxide anion, produces tissue injury (and associated inflammation) in all tissues in similar ways. Tissue injury and inflammation form the basis of many disease pathologies: ischemia and reperfusion injuries, radiation injury, hyperoxic lung damage, atherosclerosis and so forth. This provides a unique opportunity to manipulate numerous disease states with an agent that selectively removes superoxide anion.

Most of the knowledge obtained about the roles of superoxide anion in disease has been gathered using the native superoxide dismutase enzyme[23–26] and, more recently, by data generated in transgenic animals that overexpress the human enzyme (VIDE INFRA). Protective and beneficial roles of superoxide dismutase have been demonstrated in a broad range of diseases, both preclinically and clinically.[27–29] For example, preclinical studies have revealed that superoxide dismutase enzymes have a protective effect in animal models of ischemia-reperfusion injury (including heart, liver, kidneys, brain),[30–35] transplant-induced reperfusion injury,[36] inflammation,[37–38] Parkinson's disease[39,40], cancer,[41–43] AIDS[44–46] and pulmonary

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disorders including asthma, chronic obstructive pulmonary diseases[47,48] and Respiratory Syncytial Virus (RSV) infections.[49] In some situations such as stroke or Parkinson's, the native enzymes do not show efficacy since they do not penetrate (because of their large size, MW ~30 KD) the blood brain barrier (Fig. 2). Under these circumstances use of transgenic animals that overexpress the superoxide dismutase enzyme has led to some important observations. For instance overexpression of the superoxide dismutase enzyme in rats is protective in animal models of stroke or Parkinson's.[50]

Most importantly, human clinical results with, Orgotein® (bovine CuZnSOD) showed promising results as a human therapy in acute and chronic conditions associated with inflammation, including rheumatoid arthritis and osteoarthritis as well as side effects (acute and chronic) associated with chemotherapy and radiation therapy.[24,51,52] Thus, in clinical trials, the use of the native enzyme supported the concept that removal of superoxide anion had a beneficial outcome. Although, the native enzyme has shown excellent anti-inflammatory properties in both preclinical and clinical studies, in a variety of diseases, there were major drawbacks associated with its use. The main problem was the non-human origin of the enzyme: bovine. This inevitably gave rise to a variety of immunological problems, which eventually led to its removal from the market, except in Spain where it is still clinically used to prevent radiation-induced side effects.

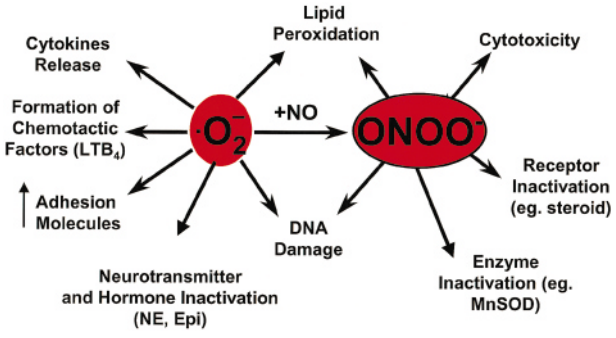

Fig. 1 Superoxide anion in inflammation.

Based on the concept that removal of superoxide anion modulates the course of inflammation, we have pursued the concept of designing synthetic, low molecular weight mimetics of the superoxide dismutase enzymes which could overcome some of the limitations associated with Orgotein (Fig. 3). This could allow the synthetic superoxide dismutase mimetics to serve as pharmaceutical candidates in a variety of diseases in which the native SOD enzyme was found to be effective.[35–38,40,44,48,53–57] This concept has proven to be one in which a number of researchers and companies have been pursuing in recent years.[58] A review of the patent literature in this arena was in fact recently published.[59]

In this short review article we will discuss findings obtained with *selective* superoxide dismutase mimetics that led to the proposal that superoxide anion is a crucial mediator of inflammation, thus, the potential use of SOD mimetics (SODm) as therapeutic agents in diseases of various aetiologies, including bronchopulmonary disorders.

DESIGN OF SODm

We have focused on the design and synthesis of Mn(II) and Fe(III) complexes, which possess high inherent chemical and thermodynamic stability, and

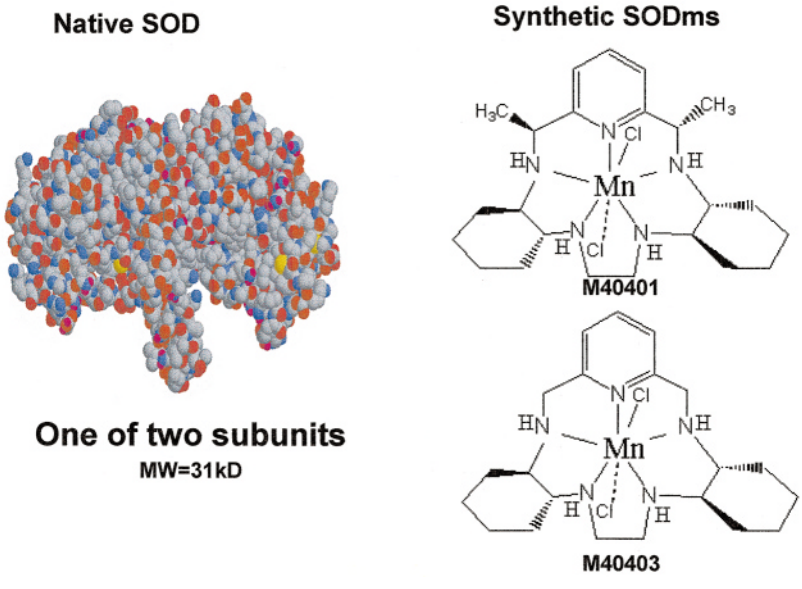

Fig. 2 Synzymes of superoxide dismutase.

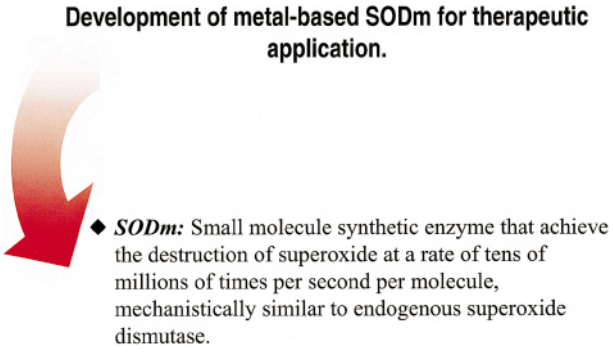

Fig. 3 Goals: superoxide dismutase mimics (SODm).

Table 1 Properties of superoxide dismutase mimics, e.g. M40403.

Manganese containing biscyclohexylpyridine
Catalytic activity equivalent if not superior to that of the native enzyme
Non-peptide small molecule: non-immunogenic
Penetrates cells
Selective for superoxide (no interaction with biologically important molecules)
Stable in vivo: no Mn dissociation
Not deactivated by peroxynitrite
Protective in various models of acute and chronic inflammation, reperfusion injury and shock

at the same time are highly effective catalysts for the dismutation of superoxide anion. This dual design goal of *high stability* and *high SOD activity* was achieved utilizing a combination of computer-aided modelling studies and synthesis activities and has led to the development of a novel class of highly active superoxide dismutase catalysts which are also very stable complexes.[60–62] These synthetic superoxide dismutase mimetics are exemplified by the prototypical complexes, M40403 and M40401 (Fig. 2), derived from the 15-membered macrocyclic ligand, 1,4,7,10,13-pentaazacyclopentadecane, containing the added bis(cyclohexylpyridine) functionalities.[63]

The framework of these two ligands coordinated to Mn(II) affords a very kinetically stable (to dissociation) and oxidatively stable Mn(II) complexes.[61–63] M40403 (see Fig. 2 and Table 1) is a stable, low molecular weight, manganese-containing, non-peptidic molecule possessing the function, and catalytic rate of $2 \times 10^{+7}$ M^{-1} s^{-1} at pH = 7.4, but unlike the native superoxide dismutase enzymes, it possesses a pH dependence in its catalytic rate so that at lower pH (e.g., pH = 6) the rate constant is in excess of $1 \times 10^{+8}$ M^{-1} s^{-1}. M40401 on the other hand was a product of our computer-aided design studies and possesses a catalytic rate constant in excess of $2 \times 10^{+9}$ M^{-1} s^{-1} at pH = 7.4 and is hence as active as the native enzymes on a molar basis and even more active at lower pH.[61] Thus, these new mimetics possess not only the catalytic activity of the native enzymes on a molar basis, but also possess the added advantage of being a much smaller molecule (MW 483 (or M40401: MW = 501) vs. MW 30 000 for the mimetic and native enzyme, respectively).[61,62] An important property of these superoxide dismutase mimetics is that they catalytically remove superoxide anion at a high rate selectively without interacting with other reactive species including nitric oxide, peroxynitrite, hydrogen peroxide, hypochlorite or oxygen[64,65] (Table 1).

What is responsible for such *selectivity* and why is this important? The unique *selectivity* of mimetics such as M40403 resides in the nature of the manganese(II) center in the complex. The resting oxidation state of the complex is the reduced Mn(II); as a consequence, the complex has no reactivity with reducing agents until it is oxidized to Mn(III) by protonated superoxide, whereupon, the complex is rapidly reduced back to the Mn(II) state by the superoxide anion at diffusion-controlled rates. Since the complex is so difficult to oxidize (+0.78 vs. (SHE)) many one-electron oxidants cannot oxidize this and its related complexes (including nitric oxide and oxygen). Further, since the superoxide dismutase mimetics operate via a facile one-electron oxidation pathway, other two-electron non-radical, but nevertheless, potent oxidants are not kinetically competent to oxidize the Mn(II) complex; e.g., peroxynitrite, hydrogen peroxide or hypochlorite. Thus, M40403 and other complexes of this class of superoxide dismutase mimetics can serve as selective probes for deciphering the role of superoxide anion in biological systems where other such relevant biological oxidants may be present.[64]

This property is not shared by other classes of 'so called and claimed classes of SOD mimetics' including several metalloporphyrins such as tetrakis-(N-ethyl-2-pyridyl) porphyrin (MnTE-2-PyP) and tetrakis-(benzoic acid)porphyrin (MnTBAP), that interact with other reactive species such as nitric oxide (NO) and $ONOO^-$ which clearly play important roles in inflammation.[66] In addition, in a rat model of lung pleurisy, the intraperitoneal treatment with Mn(III)-tetrakis (4-benzoic acid) porphyrin (MnTBAP) prior to carrageenan administration was found to suppress inflammatory responses in a dose-dependent manner.[67] The most profound effects of MnTBAP were on depressing neutrophil influx and in reducing nitrotyrosine formation, a marker of peroxynitrite formation in inflammation. Others mimetic compounds (mixed SOD and catalytic action) such as EUK 8, EUK 134, Tempol and Nitroxide SOD mimics have shown some therapeutic benefits in inflammation.[68–71]

The selectivity exhibited by M40403 and other complexes of this class of stable SOD mimetic allows us to decipher superoxide's unique role in disease. But the issue of what is the fate of the superoxide is

important to consider. Each mole of superoxide that is dismuted produces $\frac{1}{2}$ mole of oxygen and $\frac{1}{2}$ mole of hydrogen peroxide. It is important to note that superoxide is a very good reducing agent in its anionic state, but when protonated becomes a very good oxidant. Thus, hydrogen peroxide which is produced when $HO_2^{\bullet}$ oxidizes a biological target, is itself not a radical, but actually is quite an inert oxidant whose cellular toxicity is probably in the 100 μM to mM range. Hydrogen peroxide's toxicity is likely due to the generation of reduced iron (Fe(III) is the oxidation state of iron in iron storage sites) which as Fe(II) reacts with hydrogen peroxide (Fenton Rxn.) undergoing homolytic cleavage to generate Fe(III)(OH) and hydroxyl radical. Iron(III) must first be reduced to 'free' soluble Fe(II) for this reaction to occur, and one of the best reductants available in inflammatory, or reperfusion disease states is superoxide and it has been shown to be an excellent kinetically competent reductant of Fe(III) in iron stage sites liberating Fe(II) and generating oxygen.[72] Thus, superoxide, when involved as a biological reductant, becomes oxygen (not hydrogen peroxide, and is the culprit leading to generation of conditions favourable for Fenton chemistry to be initiated).

The possibility that selective mimetics (such as those discussed here) would be creating a more toxic condition by generating more hydrogen peroxide is not correct when one inspects the stoichiometry of the reactions involved. Nearly all of the oxidizing reactions which superoxide enters into ($HO_2^{\cdot}$ driven) involve hydrogen atom abstraction from a biological target molecule such as a catecholamine[14], DNA[15]. These oxidation reactions are free radical chain reactions and produce at least one hydrogen peroxide per oxidation – in fact these are all free radical chain reactions that in the presence of oxygen will yield many molecules of hydrogen peroxide with one initiation from superoxide. When superoxide is dismuted the stoichiometry is such that two superoxides and two protons generate as the net reaction one oxygen molecule and one hydrogen peroxide; thus, in effect each mole of superoxide now leads to $\frac{1}{2}$ a mole of hydrogen peroxide. So in effect by dismuting superoxide one actually decreases the potential H_2O_2 burden – not increasing it. Finally, we have actually tested our family of molecules in vitro[73] in assays designed to test the protective effects of this family in neutrophil-mediated injury of human aortic endothelial cells. Not only do the compounds protect against the activated neutrophil-mediated killing of the human aortic cells, added catalase and glutathione peroxidase had no added benefit either in the presence of the mimetics or in their absence. Our data support that hydrogen peroxide toxicity is not an issue when efficient and selective superoxide dismutation is achieved in models of inflammation.

In light of the critical roles of superoxide anion in disease and cellular signalling, these new *selective*, potent and stable synthetic enzymes of superoxide dismutase, as represented by M40403, have broad potential as therapeutic agents in the treatment of numerous diseases ranging from acute and chronic inflammation to cardiovascular diseases and cancer. In fact, we have shown over the last several years that superoxide dismutase mimetics are anti-inflammatory, protective in models of septic shock and ischemia-reperfusion injury:[64,65,74,75] the findings from the studies are summarized and discussed below.

SUPEROXIDE ANIONS, SUPEROXIDE DISMUTASE MIMETICS AND INFLAMMATION

There is no doubt that reactive oxygen species including nitric oxide, superoxide anion and the product of their reaction, peroxynitrite, are involved in acute and chronic inflammation. The relative contribution of each of these species is becoming increasingly substantiated through the development of *selective* agents that either inhibit their formation or remove them. Pharmacological use of *selective* Mn(II) mimics of the biscyclohexylpyridine class, such as M40403, has provided invaluable information as to the potential role(s) of superoxide anions in inflammation. A summary of the key anti-inflammatory effects of superoxide dismutase mimetics are shown in Tables 2 and 3 and discussed below.

An important mechanism by which superoxide dismutase mimetics attenuate inflammation is by

Table 2 Summary of the anti-inflammatory properties of M40403.

Inhibition of the up-regulation of adhesion molecules (ICAM-1, P-selectin) and PMNs infiltration at the inflamed site
Attenuation of pro-inflammatory cytokines release (TNFα, IL-1β, IL-6)
No effect or increased production of anti-inflammatory cytokines (IL-10)
Protection of the inactivation of nitric oxide and preservation of its beneficial effects

Table 3 Summary of the anti-inflammatory properties of M40403 (2).

Inhibition of lipid peroxidation and cellular protection
Attenuation of DNA damage and subsequent activation of poly-ADP-ribose polymerase (PARP)
Attenuation of peroxynitrite formation and thus subsequent peroxynitrite mediated damage
Protects against superoxide anion-driven deactivation of catecholamines, important immunoregulators
Inhibition of the activation of transcription factors (NFκB)

reducing peroxynitrite formation by simply removing superoxide anion before it can react with nitric oxide. This is important since the pro-inflammatory and cytotoxic effects of peroxynitrite are numerous.[76,77] For instance, removal of peroxynitrite by agents such as FeTMPS, a porphyrin-containing molecule that increases the rate of isomerization of peroxynitrite to nitrate[78] is cytoprotective[79] and anti-inflammatory.[19,77] Peroxynitrite also nitrates tyrosine residues in proteins, and nitrotyrosine formation, as monitored by its immunofluorescence, has been used as a marker for the detection of the endogenous formation of peroxynitrite.[80] We have found that the SODm block nitrotyrosine staining in models of inflammation, suggesting that superoxide anion-driven peroxynitrite formation is in fact responsible for the formation of nitrotyrosine and that it's inhibition could account for the anti-inflammatory effects of superoxide dismutase mimetics. This in fact was the first evidence to show in vivo a superoxide-dependent nitration, since superoxide dismutase mimetics do not react with nitric oxide or peroxynitrite. A similar pattern of immunoreactivity for nitrotyrosine is observed in a lung model of ischemia and pleurisy.[65–81]

A substantial amount of data has been generated to support the concept that peroxynitrite generation plays an important part in the pro-inflammatory roles that have been ascribed to date to nitric oxide (see Ref. [77] for review). Based on the above, we propose that superoxide dismutase mimetics should be considered as a therapeutic means to attenuate nitric oxide-driven inflammatory responses. In addition, superoxide anion by interacting with nitric oxide destroys the biological activity of this mediator[22] attenuating important anti-inflammatory and tissue protective properties of nitric oxide namely: maintenance of blood vessel tone, platelet reactivity and cytoprotective effects in numerous organs (including heart intestine and kidney), and release of anti-inflammatory and cytoprotective prostacyclin (via activation of the constitutive cyclooxygenase enzyme).[82,83] Therefore, removal of superoxide *protects nitric oxide* and reduces the formation of the *cytotoxic peroxynitrite*.

Superoxide anion and peroxynitrite induce DNA single-strand damage that is the obligatory trigger for poly-ADP-ribose polymerase activation[84,85] resulting in the depletion of its substrate NAD^+ in vitro and a reduction in the rate of glycolysis. As NAD^+ functions as a cofactor in glycolysis and the tricarboxylic acid cycle, NAD^+ depletion leads to a rapid fall in intracellular ATP and, ultimately, cell injury.[86] Furthermore, substantial evidence exists to support the fact that poly-ADP-ribose polymerase activation is important in inflammation.[86] Poly-ADP-ribose polymerase inhibitors such as nicotinamide and 3-aminobenzamide attenuate both acute and chronic inflammatory processes.[87] We have also found that superoxide dismutase mimetics reduced poly-ADP-ribose polymerase immunofluorescence and attenuated the reduction of NAD^+ in models of acute and chronic inflammation.[88] In light of the role of poly-ADP-ribose polymerase in inflammation, it is possible that poly-ADP-ribose polymerase inhibition by superoxide dismutase mimetics accounts for their anti-inflammatory response.

Superoxide anions increase neutrophil adhesion and infiltration[13,64,65,89] and generate potent chemotactic mediators such as leukotriene B_4.[90] Removal of superoxide inhibits the infiltration of neutrophils at sites of inflammation as shown by the use of the native superoxide dismutase enzyme, experiments performed in transgenic mice that overexpress the human CuZnSOD enzyme,[34,35] and by use of superoxide dismutase mimetics such as SC-55858 and M40403.[13,64,65,91] This correlates well with an attenuation of lipid peroxidation and overall attenuation of acute and chronic inflammation.[92] A possible mechanism by which superoxide dismutase mimetics attenuates neutrophil infiltration is by down-regulating adhesion molecules such as ICAM-1 and P-selectin. Thus, inhibition of neutrophil infiltration at sites of inflammation and reperfusion injury correlated well with the inhibition of both ICAM-1 and P-selectin[73,93] supporting the involvement of superoxide in the regulation of adhesion molecules (through mechanisms yet to be defined). In addition to ICAM-1 and P-selectin, other adhesion molecules may be affected by superoxide. For instance, native superoxide dismutase enzyme attenuates monocyte infiltration in glomeruli post endotoxin, an effect associated with an attenuation of the expression of various adhesion molecules including glomerular ICAM-1 and VCAM-1 and leukocyte LFA-1 and VLA-4.[94]

The release of a variety of pro-inflammatory cytokines is also regulated by superoxide. Thus, superoxide dismutase mimetics inhibit a number of inflammatory cytokines including tumor necrosis factor α, interleukin-1β and interleukin-6 (TNF-α, IL-1β and IL-6, respectively) as shown in models of acute[65] and chronic inflammation[75] as well as reperfusion-injury.[74] At present the mechanism(s) through which superoxide regulate cytokines is not known, but this is the subject of intensive research. Recent data demonstrates that superoxide anions (generated from xanthine–xanthine oxidase) can directly release TNFα from macrophages.[95] Interestingly, the anti-inflammatory cytokine IL-10 is not affected.[65]

In summary, removal of superoxide anion impacts the inflammatory cascade through at least three major pathways:

- inhibition of peroxynitrite formation and sparing of nitric oxide,

- inhibition of neutrophils infiltration at the site of inflammation,
- inhibition of pro-inflammatory cytokine release.

To date, the relative contribution of each mechanism to the contribution of the above mentioned events is not clear. Numerous ideas can be developed and explored. For instance, is the ability of superoxide anion to evoke neutrophil infiltration the consequence of generating chemotactic factors such as leukotriene B_4 or is it the consequence of superoxide anion-driven upregulation of key adhesion molecules? And if the latter were to be the key driver, then what is the intracellular transduction mechanism through which superoxide anion upregulates adhesion molecules? One possibility would be the activation of transcription factors such as NFkB or AP-1, which in turn regulate a variety of genes that encode for pro-inflammatory cytokines, chemokines, inflammatory enzymes, adhesion molecules and receptors.[96,97]

Overall, these findings support the potential use of superoxide dismutase mimetics as therapeutic agents in diseases of various aetiologies, including bronchopulmonary disorders.[98] It was not our goal to review in any length the relationship that exists between increased superoxide generation, decreased superoxide dismutase activity and its consequences in these disorders. Nevertheless, ample evidence is available to support a role for superoxide in asthma (Refs. [98–99] for reviews), chronic obstructive pulmonary disorders (Refs. [48–100] for reviews), adult respiratory distress syndrome (Ref. [101] for review) and respiratory syncytial virus infection (RSV, Ref. [49]). Additional properties of superoxide anion that have not been discussed here but that are clearly pertinent to pathological events of various bronchopulmonary disorders include for instance: release of histamine from mast cells,[102,103] damage to epithelial cells[104,105] activation of latent metalloproteinases such as pro-collagenase and pro-elastase.[106,107] The challenge in the future will be to understand the signal transduction mechanisms used by superoxide anion so as to modify key components of the inflammatory response, as this will undoubtedly elucidate important molecular targets for future pharmacological intervention.

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