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A yeast-based model of α -synucleinopathy identifies compounds with therapeutic potential

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Abstract

We have developed a yeast-based model recapitulating neurotoxicity of α -synuclein fibrilization. This model recognized metal ions, known risk factors of α -synucleinopathy, as stimulators of α -synuclein aggregation and cytotoxicity. Elimination of Yca1 caspase activity augmented both cytotoxicity and inclusion body formation, suggesting the involvement of apoptotic pathway components in toxic α -synuclein amyloidogenesis. Deletion of hydrophobic amino acids at positions 66–74 in α -synuclein reduced its cytotoxicity but, remarkably, did not lower the levels of insoluble α -synuclein, indicating that noxious α -synuclein species are different from insoluble aggregates. A compound screen aimed at finding molecules with therapeutic potential identified flavonoids with strong activity to restrain α -synuclein toxicity. Subsequent structure–activity analysis elucidated that these acted by virtue of anti-oxidant and metal-chelating activities. In conclusion, this yeast-cell model as presented allows not only fundamental studies related to mechanisms of α -synuclein-instigated cellular degeneration, but is also a valid high-throughput identification tool for novel neuroprotective agents.

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1. Introduction

Noxious α -synuclein amyloidogenesis is a common denominator in a plethora of age-related brain disorders exemplified most prominently by Alzheimer's, Diffuse Lewy Body and Parkinson's disease [1]. In these examples, insoluble aggregates of α -synuclein are found in extracellular plaques (as the non-amyloid component), in Lewy bodies and as inclusions in dopaminergic neurons, respectively. Extensive research has recognized the propensity of α -synuclein to self-polymerize as a

prominent risk factor in the pathogenesis. Compelling evidence supporting this mechanism comes from identification of dominant mutations in the α -synuclein coding region that promote aggregation and early disease onset [2,3]. Although the mechanisms of α -synuclein amyloidogenesis and its related cytotoxicity remain elusive, it appears that oxidative stress triggers oligomerization, a first step towards formation of insoluble aggregates [4]. Consistent with this view, redox-active metal ions (such as iron) are considered as major risk factors by virtue of their ability to produce reactive oxygen species (ROS) in a cellular environment [5–7]. Since α -synuclein aggregation may also promote ROS directly [8], its unrestrained amyloidogenesis supposedly leads to a vicious cycle of ROS production and aggregation. Thus, from different perspectives oxidative stress plays a prominent role in disease onset and development and therefore agents that either prevent the production of ROS

Abbreviations: MPTP, N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; SAR, structure–analysis relationship; SYN, α -synuclein

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directly and/or inhibit toxic α -synuclein aggregation would be of particular therapeutic interest.

Despite numerous studies the precise molecular events leading to cellular degeneration and the role of α -synuclein fibrilization is puzzling, perhaps most dramatically illustrated by the fact as to no consensus exists whether soluble protofibril species or matured aggregates incite neurotoxicity. In fact, it has been postulated that supramolecular protein aggregates are inert or even neuroprotective reservoirs of otherwise harmful soluble species [9]. Studies using yeast-based models [10,11] linked the formation of aggregates directly to toxicity. In yeast cells α -synuclein appear to possess membrane-binding activity [12] corroborating its presumed physiological role in synaptic vesicle binding and their recycling [13] and a genome-wide, unbiased screening effort identified genes involved in lipid metabolism and vesicle-mediated transport that appear to increase α -synuclein toxicity [14]. Collectively, these studies suggest that aggregation-prone α -synuclein misconformers may possess noxious membrane-binding properties affecting cellular integrity. Moreover, it qualified yeast as an excellent model organism to study physiological and pathological features of α -synuclein as they are found in normal and diseased brains. In this study, we developed a yeast-based system to investigate known (or suspected) environmental and genetic cues that involve α -synuclein-provoked degeneration of neuronal cells. Subsequently, this model was optimized and used in high-throughput screens of chemical libraries to search for molecules that alleviate α -synuclein-induced cytotoxicity.

2. Materials and Methods

2.1. Yeast strains, growth conditions and compound screening

Yeast media were prepared as described in [15]. Cells were grown in YPAD medium or in synthetic complete (SC) medium lacking essential nutrients for plasmid selection. Yeast strains used in this study were: BY4741, BY4743 [16]; rM101 (*BY4741 yca1::KanMX*), rM102 (*BY4743 yca1::KanMX yca1::KanMX*); W303a/ α [17]; AD1–8 [18]. This strain bears 8 deletions of so-called genes encoding for ABC transporters with xenobiotics efflux capacity. Therefore use of this strain facilitates uptake of compounds [18]. rM103 (AD1–8 *yca1::KanMX*). For metal toxicity studies ZnSO₄ (Merck) and FeCl₃ (Sigma-Aldrich) were added to the medium. Growth assays on solid medium: yeast cells were grown overnight at 30 °C in selective media until they reached late log phase. Cell density was determined using a spectrophotometer and cells were diluted to an OD₆₀₀ of 1. Ten-fold serial dilutions were spotted and incubated at 30 °C. Growth assays on liquid medium: cells were inoculated in 96 well microtiter plates (150 μ l/well) at OD₆₀₀ of 0,05 and incubated in time as indicated in the figures. Growth was followed by measuring the turbidity at 600 nm using a standard microtiter plate reader (Multiskan Ascent; Thermo Labsystems). The OD₆₀₀ measurement for growth curves is the average of triplicate measurements from several independent transformants.

For high-throughput screening a collection of 10,022 diverse compounds were tested. To this end, strain rM102 was inoculated at OD₆₀₀ of 0,0125 in SC-URA containing 2 mM FeCl₃ and subsequently dispensed in 96 well microtiter plates. Compounds were dissolved in DMSO and added to the yeast culture at a final concentration of 10 μ g/ml. Growth was followed by measuring OD₆₀₀ using a microtiter plate reader until control cultures (to which equal amounts of DMSO without compound were added) reached an OD₆₀₀ of approximately 0.15. The compounds were purchased from ComGenex (Hungary) and Sigma-Aldrich.

2.2. α -synuclein expression plasmids

cDNA's encoding human α -synuclein (wild type, A53T, A30P and Δ 66–77) were constitutively expressed in yeast cells using the triose phosphate isomerase (*TP11*) promoter and alcohol dehydrogenase (*ADH1*) terminator. To this end, a DNA fragment containing the *ADH1* terminator was cloned in the *XhoI* site of pYX212 [11] resulting in plasmid pJW212T. Subsequently, PCR fragments were generated encoding human α -synuclein and the mutant derivatives with primers: α -SYN1-F 5'-GAT CAG AAT TCC CAT GGA TGT ATT CAT GAA AGG ACT TTC-3' and α -SYN2-R 5'-GAT CAA GAT CTCT CGA GTT AGG CTT CAG GTT CGT AGT CTT G-3' using plasmids containing the relevant cDNA's as template. These PCR fragments were subsequently cloned (*NcoI/XhoI*) in vector pJW212T resulting in plasmids 212T-SYN(WT), 212T-SYN(A53T), 212T-SYN(A30P) and 212T-SYN(Δ 66–77). These α -synuclein expression plasmids were transformed to appropriate yeast strains. Construction of a plasmid 23SYN-GFP encoding α -synuclein-GFP fusion is described in [11]. Cells were viewed using a Nikon Optishot fluorescence microscope.

2.3. Western analysis

Yeast cell cultures were grown till an OD₆₀₀ of 2–3. Cells were harvested by centrifugation, washed in sterile water and the pellets were resuspended in extraction buffer containing 10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20, 10% Glycerol, 5 mM EDTA, 1 mM DTT and a mixture of protease inhibitors (Complete, Roche Molecular Biochemicals, Germany) and disrupted by vortexing for 5 min in the presence of glass beads. To remove cell debris the resulting suspension was spun down at 200 \times g and subsequently the supernatant was centrifuged at 16,000 \times g. The resulting supernatant (soluble fraction) and pellet (insoluble fraction) were separately taken up in loading buffer, fractionated by 15% SDS-PAGE and blotted onto PVDF membrane. Immunodetection was carried out using mouse anti- α -synuclein monoclonal antibody (Zymed Laboratories Inc.) and donkey anti-mouse IgG polyclonal antibody conjugated with alkaline phosphatase (Rockland, USA). Proteins were visualized using NBT/BCIP ready-to-use tablets (Roche) according to the manufacturer's instructions.

3. Results and discussion

3.1. Expression of α -synuclein cDNA in yeast cells

To construct a cellular model of α -synucleinopathy, we successfully developed several yeast strains expressing wild type α -synuclein or two corresponding clinical mutants (A30P and A53T) (Fig. 1a). When expressed as a GFP fusion, the resulting chimeric protein concentrated at the plasma membrane (Fig. 1b) indicating that it retained functionality with respect to its reported lipid bilayer association [10,19]. Although expression of α -synuclein in yeast cells did not result in an obvious growth or viability phenotype on solid medium (Fig. 1c left panel), detailed kinetic analyses of growth in liquid medium revealed a moderate growth-inhibitory effect (Fig. 1e, solid symbols).

3.2. Identification of extracellular and genetic cues that provoke α -synuclein-instigated cytotoxicity

We studied the effect of metal ions on cytotoxicity since these are considered major risk factors in developing toxic α -synuclein aggregation [5]. Addition of Zn²⁺ or Fe³⁺ ions to the growth medium led to a further α -synuclein-dependent growth inhibition (Figs. 1c, d, e, 2a) indicating that, similar to the situation in diseased brains, also in yeast metal ions

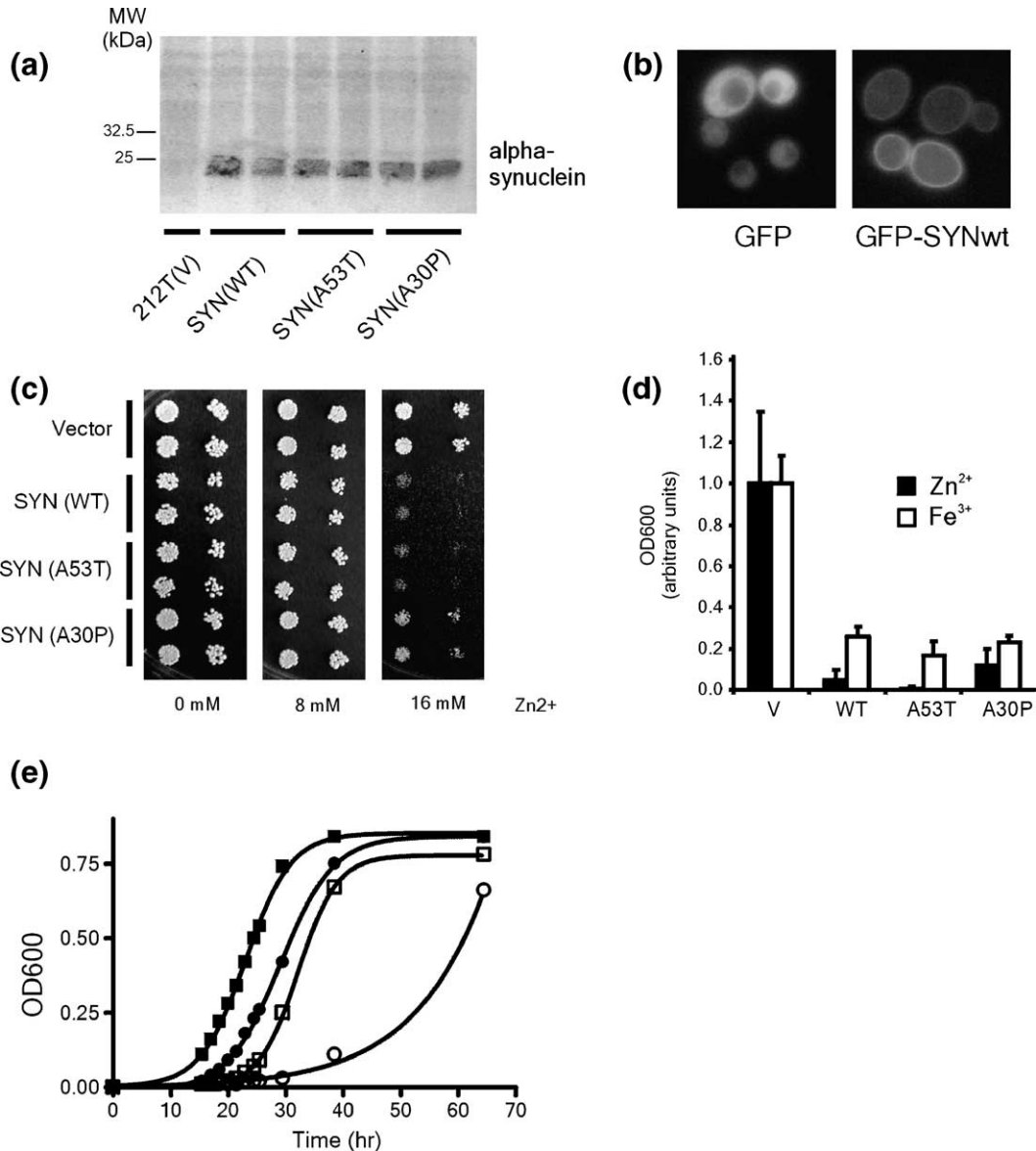


Fig. 1. Metal ions induce α -synuclein toxicity in yeast cells. (a) Western analysis of yeast cells expressing cDNA of human α -synuclein or mutants. Notations 212T(V), SYN(WT), SYN(A53T) and SYN(A30P) refer to extracts from W303a/ α yeast strain transformed with empty control vector pJW212T, or with 212T-SYN-WT, 212T-SYN-A53T, 212T-SYN-A30P, respectively. (b) Fluorescence microscopy of rM101 cells transformed with pUG23 (no insert) and 23SYN-GFP encoding GFP and α -synuclein-GFP, respectively. (c, d, e) Growth characteristics of yeast cells expressing human wild-type α -synuclein or mutants on solid c, or liquid d, media containing ZnSO₄ and FeCl₃. In panels c and d, the same strains were used as described in panel a. In panel d, black and open bars indicate growth in the presence of 16 mM ZnSO₄ and 2 mM FeCl₃, respectively. Growth is normalized to vector-containing cells. Bars indicate standard deviation. In panel e, kinetics of growth is determined of AD1–8 cells transformed with pJW212T (■, □) or 212T-SYN(WT) (●, ○) in the absence (solid symbols) or presence (open symbols) of FeCl₃.

aggravate α -synuclein toxicity. Expression of the clinical α -synuclein mutants (A53T; A30P) also led to metal-dependent toxicity but the extent of growth inhibition was more variable (Fig. 1c).

Although it has been suggested that α -synuclein aggregation may stimulate apoptosis, it remains elusive whether programmed cell death itself is directly responsible for neuronal loss, especially considering the observations that α -synuclein may also exert an anti-apoptotic activity [20,21]. This prompted us to study whether Yca1, a caspase involved in yeast apoptosis [22], modulated α -synuclein-triggered toxicity. Deletion of *YCA1*, however, did not

ameliorate cytotoxicity compared to its isogenic wild type strain but – contrary to an anticipated suppression of α -synuclein toxicity – rather displayed an even further reduction of growth (Fig. 2a, b). Interestingly, Yca1 has not been recognized before as a modulator of α -synuclein toxicity in a genome-wide screening approach [14], possibly due to the fact that significant toxicity (at least in this experimental set-up) is only observed in the presence of FeCl₃. Since *ycal* cells are unable to execute apoptosis, the observations suggest that the caspase plays another, yet unprecedented role independent of apoptosis, in preserving integrity of cells coping with α -synuclein toxicity.

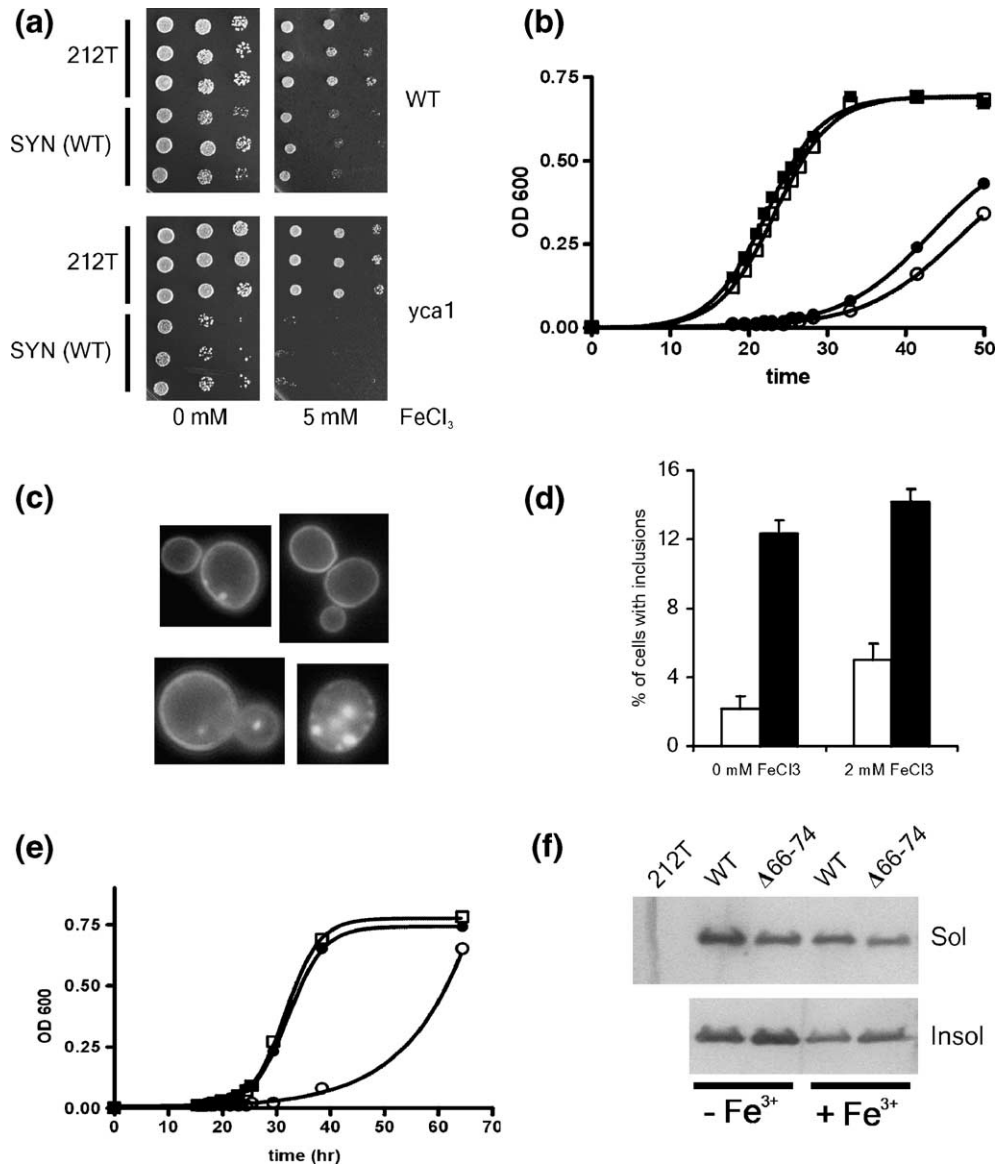


Fig. 2. Characterization of α -synuclein cytotoxicity in yeast cells. (a) Growth of wild type strain BY4743 and rM102 (*yca1/yca1*) transformed with pJW212T (no insert) and 212T-SYN-WT in presence or absence of FeCl_3 and (b) in liquid media containing 2 mM FeCl_3 . (c) Aggregation of α -synuclein-GFP in strain BY4741 (wild type, open bars) and rM101 (*yca1* mutant, black bars) transformed with 23SYN-GFP. (d) Quantification of α -synuclein-GFP aggregation in strain BY4741 (wild type, open bars) and rM101 (*yca1* mutant, black bars) transformed with 23SYN-GFP. The percentage of cells with inclusions is determined as a function of FeCl_3 and Yca1. Kinetics of growth (e) and Western analysis of soluble and insoluble fractions (f) of strain rM103 transformed with pJW212T (\square), 212T-SYN-WT (\circ) and 212T-SYN($\Delta 66-77$) (\bullet) in liquid media containing 2 mM FeCl_3 .

3.3. Fe^{3+} ions and abrogation of Yca1 activity promote α -synuclein aggregation in vivo

To gain further insight in the involvement of Yca1 in α -synuclein toxicity, we analyzed whether α -synuclein inclusions are formed in yeast cells and whether iron ions and Yca1 caspase are modulators. Wild type and *yca1* mutant cells expressing a GFP- α -synuclein fusion protein were generated and grown in the presence of iron ions. By fluorescence microscopy of actively dividing cells, we observed heterologous populations with some containing GFP- α -synuclein aggregates while most displayed plasma membrane-associated, diffuse fluorescence (Fig. 2c). Quantification of the percentage

of cells with aggregates (Fig. 2d) revealed that Fe^{3+} ions increased the percentage of inclusion-containing cells, substantiating the hypothesis that ferrous ions promote aggregation. Moreover, *yca1* mutant strains, for which expression of α -synuclein is profoundly cytotoxic, also displayed high levels of α -synuclein aggregates, even in the absence of iron ions. Thus, Yca1 caspase activity did not only protect against α -synuclein-elicited toxicity (Fig. 2a, b) but appears to play a more specific role in counteracting or inhibiting aggregation. Collectively, the data revealing ferrous ions and Yca1 as modulators of α -synuclein cytotoxicity (Fig. 2a, b) and the fluorescence studies (Fig. 2c, d) suggest a positive correlation of growth inhibition and inclusion formation. We also note an unanticipated role of

Yca1 in α -synuclein toxicity, which may become instrumental in detailing the involvement of caspases in neuronal α -synuclein pathology. Since Yca1 is thought to be responsive to oxidative stress [23], we hypothesize that α -synuclein aggregation and/or FeCl_3 may mediate increases in ROS production for activating Yca1 which in turn may trigger an as yet fully speculative activity involved in breakdown of aggregation-prone α -synuclein misconfomers. Indeed, many neurodegeneration-associated gene products (such as α -synuclein) have been recognized as substrates of caspases [24], a significant finding considering the high substrate specificity of caspases [25].

3.4. A stretch of hydrophobic amino acids (66–74) confers cytotoxicity of α -synuclein

Since cellular degeneration appears to be intimately linked with formation of inclusion bodies (Fig. 2d), we went on to study whether a previously identified stretch of hydrophobic amino acids (66–74) required for *in vitro* self-association [26] mediates cytotoxicity. The contribution of these amino acids regarding *in vivo* cytotoxicity was defined by expressing a mutant version of α -synuclein with residues 66–74 deleted ($\alpha\text{SYN}_{\Delta 66-74}$) in *yca1* cells. Expression of $\alpha\text{SYN}_{\Delta 66-74}$ caused almost no growth inhibition compared to wild type α -synuclein (Fig. 2e) suggesting that this hydrophobic motif is required for cytotoxicity (also using wild type yeast cells identical results were obtained; data not shown). To establish whether this motif affects α -synuclein solubility, we performed Western analysis of *yca1* cells expressing wild type α -synuclein or $\alpha\text{SYN}_{\Delta 66-74}$ in response to FeCl_3 (Fig. 2f). We observed that the levels of insoluble $\alpha\text{SYN}_{\Delta 66-74}$ are equal (if not higher) compared to wild type α -synuclein irrespective of the presence of extracellular FeCl_3 (note that addition of FeCl_3 as such leads to a decrease in cellular α -synuclein). These data demonstrate that, although residues 66–74 mediate toxicity, it does not lower the levels of α -synuclein in the insoluble fraction indicating that precipitation alone is not sufficient to elicit toxicity. These findings may seem surprising considering the results (see above) uncovering a correlation of (full length!) α -synuclein toxicity and *in vivo* inclusion formation. Apparently, formation of insoluble aggregates is not toxic per se (in fact, it has even been suggested that soluble protofibrils rather than insoluble aggregates of α -synuclein confer cytotoxicity [27]) suggesting that noxious α -synuclein amyloidogenesis requires specific conformational or biophysical characteristics which require residues 66–74. Thus, aggregates or protofibrils of wild type α -synuclein may affect cellular integrity negatively, while precipitation of $\alpha\text{SYN}_{\Delta 66-74}$ may not be harmful.

3.5. High-throughput screening of a small molecule library identified flavonoids counteracting α -synuclein cytotoxicity

To further characterize the mechanisms involved in α -synuclein pathogenesis, we tested about 10,000 compounds to identify molecules that counteracted cytotoxicity. The effect of the compounds on growth was evaluated using FeCl_3 -

challenged *yca1* cells (rM103) expressing wild-type α -synuclein since this was experimentally the most robust and reproducible assay (Fig. 2b). To facilitate uptake of compounds into the yeast cell, a genetic background was used with multiple genomic deletions of (*MDR1*) genes encoding xenobiotics efflux pumps. Since addition of 2 mM FeCl_3 to the medium is inherently toxic, compounds that either reduce the toxic properties of FeCl_3 or α -synuclein or both will be identified as growth enhancers. We would like to point out that suppression of inherent FeCl_3 toxicity may be of therapeutic interest since ferrous ions are considered as an important risk factor of triggering neuronal degeneration in diseased brains (at least in part by stimulating α -synuclein aggregation). Two compounds were identified, i.e., quercetin and (–)-epigallocatechin-3-gallate, that were found to increase growth of α -synuclein cells (Fig. 3) indicating a inhibitory activity of α -synuclein cytotoxicity. This effect was found, at least partly, independent of FeCl_3 since also in the absence of FeCl_3 growth of α -synuclein cells was improved.

However, also FeCl_3 -grown parental cells (open bars) displayed a modest growth improvement, although far less than of α -synuclein cells, indicating that the compounds were able – to some extent – to mitigate FeCl_3 toxicity.

Both compounds acted with EC_{50} values in the low micromolar range (Fig. 3b), indicating considerable potency towards relieving cytotoxicity. Quercetin and (–)-epigallocatechin-3-gallate belong to the large group of flavonoids that are commonly found in fruit, vegetables and green tea and they are thought to possess a broad range of pharmacological activities due to their strong anti-oxidant and metal chelation properties. The anti-oxidant activities of the compounds could explain the ability to inhibit α -synuclein-mediated cytotoxicity since its aggregation is thought to promote harmful ROS production *in vivo* [8]. The metal chelation (and anti-oxidant) properties are likely to be responsible for countering inherent FeCl_3 toxicity as it is observed with parental cells challenged with ferrous ions (see discussion above and Fig. 3a). Noteworthy in this context are *in vitro* studies that recognized flavonoids as agents with protein anti-fibrilization activity [28,29] although we could not observe a reduction of α -synuclein in the insoluble fraction of quercetin treated cells (data not shown). Nonetheless, (–)-epigallocatechin-3-gallate has been shown neuroprotective in a (MPTP-induced) animal model of dopaminergic degeneration [30] demonstrating that the identified molecules are efficacious in a relevant and well-validated model. Our data extend these conclusions considerably by demonstrating that these flavonoids counteract the cytotoxic properties of α -synuclein toxicity directly in a cellular context.

Structure–analysis relationship (SAR) studies of flavonoids [31,32] revealed metal chelation sites between 4-oxo and adjacent hydroxyl groups (3 and 5 positions), while also ortho-oriented hydroxyl-groups on the B ring appeared to have metal-binding properties. The same structural configuration was found essential for scavenging radicals, showing that metal chelation and anti-oxidant activity are intimately linked. We performed SAR studies of some quercetin analogues to test structural properties with *in vivo* relevance for inhibition of α -synuclein

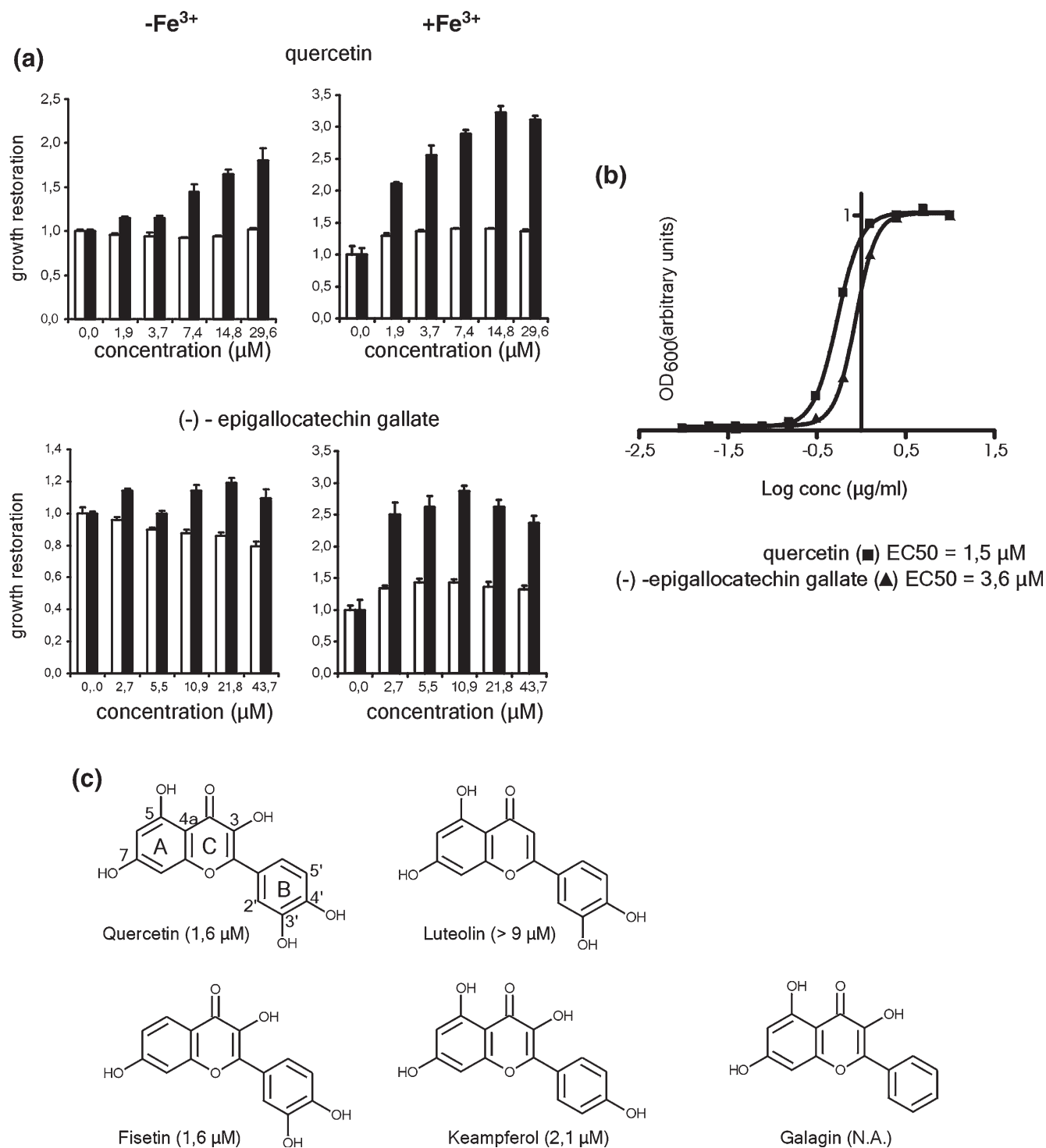


Fig. 3. Identification of polyphenols as protective agents against α -synuclein-instigated cellular degradation. (a) Growth-restoration of strain rM103 (*yca1*) transformed with pJW212T (open bars) and 212T-SYN-WT (black bars) in medium without (left panel) or with (right panel) 2 mM FeCl₃ with varying concentrations of quercetin or (-)-epigallocatechin gallate. Growth is normalized to 0 mM compound. (b) Dose–response curves of strain rM103 transformed with 212T-SYN-WT. Growth was determined as a function of the quercetin or (-)-epigallocatechin gallate concentration. (c) Structure–activity relationship analysis of several flavonoids. EC₅₀ values are indicated in parenthesis.

cytotoxicity. We observed that hydroxyl groups on the B-ring are of particular importance since their removal reduced or abolished activity (quercetin vs. keampferol and galagin, respectively) (Fig. 3c). Also the hydroxyl group at the 3 position was found important (quercetin vs. luteolin) whereas

changing the hydroxyl content of the A ring did not have any significant impact (fisetin). Clearly, the SAR study suggests that metal chelation and/or radical scavenging are important structural properties to counteract α -synuclein toxicity in vivo. We conclude that oxidative stress plays a determining

role in α -synuclein-instigated cellular degeneration and thus qualifies anti-oxidants and/or metal chelators as legitimate candidates for therapeutic intervention in α -synucleinopathies.

Collectively, the data reported here divulge a phenotype-based yeast model that recapitulates pathological properties of human α -synuclein. This cellular model allows further fundamental studies with respect to the underlying mechanisms of aggregation and cytotoxicity, and to define environmental and genetic modulators in vivo of α -synuclein-mediated cytotoxicity. The model will also allow and facilitate the search for disease-modifying agents. The identification of flavonoids as inhibitors of toxicity already dramatically illustrated the capability of this model as a powerful tool for high-throughput screening to identify, and subsequently optimize, novel molecular entities with therapeutic potential.

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