MINIREVIEW

From yeast killer toxins to antibiobodies and beyond

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Abstract

Antibiobodies are paradigmatic of yeast killer toxin (KT)-like antibodies (KAbs) mimicking the antimicrobial activity of KTs in the frame of the yeast killer phenomenon. Polyclonal, monoclonal and recombinant anti-idiotypic antibiobodies (anti-idiotypic KAbs), internal images of a wide-spectrum KT produced by the yeast *Pichia anomala* (*Pa*KT), have been produced by immunization with the idiotype of a *Pa*KT-neutralizing monoclonal antibody. Anti-idiotypic KAbs showed microbicidal activity against eukaryotic and prokaryotic pathogenic agents through the interaction with specific KT receptors (KTRs), putatively constituted by β-glucans. Natural KAbs have been found in animals and humans experimentally or naturally infected by KTR-bearing microorganisms. Recombinant KAb-derived synthetic killer peptides showed further antiviral and immunomodulatory activities. The perspectives of KAbs and killer peptides as potential sources of novel therapeutic agents, and of KTRs and idiotypes as vaccines against infectious diseases are discussed.

Introduction

In the environment, microorganisms must resort to different strategies to compete for limiting nutritional resources. Secretion of killer toxins (KTs) represents an efficient tool to eliminate competitors without direct cell-to-cell contact, a property widely distributed among yeast species/genera. KTs' genetic determinants, physical-chemical properties and mechanisms of recognizing and killing susceptible strains are heterogeneous. Killer yeasts secrete protein or glycoprotein KTs that generally kill susceptible cells in a two-step receptor-mediated manner. First, KTs bind to primary cell wall receptors (KTRs) and then translocate to the plasma membrane where they interact with secondary KTRs or enter susceptible cells, thus exerting their cytocidal effect (for reviews, see Magliani *et al.*, 1997a; Schmitt & Breinig, 2006).

Almost all the different fungal cell wall structural components (Lesage & Bussey, 2006; Ruiz-Herrera *et al.*, 2006) can act as primary KTRs. Double-stranded mycoviral RNA-encoded KTs in *Saccharomyces cerevisiae* bind to β-1,6-glucan (K1 and K2) or α-1,3-linked mannose residues in the mannoprotein layer (K28). Other KTs can interact with β-1,6-glucan, such as *Pichia membranifaciens* (PM)KT and FC-1 from *Pichia membranifaciens* (Santos *et al.*, 2007) and *Filobasidium capsuligenum* (Keszthelyi *et al.*, 2006), respectively. Zygocin from *Zygosaccharomyces bailii* (Weiler & Schmitt, 2003) binds to an α-1,3-mannoprotein, while chitin acts as a primary KTR of different heteromeric KTs from *Kluyveromyces lactis* (zymocin), *Pichia acaciae*, *Pichia inositovora* and *Wingea robertsiae*, among others (Jablonowski & Schaffrath, 2007). β-1,3-Glucan has emerged as the primary KTR/target of KTs produced by different *Pichia* and *Williopsis* species.

The second, energy-dependent step involves KT translocation to secondary membrane KTRs. Among them, few have been identified so far, such as Kre1p, a glycosyl phosphatidyl inositol (GPI)-anchored protein involved in β-1,6-glucan synthesis, for K1 (Breinig *et al.*, 2002); Cwp2p, a mannoprotein that plays a role in stabilizing the cell wall whose precursor is GPI-anchored to the plasma membrane, for PMKT (Santos *et al.*, 2007); and the four amino acid hepitope HDEL receptor Erd2p, which colocalizes to the cytoplasmic membrane, for K28 (Schmitt & Breinig, 2006).

Some KTs, such as K1, K2, zygocin and PMKT, rapidly kill susceptible cells by forming cation-selective ion channels, thus disrupting plasma membrane integrity and function. Some others interfere with essential proteins that are

involved in cell cycle control and/or chromosomal DNA synthesis. Among them, K28 is taken up by endocytosis, travels the secretion pathway in reverse to the endoplasmic reticulum and acts in the nucleus by blocking DNA synthesis and causing a cell cycle arrest at the G1/S boundary (Santos & Marquina, 2004; Schmitt & Breinig, 2006). Other KTs can arrest cell cycle. In plasmid-encoded heterotrimeric KTs, after binding to chitin, the hydrophobic β-subunit allows the subsequent import of the lethal γ-subunit into the susceptible cell. In zymocin and *P. inositovora* KT, this subunit has been shown to be an anticodon nuclease. It targets tRNAs, by causing their depletion, block in translation and G1 cell cycle arrest that prevents cell budding (Jablonowski & Schaffrath, 2007). *Pichia acaciae* and *W. robertsiae* KT γ-subunits lead to S-phase arrest and concomitant activation of the intra-S-phase DNA damage checkpoint (Klassen *et al.*, 2004).

Lethal necrotic effects are observed in susceptible cells in the presence of high concentrations of KTs. Under low-to-moderate doses, as in natural habitats, KTs can induce an apoptotic cell death, which is mediated by yeast caspase Yca1p and is characterized by production of reactive oxygen species, DNA fragmentation and phosphatidylserine flipping to the outer layer of the membrane. Thus, apoptosis could be a general KT-induced cell death mechanism under natural environmental conditions (Schmitt & Reiter, 2008).

Killer yeasts are immune to their own KTs, while remaining susceptible to others. Immunity usually appears to be closely associated with the KT-encoding genetic elements and its mechanism(s) remains one of the most intriguing aspects of the killer phenomenon. As killer yeasts bear cell wall KTRs and bind normal amounts of KT, membrane KTRs or other still unknown cellular components appear to be involved. In *S. cerevisiae* K1-producing strains, an interaction between Kre1p and the intracellular preprotoxin (pptox) has been suggested to lead to diversion of the complex from the secretory pathway to degradation in the vacuole. Because K1 binds to the plasma membrane, immunity apparently does not involve loss of Kre1p but affects a still unknown step downstream of K1 binding (Schmitt & Reiter, 2008). K28-producing strains take up their own secreted toxin and translocate it back to the cytosol, where it can form a complex with the intracellular preprotoxin molecules that have not yet been imported into the endoplasmic reticulum. The β-subunit in the complex is selectively polyubiquitinated and rapidly degraded by the proteasome, allowing KT-producing cells to inactivate the toxin before the α-subunit reaches its ultimate intracellular target. Thus, as also suggested in other killer systems such as *K. lactis* and *P. acaciae* (Paluszynski *et al.*, 2007), the most frequently applied strategy for achieving immunity appears to be disarming KTs at the intracellular stage.

Some KTs, such as K1 and K28, are characterized by a very narrow spectrum of activity limited to susceptible strains of the same species. Some others, such as chromosomally encoded KTs produced by killer strains belonging to *Pichia* and *Williopsis* species, have attracted increasing attention for their wide spectrum of activity, involving human, animal and plant pathogenic microorganisms. Most of them interfere with cell wall synthesis by recognizing β-glucans as a KTR/target. Inhibition of β-glucan synthase activity is the main effect of HM-1 and HYI, small KTs from *Williopsis saturnus* var. *mrakii* IFO 0895 and var. *saturnus* IFO 0117, respectively (Komiyama *et al.*, 1996, 1998). A strong β-1,3-glucanase activity is the primary killing mechanism of KTs such as panomycocin from *Pichia anomala* NCYC 434 (Izgü *et al.*, 2007), *Wm*KT from *W. saturnus* var. *mrakii* MUCL 41968 (Guyard *et al.*, 2002), KpKT from *Kluyveromyces phaffii* (Comitini *et al.*, 2004) and a recently described KT from a marine strain of *P. anomala* (Wang *et al.*, 2007).

A 105 kDa KT glycoprotein produced by *P. anomala* ATCC 96603 (*Pa*KT), in particular, has been shown to display peculiar properties. *Pa*KT is characterized by a wide spectrum of antimicrobial activity, inclusive of *Candida albicans*, *P. anomala*, *S. cerevisiae* and *Pneumocystis carinii* (Magliani *et al.*, 1997a). Even though its mechanism of action is still obscure, it appears to be mediated by direct interaction with specific cell wall KTRs, putatively represented by β-glucans (mainly β-1,3-glucan). Furthermore, *Pa*KT appears to be antigenically related to *Wm*KT (Guyard *et al.*, 2001). By damaging the integrity of the vital β-glucan scaffold, these KTs induce rapid cell death, by osmotic lysis, similar to that observed with echinocandins (Morrison, 2006).

Research on killer systems has allowed clarification of relevant aspects of eukaryotic cell biology, such as protein intracellular processing, maturation and secretion, virus–host cell interactions, fungal cell wall structure and synthesis. Killer yeasts and KTs have been exploited in the food and fermentation industries as biocontrol agents (Lowes *et al.*, 2000), in recombinant DNA technology (Eiden-Plach *et al.*, 2004), and in biotyping of medically and industrially important microorganisms (Buzzini *et al.*, 2007), as well as in the development of novel potential antimicrobial agents. From this perspective, a direct use of KTs as potential therapeutic agents has been discouraged by their characteristics. With few exceptions, most KTs are heat-labile, protease-susceptible macromolecules, which display their activity only within narrow pH and temperature ranges, usually below physiological limits. They are characterized by antigenicity and toxicity, as demonstrated for *Pa*KT (Pettoello-Mantovani *et al.*, 1995). To our knowledge, the demonstration that topical application of concentrated *Pa*KT was curative against cutaneous experimental infections by *Malassezia* spp. in different animal models is the

only reported evidence of the therapeutic effectiveness of a KT (Polonelli *et al.*, 1986).

The generation of immunological derivatives that mimic the antimicrobial activity of KTs, on the basis of the idiotypic network hypothesis, has been pursued. Antibiotic-like antibodies named 'antibiobodies' (Polonelli *et al.*, 1991) or killer antibodies (KAbs), i.e. antimicrobial antibodies that bear the internal image of the active site of a *Pa*KT, have been generated.

From killer toxins to killer antibodies

The anti-idiotypic innovative approach was based on the assumption that the interaction between a KT and specific antibodies able to neutralize its activity could mirror the interaction with its specific cell wall KTRs on susceptible microorganisms. In an analogous way, anti-idiotypic and anti-KTR KAbs could be functionally equivalent to the KT-active site. An anti-*Pa*KT monoclonal antibody (mAb KT4) has been produced by conventional fusion of splenocytes from mice primed with *Pa*KT, which proved to neutralize its killer activity against a reference strain of *C. albicans* (Polonelli & Morace, 1987). When mAb KT4 was used to immunize rabbits by idiotypic vaccination, anti-idiotypic KAbs were raised, which were able to compete with *Pa*KT for binding to mAb KT4 and, more importantly, to inhibit the growth of the susceptible reference strain, thereby mimicking the candidacidal activity of *Pa*KT (Polonelli & Morace, 1988). Parenteral or intravaginal idiotypic vaccination with mAb KT4 in mice or rats elicited serum or secretory anti-idiotypic KAbs, which were candidacidal *in vitro* and significantly protected animals against systemic or mucosal candidiasis (Polonelli *et al.*, 1993, 1994). The mucosal inoculation of *Pa*KT-susceptible *C. albicans* cells was able to boost secretory KAbs in animals immunized previously with mAb KT4 as well as to elicit the production of KAbs in naïve animals, thus demonstrating the functional equivalence between the idiotype of mAb KT4 and *Pa*KTR. Accordingly, natural anti-*Pa*KTR KAbs were also consistently found in the vaginal fluid of women affected by vaginal candidiasis, following natural exposition to the yeast KTRs (Polonelli *et al.*, 1996). Finally, these observations have been confirmed by the production of anti-idiotypic KAbs in the monoclonal (m-KAb) (mAb K10, IgM) and single-chain recombinant (r-KAb) (scFv H6) format, from splenocytes of rats or mice immunized with mAb KT4 (Magliani *et al.*, 1997b; Polonelli *et al.*, 1997).

Irrespective of their origin, all these KT-like antibodies display the same functional characteristics: they are able to compete with *Pa*KT for binding to mAb KT4 and *Pa*KTRs, exert a candidacidal activity *in vitro* on *Pa*KT-susceptible *C. albicans* cells and display a significant therapeutic activity in experimental animal models of systemic and mucosal candidiasis when passively transferred to normal and immunodeficient naïve animals. Interestingly, their candidacidal activity can be specifically neutralized by mAb KT4 and laminarin, a soluble β-1,3-glucan from *Laminaria digitata*, suggesting that β-glucans are involved, entirely or in part, in the structure of the *Pa*KTR. In addition, cell wall *Pa*KTRs in *C. albicans* can be easily visualized using KAbs in immunofluorescence studies; they appear to be preferentially expressed on growing cells and particularly on budding cells and germ tubes, where the cell wall is neo-synthesized and β-1,3-glucans are temporarily exposed at the cell surface, as shown in Fig. 1.

Even though the precise clinical relevance of these microbicidal antibodies in disease remains to be elucidated, their limitless availability and greater stability at different temperatures and pH, as compared with *Pa*KT, allowed their testing *in vitro* against many different microbial eukaryotic and prokaryotic pathogens and their use as therapeutic agents in experimental animal models of infection. Besides *C. albicans*, KAbs demonstrated a significant *in vitro* wide spectrum of microbicidal activity against other yeast and fungal species, such as *Candida* spp. (Manfredi *et al.*, 2005), *P. carinii* (Séguy *et al.*, 1997) and *Aspergillus fumigatus* (Cenci *et al.*, 2002) as well as pathogenic bacteria, such as multidrug-resistant *Mycobacterium tuberculosis* (Conti *et al.*, 1998), antibiotic-resistant Gram-positive cocci (Conti *et al.*, 2000), oral streptococci (Conti *et al.*, 2002) and protozoa, such as *Leishmania major*, *Leishmania infantum* (Savoia *et al.*, 2002), and *Acanthamoeba castellanii* (Fiori *et al.*, 2006). More importantly, KAbs exerted a significant therapeutic effect in experimental models of candidiasis, aspergillosis and pneumocystosis. This anti-idiotypic approach has been confirmed recently by further experimental evidence. Mice were immunized with a mAb neutralizing the candidacidal and β-1,3-glucan synthase inhibitory activities of HM-1. By recombinant DNA technology and

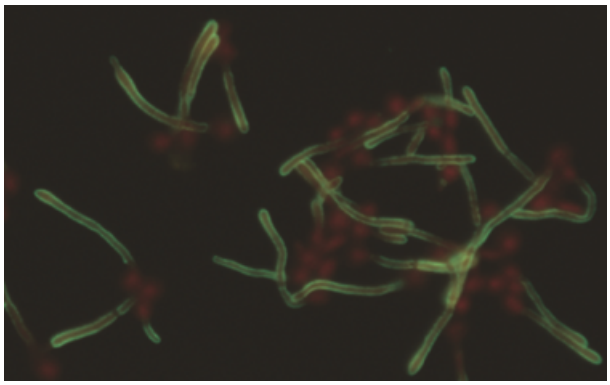

Fig. 1. Immunofluorescence visualization of *Pa*KTRs on *Candida albicans* germinating cells with affinity chromatography purified *Pa*KT-like biotinylated mAb K10 and fluorescent streptavidin (× 1000).

phage display, an anti-idiotypic r-KAb in the single-chain format has been produced that mimics HM-1 by displaying *in vitro* the same killing and inhibitory activities (Selvakumar *et al.*, 2006). The availability of genes encoding r-KAbs could allow their easy genetic manipulation, such as cloning and expression in human commensal bacteria for direct production and delivery of therapeutic anti-idiotypic r-KAbs at mucosal sites, as demonstrated in *Streptococcus gordonii* (Beninati *et al.*, 2000). Finally, sequencing of r-KAb genes could enable the synthesis and selection of potent antimicrobial antibody-derived peptides, opening further, unexpected biotechnological and therapeutic new perspectives.

From killer antibodies to killer peptides

More than 200 decapeptides that overlapped by two-residues and reproduced the entire variable regions of scFv H6 and the six peptides pertaining to its complementary determining regions (CDRs) have been synthesized and tested *in vitro* against the *C. albicans* reference strain. Among different candidacidal peptides, one decapeptide (P6) was selected for further studies because of its high activity. The final residues of its sequence (EKVTMTCSAS) were the first three of the V_L CDR1. P6 alanine scanning analysis allowed the generation of a killer decapeptide (KP, AKVTMTCSAS, MW 998.2), with A replacing E, which showed a significant increase in candidacidal activity (Polonelli *et al.*, 2003). The peptide activity, which appeared to be mediated by interaction with specific yeast cell wall β-glucan KTRs, was strongly and dose-dependently inhibited by laminarin. Moreover, the peptide completely inhibited the binding of KAbs to germinating *C. albicans* cells. Thus, KP retained the same activity of the r-KAb from which it was derived, demonstrating a rapid candidacidal effect in time-killing studies, in comparison with a scrambled control peptide named SP (MSTAVSKCAT), synthesized on the basis of the KP sequence, which showed no candidacidal activity (Magliani *et al.*, 2004a, b).

These characteristics and the easy production and testing of KP allowed the demonstration of its activity *in vitro* against all the microbial pathogens previously shown to be susceptible to KAbs, for example *C. albicans* (Magliani *et al.*, 2004b, 2007; Fiori *et al.*, 2006; Savoia *et al.*, 2006), but also against new ones such as *Cryptococcus neoformans* (Cenci *et al.*, 2004), *Paracoccidioides brasiliensis* (Travassos *et al.*, 2004) and phytopathogenic bacteria (*Pseudomonas syringae* and *Erwinia carotovora*) and fungi (*Botrytis cinerea* and *Fusarium oxysporum*) (Donini *et al.*, 2005). More importantly, KP proved to be therapeutic in normal and immunocompromised animals in experimental infections, such as vaginal and systemic candidiasis (Polonelli *et al.*, 2003), as well as disseminated cryptococcosis (Cenci *et al.*, 2004) and paracoccidioidomycosis (Travassos *et al.*, 2004). Thus, KP displayed a sufficient *in vivo* stability and its antimicrobial activity was observed irrespective of mechanisms of resistance to other conventional antimicrobial drugs and the host's response.

The antimicrobial mechanism(s) of action of KP is yet to be identified. The screening of KP activity against a comprehensive *S. cerevisiae* nonessential gene deletion collection (Euroscarf, Frankfurt, Germany) failed to detect any resistant mutant derivative. This finding might suggest that a KP-resistant phenotype is not compatible with viability (Conti *et al.*, 2008). As shown in Fig. 2, KP-treated *C. albicans* cells are characterized by the appearance of significant internal alterations, including cell wall swelling with a middle electron-dense region, plasma membrane collapse, condensation and fragmentation of nuclear material, similar to what is observed in cells treated with acetic acid, a classical apoptotic agent.

As a small molecule, KP can be easily manipulated and tested for new additional activities. KP was expressed in *Nicotiana benthamiana*, using a Potato virus X-derived vector, yielding chimeric virus particles displaying an active form of the peptide. KP-expressing plants showed enhanced resistance to bacterial experimental infections (Donini *et al.*, 2005). KP demonstrated a selective binding to murine

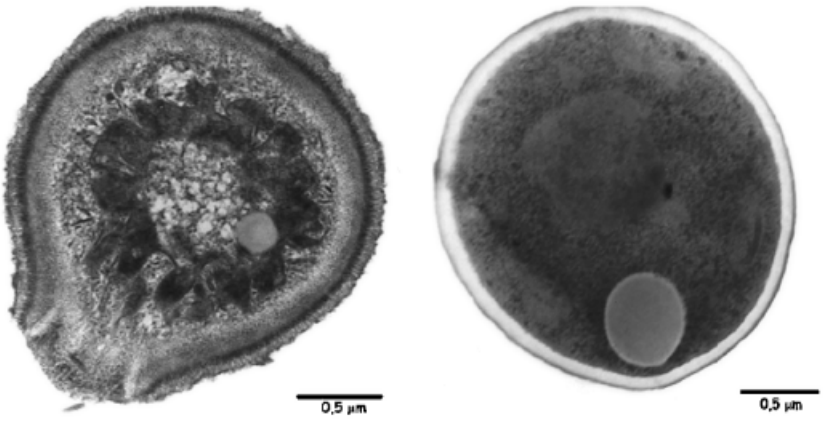

Fig. 2. TEM showing the cytotoxic effects of the KP on *Candida albicans*. Yeast cells were treated with KP (left) or with a SP (right). Major alterations can be seen as the swelling of the cell wall with an electron-dense middle layer, plasma membrane collapse, chromatin condensation and nuclear fragmentation. SP-treated cells were identical to control untreated cells (not shown).

	Potential	Limits
*Pa*KT *c.* 100 kDa	• Therapeutic by topical application	• Toxicity • High antigenicity • Lability in physiological conditions • Variableness in preparation • Difficulty of purification • Cost of production
m-KAb *c.* 900 kDa	• Therapeutic by topical application • Therapeutic by parenteral administration	• Antigenicity • Instability • Problems of purification • Safety in preparation • Cost of production
r-KAb *c.* 28 kDa	• Therapeutic by topical application • Therapeutic by parenteral administration • Therapeutic by recombinant bacteria at mucosal sites	• Low antigenicity • Instability • Scarce yield • Cost of production
KP *c.* 1000 Da	• Therapeutic by topical application • Therapeutic by parenteral administration • Expression in phytopathogen-resistant plants • Production *in planta* • Wider spectrum comprehensive of viruses • Immunomodulatory activity • Feasibility of molecular engineering	• Cost of production

Fig. 3. Potential and limits of *Pa*KT and its immunological derivatives in different formats.

dendritic cells (DCs) and to a lesser extent to macrophages, thus modulating the expression of costimulatory and MHC molecules and improving DC capacity to induce lymphocyte proliferation (Cenci *et al.*, 2006). KP was found to possess sequence homology with critical segments in gp160 V1/V2 and V3 loops of HIV-1 and was able to inhibit virus replication in peripheral blood mononuclear cells (PBMCs) either from patients in the acute phase of infection or exogenously infected with R5 or X4 strains. Downregulation of the CCR5 coreceptor and/or physical blockade of the gp120-receptor interaction have been suggested as possible mechanisms of KP activity. KP did not inhibit the *in vitro* replication of other RNA and DNA viruses (Casoli *et al.*, 2006). Thus, KP appears to be the first anti-idiotypic antibody-derived peptide displaying inhibitory activity and potential therapeutic effect against pathogenic microorganisms and viruses by different mechanisms of action.

Conclusions

Even though many questions concerning the molecular mechanisms of action and possible clinical applications of KAbs and KAb-derived peptides are still unanswered, this ongoing story of KTs and their immunological derivatives suggests some intriguing considerations and perspectives.

(1) While excluding a direct use of KTs as therapeutic agents, unravelling their mechanisms of action could result in the discovery of new potential targets for antimicrobial agents.

(2) Microbicidal antibodies can be elicited either during infections by KTR-bearing pathogenic microorganisms or following idiotypic vaccination with KT-neutralizing antibodies. Other wide-spectrum microbicidal antibodies directed to different cell targets have been described more recently, such as Mycograb, mAb C7 and anti-β-glucan antibodies, suggesting the existence of a family of microbicidal antibodies whose clinical relevance still needs to be determined (Magliani *et al.*, 2005).

(3) Immunological derivatives of KTs in different formats exhibit specific benefits and limitations compared with KTs, as described in Fig. 3. In particular, antibody-derived peptides, such as KP, can retain the microbicidal activity of the whole antibody and display antimicrobial, antiviral and immunomodulatory activities (Magliani *et al.*, 2007).

(4) β-Glucans have emerged as viability-critical microbial structures targeted by KAbs and KP, as well as by

antifungals. These carbohydrates are not biosynthesized by mammalian cells, but they are produced by a wide range of microbial species (Magliani *et al.*, 2008). Their relevance is also attested by the demonstration that a β-glucan (laminarin)-conjugate vaccine was protective against different fungal experimental infections, by eliciting fungicidal antibodies (Torosantucci *et al.*, 2005).

(5) Immunological derivatives of KTs, killer peptides in particular, lack cytotoxicity, as demonstrated for KP on PBMCs and *in vitro*-cultured cell lines (Magliani *et al.*, 2004a; Casoli *et al.*, 2006).

Overall, KTs and KT-like molecules can represent prototypal compounds for the development of new receptor-driven drugs for antimicrobial and antiviral therapy, even in combination with current anti-infective agents. On the other hand, KTRs and some idiotypes might represent candidate vaccines for immunoprevention of infections through an unconventional mechanism, such as the elicitation of antibodies with direct antimicrobial activity. Thus, new exciting perspectives could be opened in the field of human, animal and even plant infectious diseases, where antimicrobial and antiviral resistance is spreading among relevant pathogenic agents and new less susceptible/resistant species are emerging (Magliani *et al.*, 2002, 2003).

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