Design of High-Throughput Screening of Natural Extracts to Identify Molecules Bypassing Primary Coenzyme Q Deficiency in *Saccharomyces cerevisiae*

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Abstract

Coenzyme Q₁₀ (CoQ₁₀) deficiency syndrome is a rare disease included in the family of mitochondrial diseases, which is a heterogeneous group of genetic disorders characterized by defective energy production. CoQ₁₀ biosynthesis in humans requires at least 11 gene products acting in a multiprotein complex within mitochondria. The high-throughput screening (HTS) method based on the stabilization of the CoQ biosynthesis complex (Q-synthome) produced by the *COQ8* gene overexpression is proven here to be a successful method for identifying new molecules from natural extracts that are able to bypass the CoQ₆ deficiency in yeast mutant cells. The main features of the new approach are the combination of two yeast targets defective in genes with different functions on CoQ₆ biosynthesis to secure the versatility of the molecule identified, the use of glycerol as a nonfermentable carbon source providing a wide growth window, and the stringent conditions required to mark an extract as positive. The application of this pilot approach to a representative subset of 1200 samples of the Library of Natural Products of Fundación MEDINA resulted in the finding of nine positive extracts. The fractionation of three of the nine extracts allowed the identification of five molecules; two of them are present in molecule databases of natural extracts and three are nondescribed molecules. The use of this screening method opens the possibility of discovering molecules with CoQ₁₀-bypassing action useful as therapeutic agents to fight against mitochondrial diseases in human patients.

Keywords

Saccharomyces cerevisiae, coenzyme Q, coenzyme Q deficiency syndrome, high-throughput screening (HTS), natural product extracts, mitochondrial respiration, vanillic acid

Introduction

Mitochondrial diseases are a large family of genetic disorders characterized by defects in multiple biochemical pathways and cellular processes in mitochondria.¹ Mitochondria are recognized as the massive energy producers in the cell but are responsible for other essential functions not related to ATP production. The functional diversity of mitochondria explains the pleiotropic effects found in mitochondrial diseases and the difficulty in identifying definitive therapeutic procedures.²

Coenzyme Q₁₀ (CoQ₁₀ or Q₁₀) deficiency is one mitochondrial disorder that is considered a rare disease.³ CoQ₁₀ is an amphipathic lipid formed by an aromatic polar ring of quinone

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with redox properties condensed to a hydrophobic polyisoprenoid tail responsible to the membrane attachment.⁴ The primary function of CoQ₁₀ is electron transfer between complex I (NADH-quinone reductase) or complex II (succinate-quinone reductase) and complex III (ubiquinol-cytochrome *c* reductase),⁵ but it is also involved in antioxidant protection, nucleotide synthesis as the electron acceptor of dihydro-orotate dehydrogenase, the regulation of the mitochondrial permeability transition pore (mPTP), mitochondrial sulfide metabolism, and the regulation of uncoupler proteins of brown adipose tissue, as well as being an electron acceptor for reactions of β -oxidation.⁵ CoQ₁₀ deficiency is considered a rare disease because it affects less than 1 in 2000 persons. As a mitochondrial disease, it produces pleiotropic symptoms in a high number of organs and tissues,⁶ and it can be classified in primary deficiency when it is generated by alterations in genes directly involved in CoQ₁₀ synthesis,⁷ and in secondary deficiency when the affected genes are involved in other mitochondrial functions not related to CoQ₁₀ synthesis.⁸ In humans, 11 genes are needed to produce CoQ₁₀, and human CoQ₁₀ deficiency has been reported by mutations in 10 of them.⁷ These multiple origins of the disease have not facilitated the discovery of versatile therapies that apply universally to patients.

Most of our knowledge on coenzyme Q (CoQ) biosynthesis in eukaryotic cells has been provided by research with the yeast *Saccharomyces cerevisiae*, a yeast model that produces coenzyme Q₆ (CoQ₆). CoQ shows a conserved structure, a quinone ring that is similar in all organisms bound to an isoprenoid tail composed by a different number of monomer isoprene units depending on the species, 6 units in yeast (CoQ₆) and 10 units in humans (CoQ₁₀). All yeast COQ genes show significant homology with the human orthologs; each yeast gene has one counterpart in humans with the exception of yeast *COQ1*, which is homologous to PDSS1 and PDSS2 human genes, and *COQ8*, homologous to the ADCK3/COQ8A and ADCK4/COQ8B human genes.⁹ In yeast, CoQ₆ synthesis involves the assembly of a biosynthetic complex (Q-synthome) at the matrix side of the inner mitochondrial membrane.¹⁰ Q-synthome is composed of proteins encoded by the COQ genes, except for *COQ1* and *COQ2*, responsible for the hexa-isoprenoid tail synthesis and its condensation with the aromatic ring of *p*-hydroxybenzoic acid (*p*-HB), respectively. Q-synthome works as a single enzyme catalyzing the conversion of the Coq2 product, hydroxy-hexaprenyl benzoate (HHB), to CoQ₆, although the exact composition of the Q-synthome is not clearly established and its catalytic activity has certainly never been measured. However, a shared feature for null mutant strains in COQ genes implicated in the Q-synthome assembly has been reported; the absence of one of the CoQ proteins blocks the complex formation, leading to the accumulation of the first precursor, HHB, instead of the expected substrate of the lacking enzyme.¹¹ In strains harboring missense mutations of COQ genes, the Q-synthome is assembled and the expected precursor is accumulated as has

been demonstrated for missense mutations in the *COQ7* gene.¹² We have previously demonstrated that *COQ8* gene overexpression in a null *COQ7* mutant (*coq7* Δ) restores the synthesis of 5-demethoxy-CoQ₆ (5-DMQ₆),¹³ the Coq7 precursor. 5-DMQ₆ is synthesized by the stabilization of components of the Q-synthome.¹⁰

COQ8 overexpression became an effective strategy for dissecting the yeast CoQ₆ biosynthetic pathway. The complex stabilization in the absence of specific CoQ proteins makes it possible to detect and identify diagnostic CoQ₆ precursors.¹⁴ The second application of *COQ8* overexpression was the identification of molecules bypassing the CoQ₆ biosynthesis defects of *COQ* null mutant yeasts.¹⁵ Small molecules analyzed using this approach, such as vanillic acid (VA), have been proven successful in null *COQ6* mutants (*coq6* Δ) expressing human COQ6 patients' alleles.¹⁶ Moreover, VA is able to restore CoQ₁₀ biosynthesis and ATP production in human HEK cells lacking the COQ6 gene.¹⁷ Other molecules similar to VA, such as 3,4-dihydroxybenzoic acid, can restore CoQ₆ synthesis in *coq6* Δ mutants overexpressing the *COQ8* gene. Greater interest has been aroused by β -resorcylic acid (β -RA) or 2,4-dihydroxybenzoic. β -RA restores CoQ₆ synthesis in *coq7* Δ mutants overexpressing the *COQ8* gene;¹⁴ partially restores coenzyme Q₉ (CoQ₉) levels in a murine model of induced CoQ₉ deficiency improving mitochondrial respiration and lifespan;¹⁸ rescues the encephalopathic phenotype in Coq9^{R239X} mice, a CoQ₉ deficiency model with a defective Coq9 protein;¹⁹ and blocks renal fibrosis in podocyte-specific Coq6 knockout mice.²⁰ The most promising effect of β -RA is the rescue of mitochondrial activities and CoQ₁₀ levels in fibroblasts from CoQ₁₀-deficient patients caused by mutations in *COQ7* and *COQ9* genes.^{21,22} The existence of bypassing compounds opens a therapeutic pathway for CoQ deficiency treatment that is more affordable than other methods.²³ However, the known bypassing molecules are not a general therapeutic solution for all causes of primary CoQ₁₀ deficiency, and in fact, they are specific for each mutated gene.

The goal of this study was to design and configure a high-throughput screening (HTS) method based on the specificity and robustness of the *COQ8* overexpression approach in COQ null mutant yeasts to detect new bypassing small molecules showing a general effect in all cases of primary CoQ₁₀ deficiency. Our results demonstrate that this strategy is successful and leads to discovering molecules with potential value for CoQ₁₀ deficiency treatment, based on an HTS method applied to a pilot subset of 1200 natural product extracts.

Materials and Methods

Strains, HTS Medium, and Reagents

The *S. cerevisiae* strains used in this work are described in **Supplemental Table SI**. Yeasts were grown in rich YPD

medium (1% yeast extract, 2% peptone, 2% dextrose), YPG medium (1% yeast extract, 2% peptone, 3% glycerol), YPE medium (1% yeast extract, 2% peptone, 2% ethanol), YPL medium (1% yeast extract, 2% peptone, 3% lactate), or SDc-ura 2% dextrose medium (YNB 6.7 g/L and CSM -ura dropout medium). Agar plates were prepared with the same medium composition plus 2% agar. Yeasts were cultured overnight in YPD or SDc-ura media depending on the strain used before the specific experiment. VA stock solution (W398802, Sigma, St. Louis, MO) at 20 mg/mL in Milli-Q water sterilized by filtration was used as a positive control. Yeast cell growth was tested in the presence of 2% DMSO, that is, the final concentration of DMSO after the extract addition.

HTS Conditions

HTS was analyzed in sterile 96-well polystyrene microplates. Aqueous extracts (10 μ L per well) from the Fundación MEDINA's collection²⁴ (<http://www.medinadiscovery.com>) were deposited in each microplate using the automated Biomek FX workstation (Beckman Coulter, Indianapolis, IN). Then, 90 μ L of the yeast suspension in YPG medium was added per well. Plates were incubated at 30 °C with shaking. Control wells were distributed in the left and right columns. Four controls per microplate were used in this study: (1) culture media controls (the two bottom wells of the right column without yeast [YPG]), (2) positive controls (the six top wells of this column [three top YPD and another three YPG + VA 2 mM]), (3) negative controls (the four top wells of the left column without VA), and (4) other positive controls (the four bottom wells of this column [YPG + VA 0.5 mM]). Microplates were analyzed with 80 extracts per plate (bacterial and fungi samples). All the experiments were performed in duplicate with an initial absorbance of 0.08 U/mL. Extracts were considered positive when the absorbance was at least double the initial absorbance or negative control, and the Z' score of the plate was higher than 0.85.

Fundación MEDINA's Natural Products Library Subset

For the primary screening (crude extracts), a subset of 1200 microbial extracts from different modules of the MEDINA natural product collection was used. The microbial extracts were obtained from bacterial and fungal strains cultivated in different nutritional conditions and extracted with acetone (1:1) for 1 h in an orbital shaker. Extracts were then centrifuged at 1500g for 15 min and the supernatant concentrated to half of the original volume (2XWBE) in the presence of a final concentration of 20% DMSO. Extracts were stored at -20 °C in 96-well ABgene V-bottom plates until needed.

Measurement of the Respiratory Rescue

For the primary screening (extracts), the yeast culture density in a nonfermentable carbon source (YPG) was monitored by determining the optical density (OD₆₀₀) using an EnVision multilayer plate reader (PerkinElmer, Boston, MA) before and after 120 h of culture at 30 °C with shaking (1000 rpm) in a BIOSAN PST-60HL-4 thermo-shaker (Riga, Latvia). In the second screening (fractions), a continuous mode of OD₆₀₀ monitoring was used from the beginning of the culture (TECAN SPARK 10M reader, Männedorf, Switzerland). Yeasts were cultured in 96-well plates at 30 °C with shaking (510 rpm).

Data Analysis

Extract activities were measured by OD₆₀₀ and the growth of each sample (extract or compound) was determined by the following equation:

$$\text{Growth} = (\text{SampleTf} - \text{SampleTo}) - (\text{ControlTf} - \text{ControlTo}) \quad (1)$$

where Tf is the OD₆₀₀ at the end time and To is the OD₆₀₀ at time zero. An extract was considered to have activity when its growth at least doubled the initial OD. The Z' factor predicts the robustness of an assay by taking into account the mean and standard deviation of both positive and negative controls.²⁵ In all experiments performed in this work, the Z' factor obtained was between 0.92 and 0.99.

Bioassay-Guided Extract Fractionation

Culture broths generated from positive extracts were extracted by addition of a 1:1 volume of acetone. After shaking for 2 h at 220 rpm, centrifugation at 2700 rpm, and filtration, the acetone was removed under a nitrogen stream and the remaining solution was loaded onto an SP207ss cartridge (1.5 $\times$ 5 cm) that was washed with water (100 mL) and eluted with methanol (40 mL) and acetone (60 mL). The combined organic eluates were evaporated, the residue was dissolved in DMSO (400 μ L), and 200 μ L of this volume was subjected to semipreparative high-performance liquid chromatography (HPLC) on a Gilson GX-281 system equipped with a Zorbax SB C8 column (9.8 $\times$ 250 mm) at a flow rate of 3.6 mL/min, using a gradient of H₂O:CH₃CN (5% CH₃CN for 1 min, then up to 100% CH₃CN for 35 min, held at 100% CH₃CN for 7 min, and back to 5% CH₃CN in 1 min), and UV detection at 210 and 280 nm was monitored. Eighty fractions were collected every 30 s from minutes 2 to 42. These fractions were evaporated, dissolved to a final concentration of 20% DMSO, and subjected to a bioactivity test in the second screening.

LC-MS and Database Matching of Known Secondary Metabolites

Liquid chromatography–mass spectrometry (LC-MS) analysis in the low-resolution (LR) or high-resolution (HR) mode was performed as previously described.^{26,27} A database search was performed against the MEDINA proprietary database of microbial metabolites and the commercial Chapman & Hall Dictionary of Natural Products (v25.1).

CoQ₆ Extraction and Quantification from Whole Yeast Cells

Yeast cells were lysed in a breaking buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, and 1 mM dithiothreitol) by vortexing with glass beads and centrifuged (10 min at 700g) to remove debris. The supernatant was centrifuged (60 min at 100,000g) to obtain a total membrane pellet. After protein quantification, samples equivalent to 1 mg of protein were extracted with a hexane method and separated, and CoQ₆ was identified with an HPLC–electrochemical detection (ECD) method as previously described.²⁸

COQ8 Cloning in pCM189 Plasmid

Plasmids and primers used in this study are shown in **Supplemental Table SI**. The *COQ8* gene was obtained by PCR amplification from genomic DNA of the BY4741 strain. Host pCM189 and *COQ8* amplicons were digested with *Bgl*III and *Not*I enzymes, ligated, and transformed in *Escherichia coli*. Positive plasmids were purified from bacteria using QIAprep Spin Miniprep Kit (Qiagen). The DNA sequencing was performed by the MWG-Biotech AG Sequencing Service (Ebersberg, Germany). Yeast cells were transformed with plasmids using the lithium acetate–polyethylene glycol (PEG) method.

Results

COQ8 Overexpression Strategy to Detect CoQ Deficiency-Bypassing Molecules

The HTS approach designed to detect molecules that bypass defects of CoQ₆ synthesis in yeast takes advantage of the Q-synthome stabilization produced by *COQ8* gene overexpression. This strategy is depicted in **Figure 1**. In wild-type strains, Q-synthome is assembled and the initial complex substrate (HHB), produced by Coq2, is readily converted into CoQ₆. The lack of the monooxygenase in the null mutant *coq6Δ*, responsible for hydroxylation at position C5 of the aromatic ring, blocks the Q-synthome assembly and HHB is accumulated. Coq8 overexpression unlocks Q-synthome assembly and HHB is initially accumulated since Coq6 is the first enzyme acting in the pathway.

However, the rest of Q-synthome enzymes are present and finally convert HHB into 3-hexaprenyl-4-hydroxyphenol (4-HP₆). When a bypassing molecule is present, such as VA, it can be used to produce CoQ₆. VA is a special case of bypassing molecule; it can only bypass defects in the *COQ6* gene because it already contains the hydroxyl group at the C5 position, that is, the hydroxylation to be carried out by Coq6. In this study, we have used two models of *COQ8* overexpression, a *coq6Δ* null strain to work as a control together with VA, and a *coq4Δ* null strain. The *COQ4* gene encodes a regulatory or structural protein belonging to the Q-synthome. We expect to identify molecules restoring defects in either *coq6Δ* or *coq4Δ* with natural extracts from the Fundación MEDINA. Molecules restoring both cell models could be recognized as general bypassing molecules of CoQ₆ biosynthesis.

VA and Glycerol Growth Constitute a Valuable Approach for Detecting CoQ Deficiency-Bypassing Molecules

In order to be successful, the strategy adopted by the HTS requires identifying molecules recovering CoQ₆ biosynthesis, taking advantage of Q-synthome stabilization produced by the *COQ8* gene overexpression. CoQ₆ biosynthesis recovery will be analyzed using the growth in glycerol, a nonfermentable carbon source to activate the respiratory metabolism. In the yeast model, glycerol has been extensively used to detect respiratory-deficient mutants²⁹ and CoQ₆-deficient yeast mutants.³⁰ In this screening approach, CoQ₆ deficiency strains (*coq4Δ* and *coq6Δ*) are unable to grow in YPG, a rich medium containing glycerol as a carbon source (**Fig. 2A**). Glycerol is assimilated after the phosphorylation catalyzed by a glycerol-kinase encoded by *GUT1*,³¹ followed by oxidation to dihydroxyacetone phosphate (DHAP), driving electrons to mitochondria by Gut2.³² Gut2 introduces electrons from glycerol-phosphate to reduce CoQ₆ at the level of the succinate dehydrogenase (SDH) complex.³³ Both growth in glycerol and CoQ₆ synthesis in *coq4Δ* and *coq6Δ* mutant strains can be restored by the episomal expression of *COQ4* and *COQ6* yeast genes, and in *coq6Δ* mutant strains with VA after *COQ8* overexpression, which stabilizes the Q-synthome (**Fig. 2A–C**). *COQ8* overexpression is required in this HTS to allow the bypassing effect of molecules present in the natural extracts analyzed. The supplementation with VA is a good control for the HTS because the presence of the methoxy group on VA serves to bypass the hydroxylation step mediated by Coq6³⁴ (**Fig. 1D**). Several concentrations of VA were used to supplement cultures of the *coq6Δ:COQ8* strain with glycerol as the carbon source (**Fig. 2D–E**). In a dose–response analysis, VA restored growth in a directly proportional way with the greatest effect at 2 mM. Higher concentrations result in a

Figure 1. Biological foundations of the CoQ₆-bypassing HTS method. **(A)** In wild-type yeast (BY4741) mitochondria, proteins involved in CoQ₆ synthesis that are not a component of Q-synthome (Coq1 and Coq2) produce the first CoQ₆-like intermediate, HHB, that is readily converted into CoQ₆ by the action of Q-synthome. **(B)** In a null mutant for the *COQ6* gene (*coq6Δ*), HHB is accumulated but the lack of Coq6 blocks the Q-synthome assembly, and therefore CoQ₆ is not synthesized while HHB is converted into 4-HP₆. **(C)** *COQ8* overexpression in the *coq6Δ:COQ8* yeast strain stabilizes the Q-synthome, but the absence of Coq6 prevents the C5 hydroxylation required to produce CoQ₆. In these conditions, the existence of the Q-synthome converts HHB into 6-demethoxy coenzyme Q₆ (6-DMQ₆). **(D)** VA supplementation provided with an intermediate, hexaprenyl-2-vanillic acid (HVA), that has a hydroxyl group in the C5 position, enabling CoQ₆ synthesis in the *coq6Δ:COQ8* yeast strain in the absence of Coq6. **(E)** Structures of hexaprenyl-2-hydroxybenzoate (HHB), 4-HP₆, HVA, CoQ₆, and 6-DMQ₆.

lower growth, likely caused by a toxic effect. Two different VA concentrations (0.5 and 2 mM) were selected to analyze the effect in alternative nonfermentable carbon sources, such as ethanol (YPE) and lactate (YPL) (Suppl. Fig. S1A,B). Ethanol is converted in acetyl-CoA by alcohol dehydrogenase (Adh2), aldehyde dehydrogenase (Ald1), and acetyl-CoA synthetase (Acs1). Lactate is oxidized by L-lactate cytochrome *c* oxidoreductase (Cyb2) and D-lactate cytochrome *c* oxidoreductase (Dld1).³⁵ In all the media analyzed, VA supplementation allowed the growth of the *coq6Δ:COQ8* strain but *coq4Δ:COQ8* growth was not restored (Fig. 2F and Suppl. Fig. S1A,B, black lines), corroborating the specificity of VA to recover CoQ₆ synthesis in *coq6Δ* mutants. In all the carbon sources analyzed, 2 mM VA resulted in a more robust growth compared with 0.5 mM. Glycerol media was selected to perform HTS because the growth window was wider compared with that for ethanol and lactate, and its assimilation is directly related to CoQ₆, while the other carbon sources need it indirectly. These results supported 2 mM VA as the positive control of the HTS. This combination of conditions, YPG, *COQ8* overexpression, and 2 mM VA, was selected to perform the HTS because they are useful to recover CoQ₆ synthesis in *coq6Δ:COQ8* mutants (Fig. 2B,C).

First Screening Detection of Positive Extracts

The screening was divided into two parts; the first was designed to detect positive extracts recovering the growth in YPG, and the second was performed to detect positive fractions derived from the first screening. A scheme of the full HTS is depicted in Figure 3. In the first screening, 1200 natural product extracts from the Fundación MEDINA, arranged in 15 microplates with 80 extracts per plate, were tested in duplicate against the *coq6Δ:COQ8* yeast strain, during 120 h with shaking at 30 °C. One extract was considered positive when both replicas grew in YPG with a final absorbance that was at least double the initial absorbance. The initial screening resulted in 29 positive extracts (Table 1) on nine plates. These plates were tested in similar conditions against the *coq4Δ:COQ8* yeast strain, resulting in 32 positive extracts (Table 1). Positive extracts from both yeast strain screenings in the first screening were collected (cherry-picking) in a new microplate for their analysis in similar screening conditions, which resulted in 11 positive extracts for the *coq6Δ:COQ8* yeast strain and 12 for the *coq4Δ:COQ8* yeast strain. Nine of these extracts were common extracts for both yeast strains. The raw absorbance data of these nine positive extracts and from negative controls are shown in Table 2.

Figure 2. Glycerol growth and VA supplementation are basic components of the CoQ₆-bypassing HTS method. **(A)** The indicated strains were cultured in SDc-ura glucose media for 24 h with shaking at 30 °C. Cell solutions prepared at 2 OD units/mL were diluted three times (1/10) sequentially in sterile water. YPD and YPG plates were used to deposit 3 μ L of the initial solution and its three dilutions. Plates were incubated for 48 h at 30 °C. **(B)** Total homogenates of the indicated yeast strains were subjected to CoQ₆ extraction, separation, identification, and quantification by HPLC-ECD. Data are expressed as pmol of CoQ₆/mg protein·min and correspond to the average $\pm$ SD of two independent extractions. **(C)** Chromatograms corresponding to the analysis in panel B used to quantify CoQ₆ from yeast homogenates. The structure of two molecules is indicated in the figure, CoQ₆ at the right and 6-DMQ₆ at the left. **(D)** The yeast strain *coq6Δ:COQ8* was used to inoculate YPG media at 0.1 OD units/mL in a microplate and was supplemented with the indicated amounts of VA. The plate was incubated for 120 h at 30 °C with shaking. Cell growth was monitored following absorbance at 600 nm in continuous mode. Data correspond to the average $\pm$ SD of 12 wells, four replicas of three different colonies. **(E)** The slope of the growth curve measured in the linear phase of previous dose–response analysis (panel D). Data correspond to the average $\pm$ SD of 12 wells, four replicas of three different colonies. *Data are significantly different from other data, $p < 0.001$. **(F)** BY4741, *coq6Δ*, *coq4Δ*, *coq6Δ:COQ8*, and *coq4Δ:COQ8* strains with or without supplementation with VA (0.5 and 2 mM) were inoculated in a microplate with YPG media, at 30 °C with shaking for 144 h. Cell growth was monitored following absorbance at 600 nm in continuous mode. All strains and conditions showing no growth are indicated by the black curve. Data correspond to the average $\pm$ SD of 15 wells, five replicas of three different colonies.

Second Screening with Fractionation of Positive Extracts Detected Molecules with CoQ₆-Bypassing Activity

The first screening resulted in the finding of nine extracts common to both strains analyzed. Three of them (UPO-04, UPO-05, and UPO-06) were subjected to chromatographic fractionation into 80 fractions arranged in microplates that were used in the second screening (Fig. 3). All extracts analyzed can restore the growth in YPG in CoQ₆-deficient strains, *coq6Δ* and *coq4Δ* only after the *COQ8* gene overexpression, indicating that Q-synthome assembly is a required condition to support respiratory growth (Suppl. Fig. S2A–C). The ability

of positive extracts to restore YPG growth in CoQ₆-deficient yeast strains is also extended to other mutants, such as the case of *coq7Δ* (Suppl. Fig. S6A). Fractionation plates of selected extracts were probed against the *coq6Δ:COQ8* yeast strain using similar conditions to those used in the screening, with the exception that the growth was monitored in a continuous mode. Results are shown in Table 1. In the UPO-04 extract, eight positive fractions were found, two of them with better results than the positive control (Fig. 4A and Suppl. Fig. S3). In the UPO-05 extract, nine positive fractions were found (Fig. 4B and Suppl. Fig. S4). In the UPO-06 extract, eight positive fractions were found (Fig. 4C and Suppl. Fig. S5). Positive fractions (25) were collected and placed in a common

Figure 3. Flowchart of the CoQ₆-bypassing HTS method. The first screening performed cell growth measurement in duplicate by an endpoint method using the *coq6Δ:COQ8* yeast strain. Extract plates with positive results were probed against the *coq4Δ:COQ8* yeast strain. An extract was considered positive when the final OD of the culture was at least double the initial OD. The Z' score as a robustness indicator must be above 0.85. Positive extracts were selected and allocated together in a new plate that was probed in duplicate (cherry-picking analysis). Three positive extracts common for both strains (*coq6Δ:COQ8* and *coq4Δ:COQ8*) were selected for chromatographic fractionation. In the second screening, fractions were probed against *coq6Δ:COQ8* in duplicate over 120 h and monitored in continuous mode. Positive fractions were collected and probed in the same conditions against the *coq4Δ:COQ8* strain. Common positive fractions (11) were analyzed by LC-MS to identify new or already known molecules (5).

microplate to be probed against the *coq4Δ:COQ8* yeast strain (Fig. 3) in similar conditions to those described for *coq6Δ:COQ8* yeast strain screening. Eleven fractions were common positives for both yeast strains, two from the UPO-04 extract (Fig. 4D and Suppl. Fig. S3), seven from the UPO-05 extract (Fig. 4E and Suppl. Fig. S4), and two from the UPO-06 extract (Fig. 4F and Suppl. Fig. S5). Yeast grown with common positive fractions was collected from the microplate and pooled together to detect CoQ₆ presence. HPLC-ECD analysis demonstrated that positive fractions recovered the

growth in YPG, restoring CoQ₆ synthesis to an extent that is comparable to that of wild-type yeast cells (Suppl. Fig. S6B).

Identification of Potential Bypassing Molecules with Mass Spectrometry

Common positive fractions (11) collected from fractionated extracts were subjected to LC-MS analysis. The identification resulted in the finding of two possible active molecules with a known structure and three molecules (Suppl. Table

Table 1. Summary of Screening Results.

Analysis	Extracts Analyzed	Positive	Z' Score Max	Z' Score Min
BY4741 <i>coq6Δ:COQ8</i>	1200	29	0.99	0.93
BY4741 <i>coq4Δ:COQ8</i>	720	32	0.98	0.92
Cherry-picking BY4741 <i>coq6Δ:COQ8</i>	29	11	0.97	0.97
Cherry picking BY4741 <i>coq4Δ:COQ8</i>	32	12	0.97	0.96
Common Extracts	Number			
Total	9			
Fractionated	3			
Fractionated Extracts	Fractions	Common Hits	Z' Score	
UPO-04	80	2	0.95	
UPO-05	80	7	0.95	
UPO-06	80	2	0.96	

Table 2. Raw Absorbance of Common Positive Extract.

Extract		Initial Analysis				Cherry-Picking Analysis			
Assay	I	2	VA 0.5 mM	VA 2 mM	I	2	VA 0.5 mM	VA 2 mM	
Analysis in <i>coq6Δ:pCM189-COQ8</i> ^a									
UPO-01	0.324	0.323	0.239	0.703	0.325	0.325	0.231	0.689	
UPO-02	0.398	0.398	0.239	0.703	0.404	0.404	0.231	0.689	
UPO-03	0.359	0.359	0.239	0.703	0.359	0.359	0.232	0.689	
UPO-04	0.312	0.312	0.239	0.703	0.330	0.332	0.232	0.689	
UPO-05	0.336	0.335	0.239	0.703	0.313	0.312	0.232	0.689	
UPO-06	0.346	0.345	0.239	0.703	0.337	0.335	0.232	0.689	
UPO-07	0.360	0.360	0.227	0.695	0.358	0.357	0.240	0.690	
UPO-08	0.390	0.390	0.227	0.695	0.400	0.400	0.240	0.690	
UPO-09	0.375	0.370	0.227	0.695	0.343	0.340	0.240	0.690	
Analysis in <i>coq4Δ:pCM189-COQ8</i> ^b									
UPO-01	0.310	0.310	0.234	0.684	0.309	0.310	0.241	0.684	
UPO-02	0.295	0.295	0.234	0.684	0.282	0.282	0.241	0.684	
UPO-03	0.291	0.291	0.234	0.684	0.284	0.281	0.240	0.684	
UPO-04	0.301	0.302	0.234	0.684	0.324	0.320	0.240	0.684	
UPO-05	0.316	0.319	0.234	0.684	0.317	0.317	0.240	0.684	
UPO-06	0.335	0.334	0.234	0.684	0.322	0.322	0.240	0.684	
UPO-07	0.354	0.353	0.224	0.682	0.323	0.323	0.224	0.679	
UPO-08	0.334	0.332	0.224	0.682	0.312	0.312	0.224	0.679	
UPO-09	0.344	0.344	0.224	0.682	0.344	0.340	0.224	0.679	

^aAverage $\pm$ SD of negative control (without extracts): 0.1052 ± 0.0036 ($n = 36$).

^bAverage $\pm$ SD of negative control (without extracts): 0.1316 ± 0.0057 ($n = 36$).

SII) not present in the MEDINA proprietary database of microbial metabolites or in the Chapman & Hall Dictionary of Natural Products (v25.1). All the molecules detected, as well as the mass spectrometry analysis, is detailed in **Supplemental Table SII**.

Discussion

The new strategy described in this study tried to configure an HTS approach to detect molecules produced by bacteria or fungi bypassing CoQ₆ deficiency. This activity is the ability to act as an intermediary for the CoQ₆ biosynthetic complex or Q-synthome in null mutants when the complex is stabilized by *COQ8* gene overexpression. Despite the results obtained corresponding to a pilot study, the strategy followed proved that this HTS method will be useful to identify new and universal molecules bypassing CoQ₆ deficiency.

The effectiveness of this HTS approach could be evaluated by previously known CoQ-bypassing molecules such as VA,^{16,36} which supports the growth of the *coq6Δ:COQ8* yeast strain in nonfermentable carbon sources at 2 mM. The specific design of this approach makes it possible to detect positive extracts recovering the growth in mutant yeasts

that are defective in one specific enzyme, such as Coq6,³⁴ which are also effective in mutants of a regulatory protein required for the right assembly of Q-synthome, such as Coq4.³⁷ This property opens the possibility of detecting CoQ-bypassing molecules as therapeutic agents useful for patients with CoQ₁₀ deficiency harboring mutations in all genes involved in CoQ₁₀ synthesis. Several experimental pieces of evidence support this possibility. The crude extracts analyzed (UPO-04, UPO-05, and UPO-06) restore the growth in *coq6Δ* and *coq4Δ* mutants only after *COQ8* expression, indicating that Q-synthome is required for the extract's action. The requirement of Q-synthome allows us to discard that the growth in YPG was supported by some type of CoQ molecule present in the extract. Other evidence reinforces the lack of CoQ in the extracts; extracts were obtained with an acetone–water mixture, which is not useful to purify molecules with the polarity of CoQ, and CoQ molecules were not detected in the mass spectrometry analysis performed in the positive fractions of the extracts analyzed. The UPO-06 crude extract not only restores the growth in glycerol media for *coq4Δ:COQ8* and *coq6Δ:COQ8* yeast strains but also is able to recover the growth in the *coq7Δ:COQ8* strain, supporting the idea that the HTS described here is useful for identifying molecules

Figure 4. Growth curves corresponding to the second screening of CoQ₆-bypassing molecules with fractionated extracts. Plates containing fractionated positive extract (80 samples plus controls) were probed against the *coq6Δ:COQ8* yeast strain in duplicate for 120 h with shaking at 30 °C. Yeast growth was monitored measuring OD at 600 nm in continuous mode. Data correspond to the average $\pm$ SD of both replicas. Only positive wells and controls were included in the plot. Names of fractions correspond to the well position: (A) UPO-04 extract, (B) UPO-05 extract, and (C) UPO-06 extract. Positive fractions obtained with the *coq6Δ:COQ8* yeast strain were probed against the *coq4Δ:COQ8* yeast strain using similar growth conditions. Data correspond to the average $\pm$ SD of both replicas. Only positive wells and controls were included in the plot. Names of fractions correspond to the well position: (D) UPO-04 extract, (E) UPO-05 extract, and (F) UPO-06 extract.

with a universal bypassing action. The definitive evidence of a molecule bypassing CoQ₆ deficiency is the ability to synthesize CoQ₆ in deficient cells. CoQ₆ was detected in the pooled yeasts grown with the positive fractions from the second screening. This means that not only the growth in YPG has been restored, but also CoQ₆ biosynthesis is in operation at a wild-type level.

The strength and robustness of this HTS can be deduced by the extrapolation of results obtained from the full collection of natural extracts of the Fundación MEDINA. This Natural Products Library is composed of more than 200,000 extracts, from which only a representative subset of 1200 have been used in this study. Despite the stringency parameter selected in the HTS design, that is, eight replicas per positive extract analyzed, the minimal growth threshold, and the Z' score measured for all plates, from three positive extracts, 11 fractions with CoQ₆-bypassing activity were detected and 5 potential molecules were identified by LC-MS. It is not possible to perform a simple extrapolation, but these results support the potential of the HTS described in this study.

With this limited number of fractionated positive extracts, five molecules have been detected. Even though we have found a high number of common positive fractions (11 in three positive extracts), the number of identified

molecules is lower. This apparent low efficiency can be explained by the existence of contiguous positive fractions showing the same molecule, and that the three fractionated extracts belong to phylogenetically related organisms or to the same microorganisms growing under different culture conditions.

The future use of this HTS approach will require increasing the number of natural extracts to be analyzed. However, in the short term, the analysis of the remaining six positive extracts, the identification of the active molecules present in all the extracts, and the test in cellular models derived from patients' fibroblasts affected by CoQ₁₀ deficiency will allow us to obtain data of the real therapeutic capacity of the identified molecules. Despite the differences between human and yeast, the high similitude in the CoQ biosynthetic machinery⁹ and the existence of known molecules working in both models as bypassing agents, such as VA or dihydroxybenzoic acid,^{14,18,19} support the hypothesis that molecules identified in this study could also be effective in humans.

A larger number of positive extracts detected is not an additional complication, but rather the opposite since it will enable the introduction of new yeast mutants in the first screening, in order to decrease the number of common positive extracts while improving the versatility of the molecules identified to restore the CoQ₁₀ synthesis in patients. A

second benefit of extending the analysis to more natural extracts is to eliminate the effect produced by the potent metabolic capacity of the yeast compared with the human model. Compounds with bypassing capacity in yeast may not work properly in humans and may even produce harmful effects since human cells do not have the appropriate enzymatic machinery to redirect the target molecule to a metabolite involved in the CoQ₁₀ synthesis pathway.

The primary CoQ₁₀ deficiency is a rare disease. Recent studies have estimated that the number of potential cases worldwide is 1665 taking into account the known pathogenic variants, or even 123,789 when predicted pathogenic variants are also included in the prediction study,³⁸ supporting the idea that primary CoQ₁₀ deficiency is an underdiagnosed disease. It is also necessary to take into account patients with secondary CoQ₁₀ deficiency, for which the number of potential cases is still impossible to estimate. Both primary and secondary CoQ deficiency produce common clinical symptoms, and patients can be treated with CoQ₁₀ supplementation. This therapy has showed some cases of success in the past³⁹ but in general is ineffective; CoQ₁₀ shows a poor uptake by most affected organs (skeletal muscle and nervous system), and only crosses the blood–brain barrier with larger doses.⁴⁰ The use of CoQ-bypassing molecules, such as the ones that have been identified in this study, circumvents most CoQ₁₀ supplementation defects. They are small and polar molecules that are able to cross the blood–brain barrier and usually are non-toxic and cheaper. The requirement of the Q-synthome assembly is not an obstacle, since patients maintain the ability to produce CoQ₁₀. The molecular similarities of the CoQ synthesis processes between yeast and humans open the possibility that molecules identified in a more ambitious HTS than that based in this pilot study could be potential therapeutic agents to combat CoQ₁₀ deficiency in humans, a mitochondrial rare disease that does not have a definitive and universal therapeutic strategy.

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