Polyglutamine diseases comprise a class of familial neurodegenerative disorders caused by expression of proteins containing expanded polyglutamine tracts. Great progress has been made in elucidating the molecular mechanisms contributing to polyglutamine pathology, and in identifying potential drug targets. Although much remains to be learned, these advances provide an opportunity for rational approaches to target-based drug discovery.

Therapeutic opportunities in polyglutamine disease

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Despite enormous progress in elucidating the molecular pathology of Huntington disease (HD), the prognosis for patients has improved little since the first description of this disease in 1872. Nor have effective treatments been developed for other diseases caused by expanded polyglutamine repeats. However, in the past decade, non-human primate, mouse, *Drosophila*, *Caenorhabditis elegans*, yeast and tissue culture models have provided insights into the pathogenic mechanisms of these diseases. The purpose of this review is to summarize these analyses of polyglutamine disease models—with particular attention paid to how they might guide the development of therapeutic strategies—and to discuss drug discovery efforts currently underway.

A variety of proteins contain uninterrupted tracts of glutamine residues, encoded by CAG triplet repeats, which are prone to instability and expansion. Polyglutamine tract expansion is non-pathogenic up to a threshold length, but larger expansions cause neurodegenerative diseases such as HD, spinocerebellar ataxias (SCA), spinobulbar muscular atrophy (Kennedy disease) and dentatorubropallidoluysian atrophy (DRPLA). Polyglutamine diseases are dominantly inherited, typically late-onset, fatal neurodegenerative disorders. Patterns of affected brain regions vary among the diseases, but common features include progressive neuronal cell loss and decline in motor and cognitive functions[1]. The normal functions of the proteins causing polyglutamine diseases are not known to be related, and sequence alignment of these proteins reveals that the only region of homology is the polyglutamine tract itself and, in some cases, an adjacent proline-rich region.

The observation that disease age-of-onset correlates with the length of polyglutamine expansion provided a strong indication that a novel toxic property of the mutant protein is the primary driver of pathology. A wealth of experimental evidence has demonstrated that this is indeed the case. The challenge now is to determine which cellular pathways are vulnerable to the toxic insults exerted by expanded polyglutamine, how these responses account for the clinical manifestation of disease and ultimately how this knowledge can facilitate the development of drugs.

The dominant pattern of inheritance in polyglutamine diseases has profoundly shaped thinking about causes and cures. Genetic diseases caused by a toxic gain-of-function mechanism provide unique challenges to those who would devise therapies. When genetic pathology is caused by the loss of some critical factor, identification of the gene presents an immediate goal of augmenting or bypassing the need for the missing factor. However, in the gain-of-function situation, one is faced with the task of impeding a pathogenic rogue activity. This scenario is somewhat similar to the chemotherapeutic approaches to the treatment of cancer and infectious disease; however, in this case the ultimate goal requires preserving a target cell rather than preferentially destroying it. For polyglutamine diseases, one obvious goal is to develop agents that specifically inhibit the mutant protein. Although loss versus gain-of-function is a useful distinction in genetic analysis, it should not limit the scope of our thinking about treatment strategies. The pathogenic forces exerted by expanded polyglutamine almost certainly involve diminished or compromised activities of essential cellular functions such as transcription or neurotransmission. Effective therapies may require augmentation of these functions in conjunction with interventions meant to address more primary defects rooted in the biophysical misbehavior of expanded polyglutamine.

Protein folding and turnover

Misfolding and altered solubility of proteins is a fundamental defect conferred by polyglutamine expansion. It is therefore not unexpected that the chaperone system is involved in the cellular response to polyglutamine pathology. The chaperones comprise a group of conserved enzymes whose roles include facilitating protein folding *in vivo*. Chaperone activity is particularly important under conditions of cell stress in which protein folding is impaired.

Chaperones colocalize with polyglutamine aggregates in both cell-based polyglutamine expression systems and patient tissues. Human DNAJ (HSP40) associates with ataxin-1 aggregates in cell culture and in SCA1 patient tissue[2]. HSP40 and HSP70 proteins colocalize with aggregates of expanded ataxin-3 and androgen receptor in cell culture models[3–5]. Chaperone overexpression suppresses polyglutamine aggregation in cell culture, yeast, *C. elegans* and *Drosophila*[2,5–9]. Most strikingly, chaperone overexpression has been demonstrated to suppress polyglutamine-mediated neuronal degeneration in three *Drosophila* models[9–11]. In two of these studies, however, suppression of neurodegeneration was not accompanied by suppression of aggregation[10,11]. The relationship between chaperone expression and polyglutamine aggregation in mammalian cells is also less than straightforward. Overexpression of HSP40 (HSDJ) in COS-7 cells increases the aggregation of expanded HD exon 1, but has no effect on aggregation in either PC12 or SH-SY5Y cells[12]. Although there seems to be a relationship between chaperone activity, polyglutamine aggregation and toxicity, much remains to be learned about what effect this may have in the context of disease.

The ubiquitin/proteasome system also seems to have a role in the cellular response to expanded polyglutamine. This pathway is responsible for the normal regulation of protein metabolism, as well as the destruction of misfolded or damaged proteins. Ubiquitin is present in protein aggregates associated with neuropathological features such as Lewy bodies and neurofibrillary tangles[13]. Ubiquitin and proteasome components are associated with polyglutamine inclusions in both disease models and patient tissues[14]. The persistence of ubiquitinated forms of polyglutamine associated with proteasome components indicates that cells are attempting to destroy polyglutamine-containing proteins, and that these substrates may be resistant to proteasomal degradation. Investigation of the influence of polyglutamine length on the processing of ataxin-1 showed that although ubiquitination is unaffected, proteasomal degradation is inhibited for an expanded form of the protein[15]. The proteasome inhibitor lactacystin also causes an increase in expanded polyglutamine aggregates, indicating that

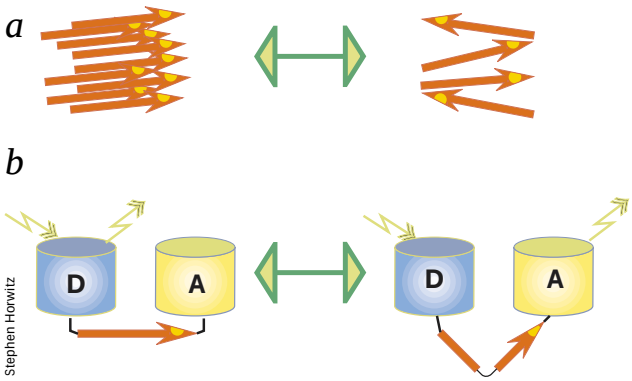

Fig. 1 *In vitro* assay systems for polyglutamine disease drug discovery. ***a***, *In vitro* assays could identify compounds that increase or decrease solubility of expanded polyglutamine-containing proteins or peptides (red arrows) so that they don't aggregate. ***b***, Tandem FRET sensor can detect conformational changes in polyglutamine proteins. This is achieved by monitoring the proximity between two fluorescent reporters by detecting the distance-dependent transfer of energy between donor (D) and acceptor (A) fluorophores. *In vivo* drug discovery assay strategies are described in the text.

proteasomal processing of ubiquitinated substrates is a clearance mechanism counterbalancing aggregate formation[3,12]. Two studies suggest that ubiquitination has protective effect in the progression of polyglutamine diseases. In a mutant huntingtin-expressing cell culture system, inhibition of the ubiquitin pathway increases huntingtin-induced apoptotic cell death[16]. In SCA1 transgenic mouse model, inhibition of ubiquitination, accomplished by crossing the expanded SCA1 transgene into a mouse lacking the E6-AP ubiquitin ligase, accelerated disease progression while diminishing inclusion formation[15].

The capacity of chaperones to facilitate protein folding, coupled with the cell's ability to degrade mutant polyglutamine proteins, probably has an important role in maintaining neuronal function. The presence of ubiquitinated polyglutamine proteins in the polyglutamine diseases indicates that ubiquitination is not the rate-limiting step in the destruction of these proteins, and therefore small molecules that enhance clearance by the proteasome may be of benefit.

Expanded polyglutamine causes aberrant protein interactions

The functional significance of the non-expanded polyglutamine tract is not known. One possibility is that this tract plays a role in facilitating protein–protein interaction. Through the use of two-hybrid screens, a number of proteins have been identified that interact with proteins containing polyglutamine; some of these interactions are sensitive to polyglutamine-tract length[17,18]. In several polyglutamine-containing proteins, the polyglutamine region is adjacent to a polyproline tract; in huntingtin, the polyproline region interacts with SH3-domain– and WW-domain–containing proteins[19,20]. A recent survey of all *S. cerevisiae* proteins containing polyglutamine tracts showed that a significant majority of these tracts are in proteins involved in transcription and signal transduction[21]. An intriguing feature of these two functional categories is that both involve combinatorial assembly of transient multisubunit protein complexes. If this functional clustering holds for the human proteome, we might also expect that the inappropriate protein–protein interactions mediated by polyglutamine will also involve proteins that function in these processes.

A growing body of evidence indicates that polyglutamine disease proteins interact directly with transcription factors. A current list of factors reported to physically associate with the mutant disease proteins includes TATA-binding protein (TBP), the *Drosophila* eyes-absent protein (EYA), CREB-binding protein (CBP), p53, nuclear receptor co-repressor (N-CoR), mSin3A and $TAF_{II}130$ (refs. 22–27). Support for the idea that some of these interactions have a role in disease comes from the fact that human TBP is localized to nuclear inclusions in human SCA3-disease brain and $TAF_{II}130$ to inclusions in DRPLA and HD (refs. 22,27). In brain from HD patients, N-CoR is mislocalized and mSin3A is present in nuclear inclusions[23]. CBP is localized to androgen-receptor inclusions in SBMA patient tissue[26]. In some cases, these interactions with transcription factors are known to inhibit function; for example, mutant huntingtin represses expression from two different p53-regulated promoters and from $TAF_{II}130$ promoters[25,27]. Further evidence linking the transcription machinery with polyglutamine toxicity has been provided by a *Drosophila* model of SCA1. A genetic screen for enhancers and suppressors of SCA1 toxicity identified the transcriptional cofactors *Sin3A*, *Rpd3*, *dCtBP* and *dSir2* (ref. 9). It is interesting to note that a number of transcriptional cofactors shown to interact with polyglutamine, or to influence polyglutamine toxicity, are known to be involved in histone acetylation or deacetylation. This raises the possibility that perturbation of these pathways might be a primary cellular defect in polyglutamine disease.

Polyglutamine effects on the transcriptome

Based on the physical interaction between polyglutamine-containing proteins and transcription factors, it is not surprising that levels of some mRNAs are altered in polyglutamine diseases[28]. The scope of transcriptional perturbation has been assessed by microarray technology and other comprehensive approaches[29,30]. In both SCA1 and HD mouse models, mRNAs encoding proteins involved in neuronal signal transduction and calcium homeostasis were preferentially decreased. In the HD model, transcripts encoding neuronal cytoarchitecture and cell-adhesion proteins were also decreased, whereas mRNAs encoding heat shock, proteasome and other stress-related proteins are increased. For the small number of mRNAs that have been evaluated in human postmortem tissue, the human data are consistent with the mouse models[30].

Advances in genomic technologies (for example, DNA microarrays) allow global analyses of cellular responses to insult, and thus offer opportunities for developing comprehensive models of cellular pathology. However, understanding the wealth of data generated in such experiments remains challenging. On a most basic level, it remains to be seen to what degree changes in mRNA expression correlate with changes in protein expression. Moreover, some of the gene expression changes observed are likely pathogenic, whereas others may be compensatory, or simply markers of altered cellular physiology. Assigning individual genes or pathways into these categories by theoretical means is not straightforward, and will likely instead require the application of conventional genetic and biochemical analyses. However, the patterns of gene expression changes reported in polyglutamine disease models present testable hypotheses, as well as strategies for pharmacologic intervention.

Intercellular events may contribute to neurodegeneration

Although much attention has focused on cell-autonomous events,

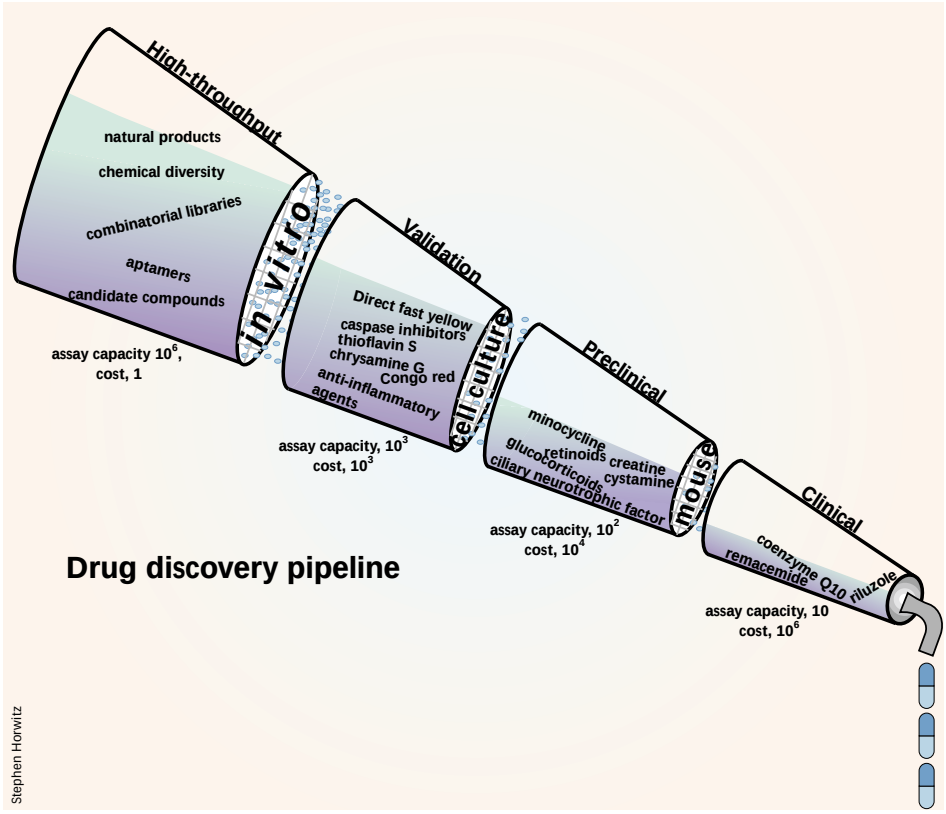

Fig. 2 The drug-discovery pipeline for potential therapeutics for the treatment of polyglutamine disease. A process of drug discovery from high-throughput screening of random chemical libraries to clinical trials of the most promising candidates flows from left to right. Assays and polyglutamine disease models described in the text will be most useful at particular segments in the pipeline. At each segment, the number of compounds screened will decrease, while the cost of screening each candidate increases. Examples of compounds (or classes of compounds) currently being evaluated in the various stages are listed in the segments.

pathogenic signaling between neurons was the first credible hypothesis advanced to explain neurodegeneration in HD (refs. 31–33). This idea is supported by the observation that the neurodegenerative changes observed in HD can be mimicked by administration of excitotoxic neurotransmitter agonists in animal models. Excessive signaling by excitatory amino acid agonists induces an excitotoxic cascade that involves calcium flux into the neuron, free radical formation, mitochondrial respiratory chain blockade and in some cases, cell death[34]. The same pattern of neurodegeneration caused by glutamatergic agonists can be replicated by systemic administration of mitochondrial poisons, indicating that blockade of the respiratory chain, regardless of upstream events, is sufficient to cause neuronal cell death[35]. Glutamate receptor antagonists and drugs that enhance mitochondrial function protect neurons in mouse, rat and non-human primate models of neurodegenerative diseases[33,36]. These findings have led to clinical trials of ketamine and remacemide (NMDA antagonists), baclofen and lamotrigine (glutamate-release inhibitors) and coenzyme Q10 and creatine (mitochondrial support agents)[36–40].

With regard to therapeutic targets, a relatively unexplored pathogenic feature of polyglutamine disease may be the presence of activated microglia. In contrast to astrocytes, which have a role in neuronal health maintenance, activated microglial cells may be harmful to neurons[41]. Microglia are bone-marrow–derived monocytic cells present in basal ganglia and other regions of HD brains[42]. The potential for targeting microgliosis in HD is supported by a recent report in which a patient treated with cyclosporin for immunosuppression related to fetal stem-cell transplant had no gliosis on autopsy (after cardiac-related death), despite advanced HD (ref. 43). The role of microglia in pathogenesis of nonpolyglutamine diseases is more widely appreciated. Cyclooxygenase (COX) inhibitors have been shown to reduce microgliosis in an animal model of Alzheimer disease and are now in clinical trial for that disease[44,45]. As cyclooxygenase inhibitors and cyclosporin are FDA approved and have been safely used in patients with neurodegenerative disease, clinical trials could be established to test efficacy of these drugs in polyglutamine disease patients.

Caspase cleavage, toxic fragments and programmed cell death

Apoptosis is a highly regulated cellular death pathway that has been shown to have a role in such neurodegenerative diseases as Alzheimer disease, amyotrophic lateral sclerosis and polyglutamine disease[46]. Execution of the apoptotic program involves the activation of a cascade of proteases known as caspases. Evidence for caspase activation has been observed in the HD brain[47,48], and expression of expanded polyglutamine in animal cell culture promotes apoptosis[49]. The relationship between expanded polyglutamine and the apoptotic pathway is complex because in addition to causing stresses that activate the apoptotic program, some polyglutamine-containing proteins are caspase substrates themselves[50]. Truncation of huntingtin increases its cellular toxicity, leading to the idea that proteolytic cleavage of huntingtin is a necessary step in the initiation of HD. The presence of truncated forms of huntingtin raises the question of which protease is responsible for the cleavage. Mutation of caspase-3 cleavage sites in the huntingtin reduces toxicity in a cell-culture model, indicating that proteolysis of huntingtin by caspase-3 may contribute to HD progression[51]. Caspase-8 is required for polyglutamine toxicity in a cell-culture model, and is directly activated by polyglutamine-mediated aggregation[52].

These data indicate a complex and cooperative association between expanded polyglutamine-containing proteins and caspases as agents of cellular dysfunction and death. Thus, caspases may represent a useful target for therapeutic intervention. In the R6/2 mouse HD model, toxicity of the expanded huntingtin transgene is reduced in a caspase-1 dominant-negative background, and direct infusion of a caspase inhibitor (zVAD-fmk) into the brains of the R6/2 mice also slows disease progression[48]. The caspase inhibitor minocycline also slows disease progression in the R6/2 mouse[53]. However, it remains unclear whether the modest clinical improvement in minocycline-treated mice is due

to caspase inhibition or the drug's anti-inflammatory or calcium-chelation activity.

Strategies for drug discovery

Our understanding of the molecular pathology of polyglutamine disease and cellular responses to expanded polyglutamine expression has matured to the point where rational strategies for drug discovery become feasible. One approach is high-throughput screening for small molecules that affect specific molecular targets. Assays for target-driven drug discovery can be done *in vitro* and *in vivo*, with each approach having specific advantages (Fig. 1). *In vitro* assays can be sensitive, quantitative and easily automated. They also provide confidence that the 'hits' identified act directly on the target used in the assay. Cell-based assays, on the other hand, can be designed to screen for compounds with favorable permeability and toxicologic properties, and can potentially yield useful hits that act through unanticipated mechanisms.

Potential targets for high-throughput chemical screens include chaperones, caspases, the ubiquitin/proteasome machinery, transcription factors and the offending polyglutamine-containing proteins themselves. The success with the caspase inhibitors zVAD-fmk and minocycline warrants further study of caspase inhibitors. Because HSP70 overexpression abolishes toxicity in several models, agents that enhance HSP expression might also be useful. Pharmacologic agents reported to potentiate chaperone expression have been described[54].

Much attention has focused on screens for drugs which prevent aggregation of expanded polyglutamine (Fig. 1*a*). Although a lively controversy exists regarding the role of aggregates in disease, the tendency toward aggregation is a well-established molecular property conferred by polyglutamine expansion. Moreover, this property is shared by all mutant pathogenic polyglutamine proteins identified to date. Despite demonstrations that the presence of visible aggregates can be uncoupled from pathology in several cases, the formation of lower order microaggregates, or the ability of the mutant protein to engage in aberrant protein-protein interactions, remains a hypothetically viable pathogenic mode of action.

Efforts are underway to identify compounds that inhibit aggregation of expanded polyglutamine. In an *in vitro* assay based on filter retention of SDS-insoluble polyglutamine aggregates, micromolar concentrations of Congo red, thioflavine S, chrysamine G and Direct fast yellow were able to inhibit the aggregation of a huntingtin-derived expanded polyglutamine tract. Congo red also suppresses aggregation in a cell-culture model[55]. These compounds all have affinity for amyloid β-sheet structures, and may act by preventing aggregate nucleation. Ron Wetzel has developed another *in vitro* assay that uses preformed polyglutamine aggregates immobilized on a solid phase (pers. comm.). This assay monitors the aggregation process by measuring the incorporation of labeled polyglutamine from the solution phase into the immobilized aggregate. Because this assay monitors aggregate growth rather than nucleation, it may yield a different class of inhibitors.

In a screen designed to detect compounds that act earlier in the aggregation pathway, Brian Pollok and coworkers at Aurora Biosciences are currently developing assays using protein–peptide reporters to discover compounds that selectively recognize structural features of the pathological length-huntingtin protein (pers. comm.). These reporters will be used in assays with fluorescence resonance energy transfer (FRET) readout. In one screen, tandem fluorescent protein sensors will be generated to detect intramolecular changes in protein structure using FRET (Fig. 1*b*). Polyglutamine peptides of varying lengths will be integrated into the tandem green fluorescent protein (GFP) sensor, with FRET changes monitored as a function of polyglutamine length and environmental perturbation. The goal of these biochemical screens is to identify compounds that recognize and potentially disrupt the structure of expanded huntingtin protein.

In vivo screens are also being planned or under way. Housman and co-workers have shown that detergent-resistant insoluble fluorescent aggregates of expanded polyglutamine fused to GFP can be detected in tissue-culture lysates. Automated fluorescence detection of these aggregates can be used to assay chemical libraries for agents that suppress aggregation *in vivo*[24]. Efforts are also underway to develop microbial assays for polyglutamine aggregation. One of us (R.E.H.) has developed a yeast assay system based on the lethality conferred by polyglutamine-mediated aggregation of an essential protein (unpublished observations), enabling high-throughput screens for chemicals able to suppress this lethality. Similarly, Leslie Thompson, Larry Marsh and co-workers have developed a *Drosophila* strain that exhibits lethality conferred by expression of expanded polyglutamine throughout the nervous system[56]. This strain will be used in a screen that exposes developing transgenic *Drosophila* to candidate drugs and chemical libraries (pers. comm.).

Hundreds or perhaps even thousands of compounds will potentially be identified in the initial screens described above. Given the resources required to evaluate compounds in murine models, strict criteria will have to be applied to determine which compounds should advance to evaluation in mice. Ideally, the screening efforts can be coordinated so that compounds with activity in multiple screening models can become candidates for evaluation in murine models.

Perspective

The cellular pathology of polyglutamine disease seems to involve processes that are shared by degenerating neurons and unaffected cells. One of the compelling mysteries in the field of polyglutamine disease is the mechanism underlying selective neuronal degeneration in response to ubiquitously expressed expanded-polyglutamine proteins. Understanding this in molecular terms may be instrumental for defining cell-specific therapeutic targets. Therapeutic perturbations of common cellular pathways (that is, 'housekeeping' functions) may protect nerve cells, but simultaneously interfere with these important processes in other tissues. Because of the potential toxic side effects associated with pharmacologic intervention in common cellular pathways, much attention has focused on expanded polyglutamine itself as a key drug target. Chemical agents that specifically inhibit the ability of expanded polyglutamine to associate with other proteins could potentially act upstream in multiple pathways of cellular pathology, and depending on their sensitivity to the polyglutamine protein context, could be therapeutics for the general class of polyglutamine diseases.

Over the past decade, the number of strategies for treatment of polyglutamine diseases has increased steadily. With the initiation of small molecule screening programs, it is now possible to conceptualize therapeutic opportunities in terms of a 'drug pipeline' (Fig. 2). Because the resources required to advance a compound toward clinical use increase significantly in each segment of the pipeline, it is important to apply thoughtful criteria when advancing an agent from one phase to the next.

Additional objective, noninvasive measures of disease progression must also be developed so that drug-mediated improvements can be more readily detected in clinical trials. As numerous labs establish drug screening and murine drug testing programs, clinical investigators might be faced with too many drugs at the threshold of human clinical trial. This is a welcome problem in the polyglutamine disease field, but also provides challenges for the design of trials that can detect what may be subtle changes in chronic late-onset disease.

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