The MFS-type efflux pump Flr1 induced by Yap1 promotes canthin-6-one resistance in yeast

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Abstract

Screening for suppressors of canthin-6-one toxicity in yeast identified Yap1, a transcription factor involved in cell response to a broad range of injuries. Although canthin-6-one did not promote a significant oxidative stress, overexpression of YAP1 gene clearly increased resistance to this drug. We demonstrated that Yap1-mediated resistance involves the plasma membrane major-facilitator-superfamily efflux pump Flr1 but not the vacuolar ATP-binding-cassette transporter Ycf1. FLR1 overexpression was sufficient to reduce sensitivity to the drug, but strictly dependent on a functional YAP1 gene.

1. Introduction

Canthin-6-one alkaloids are naturally produced by plants belonging to the Simaroubaceae, Zygophyllaceae and Rutaceae families [1–3]. They exhibit a broad spectrum of antimicrobial [4] and antifungal activities [3]. Antiparasitic properties have also been reported [5–7] and decoctions of canthin-6-one producing plants are used in the treatment of malaria and leishmaniasis in South America. Traditional medicine also uses these natural products as antipyretic agents and for the treatment of gastric ulcers and diarrhea [8]. One of these compounds, 1-methoxy-canthin-6-one, displayed cytotoxic and antiproliferative effects in human cell cultures [9] and induced apoptosis via a JNK-dependent mechanism [10]. Considering this wide range of biological activities as well as their very low toxicity on normal cells, these molecules may represent a potent alternative to current chemotherapies that display numerous drawbacks. Yet very little is known about the mechanism of action of these molecules, their cellular targets, nor the way cells may cope with them to reduce or suppress their efficacy. A link between the antifungal activity of canthin-6-one and fatty acid metabolism has been reported [11] in the yeast Saccharomyces cerevisiae: upon treatment with a sub-inhibitory concentration of the drug, the proportion of unsaturated fatty acids tended to increase, more particularly 9-hexadecenoate (C16 :1), suggesting that canthin-6-one stimulated desaturase enzyme systems. This hypothesis was not addressed, however, nor was the status of the sole alkyl chain fatty acid desaturase of yeast (Ole1) examined after drug treatment. Consistent with a putative effect on lipid metabolism, a fluorescent derivative of canthin-6-one co-localized with cytoplasmic lipid droplets after rapid uptake by yeast cells [11]. This subcellular localization, if confirmed in human cells, may be in relation with the analgesic, antipyretic and anti-inflammatory effects of these drugs. Indeed cyclooxygenase-2 (COX-2) and prostaglandin-E2 (PGE2) synthesis involved in the inflammatory response and responsible for the elevated body temperature and fever was shown to reside in lipid bodies of colon cancer cells [12]. Thus one can speculate that these molecules when accumulated in lipid droplets may interfere with PGE2 production. This hypothesis is not in contradiction with a recent study showing that anti-ulcer activity of canthin-6-one does not involve PGE2 synthesis in animal models whereas treatment with this drug rather resulted in a mild decrease of PGE2 production [8]. Considering that inhibition of PGE2 synthesis with aspirin significantly reduced cell proliferation of the CACO-2 colon cancer cell line [12], this putative effect of canthin-6-one derivatives on PGE2 production would certainly deserve to be addressed.

In order to get a better insight into the mechanism of action of these drugs, we screened a yeast genomic library in S. cerevisiae to isolate high-copy-number suppressors of canthin-6-one sensitivity. Among the genes selected with this strategy we isolated the
yeast AP1 homologue (YAP1), a gene involved in cell response to various stress, more particularly oxidative stress [13,14], and pleiotropic drug resistance in high-copy number [15,16]. More recently, the involvement of Yap1 in response to genotoxic insults has also been reported [17]. In this study we showed that plasmid-borne overexpression of YAPI leads to canthin-6-one resistance via the major-facilitator-superfamily (MFS) transporter Flr1 involved in the efflux of a broad range of cytotoxic molecules [15,18,19].

2. Materials and methods

2.1. Yeast strains, culture conditions and plasmids

All S. cerevisiae strains used in this study are listed in Table 1. Cells were plated on YPD complete medium and strains containing plasmids were plated on YNB minimal medium supplemented with appropriate nutrients for maintenance of plasmids and grown routinely at 28 °C. Culture media containing canthin-6-one were buffered at pH 6 with sodium phosphate. The yeast genomic DNA library constructed in YEp13 was a kind gift from Dr. Roger Schneiter (Fribourg University). Plasmids pRS426-Yap1-Myc for overexpression of YAP1, pRS314-GFP-Yap1 allowing production of a GFP-tagged Yap1 and pFLR1-K2 for overexpression of FLR1 were generously provided by Dr. Michel Toledano (Commissariat à l’Energie Atomique et aux Energies Alternatives, France), Dr. Yoshiharu Inoue (Kyoto University) and Dr. Helmut Jungwirth (Graz University) respectively.

2.2. Chemicals

Canthin-6-one was provided by Alpha Chimica (Châtenay-Malabry, France). It was prepared in DMSO at 80 mM and stored at −20 °C.

2.3. Measurement of growth inhibition by canthin-6-one

To study the effects of canthin-6-one on growth rate, strains were pre-grown overnight and then inoculated in fresh medium to an OD600nm of 0.2 with canthin-6-one concentration of 20, 40 or 80 μM and DMSO 0.2% in control conditions. Growth rate was monitored by recording absorbance over time (OD600 nm).

2.4. Spotting assays

For spotting assays, single colonies of S. cerevisiae from plate cultures were suspended in sterile water, serially diluted (10−2, 10−3, 10−4, 10−5) and spotted on YPG pH 6 containing 10, 20, 30 and 40 μM canthin-6-one or 0.2% DMSO for control conditions and grown for 5–7 days at 28 °C.

2.5. RNA extraction

Three independent cultures of wild-type strain with and without the plasmid for YAPI overexpression were pre-grown overnight and then inoculated in fresh medium to an initial OD600nm of 0.1. When cultures reached 0.5 OD/ml they were treated for 1 h with 40 μM canthin-6-one or 0.2% DMSO for control conditions. Total RNA extraction was performed as previously described by Li et al. [20]. Briefly, cells (equivalent to 2.5 OD600nm units) were collected by centrifugation and washed in water before suspension in isolation buffer (10 mM EDTA; 50 mM Tris–HCl, pH 6). Then, cells were lysed by incubation for 5 min at 65 °C. The cell lysate was immediately placed on ice before adding 200 μl of 0.3 M KCl. Supernatant was recovered by centrifugation (12000 rpm for 10 min at 4 °C) and total RNA was extracted with phenol/chloroform/isooamyl alcohol (25:24:1). RNA precipitation was performed overnight at −80 °C in 3 M sodium acetate pH 5.2 (0.1 vol) and isopropanol (2.5 vol). RNA pellet was recovered by centrifugation (13000 rpm for 20 min at 4 °C), washed with 70% cold ethanol, dried at room temperature and resuspended in DEPC-treated water. RNA quantification was performed by absorbance analysis at 260 nm and adjusted to 0.25 μg/μl. For CDNA synthesis, 1 μg of total RNA was used for RT with 50 ng random hexamers (GE Healthcare Europe, Saclay, France) and Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT; Promega) for 2 h at 37 °C. The RT reaction was terminated on ice by 1/5 dilution in double-distilled water.

Table 1

<table>
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<th>Strain</th>
<th>Genotype</th>
<th>Characteristics</th>
<th>Origin</th>
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<td>WT-C176</td>
<td>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0</td>
<td>WT strain derived from S288c</td>
<td>EUROSCARF (Y10000)</td>
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<td>ybp1Δ</td>
<td>MATα his31 leu2Δ0 lys20 ura3Δ0 YBR216c:kanMX4</td>
<td>Deleted in the Yapi-Binding Protein. S288c background</td>
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<td>gpx3Δ</td>
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<td>Deleted in Gpx3 thiol peroxidase. S288c background</td>
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<td>ycf1Δ</td>
<td>MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 YDR135C:kanMX4</td>
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<td>fpr1Δ</td>
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<td>Deleted in the plasma membrane transporter Fluconazole Resistance 1. S288c background</td>
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<td>pRS426-Yap1-Myc</td>
<td>Overexpression of myc-tagged YAPI. Derived from pRS426 (2 μ origin, multicopy, URA3 marker)</td>
<td>Gift from Dr. Roger Schneiter (Fribourg University)</td>
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<td>pRS314-GFP-Yap1</td>
<td>Expression of GFP-tagged YAPI. Derived from pRS314 (CEN6/ARS4, monocopy, TRP1 marker)</td>
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<tr>
<td>pFLR1-K2</td>
<td>Expression of FLR1. Derived from YEp351 (2μ origin, multicopy, LEU2 marker)</td>
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<td>pHDEL-RFP</td>
<td>Expression of HDEL-RFP, marker of the nuclear envelop/ER membrane (URA3 marker)</td>
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2.6. Real-time qPCR

The coding regions of the target genes were recovered from Yeast Genome Database (http://www.yeastgenome.org/). The primer design was undertaken by the on-line Genscript Primer Design in advanced mode (http://www.genscript.com/cgi-bin/tools/primer_genscript.cgi) using the following parameters: size of the primers between 20 and 25 bases, $T_m$ values of 59°C and amplicon size between 80 and 110 bp. The primer pairs were verified with BLAST (http://www.blast.org) and by in silico PCR (http://genome.ucsc.edu/cgi-bin/hgPcr) using the genome of S. cerevisiae as a template. Primers pairs used in this study (listed in Table 2) were validated as follows: they gave a single PCR product as verified by melting curve analysis and agarose gel electrophoresis; the distribution of PCR sigmoids was linear ($r > 0.99$) over 5 logs of template concentration with an efficiency of 1.85–1.98. The primers were provided by Invitrogen™ (Life Technologies, France). Real-time qPCR was performed on a LightCycler® (Roche Applied Sci-

<table>
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<th>Primers R</th>
<th>qPCR product (bp)</th>
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<td>AGGCGACATTTTTATGACC</td>
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<td>TRX2</td>
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<td>TCATTTATGCATGCCACAC</td>
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Table 2: Primers used for real time qPCR.

Fig. 1. Selection of YAP1 as a suppressor of canthin-6-one inhibition. (A) Sensitivity of G175, transformed with empty plasmid [YEp13], was compared to canthin-6-one resistant (CR) transformants selected from the library. G175 and CR clones were spotted on a medium containing 20 μM canthin-6-one. (B) G175 overexpressing YAP1 was compared to the reference strain YPH250 by spotting cell suspensions on a medium containing 10–30 μM canthin-6-one. (C) Growth of YAPI-deleted strain yap1Δ was compared to the isogenic strain YPH250 in liquid medium with 40 μM canthin-6-one and their sensitivity were tested by spotting them on a medium with 10–30 μM canthin-6-one. In all cases, the control condition refers to a medium containing 0.2% DMSO.
ence, Meylan, France) detection system using SYBR® Green mix (Takara-Ozyme, Montigny-le-Bretonneux, France). The amplification conditions were: enzyme activation step at 95 °C for 30 s followed by 45 cycles comprising 10 s at 95 °C, 10 s at 60 °C, and 10 s at 72 °C. PCR reactions on non-reverse-transcribed RNA samples from each condition produced no detectable amplification, thus verifying the absence of genomic DNA contamination. ALG9 was used as reference gene as described by Teste et al. [21]. Reverse transcripts copy numbers were calculated from standard curves established with known quantities of each template. Results are expressed as the ratio of number of copies of the target template per $10^5$ copies of ALG9 template following 40 μM canthin-6-one treatments versus 0.2% DMSO (fold increase).

Quantitative data are expressed as mean ± standard error of the mean (±S.E.M.) of 3 independent experiments. The difference between groups was analyzed by a paired Student’s t-test and was considered significant when $P < 0.05$.

3. Results and discussion

3.1. High-copy-number suppression of canthin-6-one sensitivity

A strategy in yeast to isolate cellular targets of a given toxic molecule consists in overproduction of the target itself or of genes involved in its production. Indeed the use of a library of wild-type yeast genomic DNA in a high-copy-number plasmid allows increased gene dosage in order to modify the stoichiometry between the target and the toxic molecule thereby reducing its effect(s). This approach may also lead to the identification of an alternative route(s) to the pathway inhibited by the molecule. A decrease in drug efficiency may also result from an enhanced degradative process, or from the activation of efflux pumps, both mechanisms leading to lower intracellular concentration of the toxic molecule. Although these two latter processes do not provide information on the mechanism of action of the drug, their identification may be important for future therapeutic development of this drug. The screening of the library was performed with the G175 reference strain on a medium containing 20 μM canthin-6-one. We noticed that growth was more severely affected on solid medium than in liquid medium (not shown) and that less sensitive clones appeared at a relatively high frequency (illustrated in Fig. 1A and B, see G175[Yep13]). G175 was transformed to Leu+ with the library DNA (LEU2 marker, 2 μ origin, mean insert size 5–6 kbp) and the transformants (about 25000) growing on 20 μM canthin-6-one were selected. Among a total of nine clones still significantly positive after replica plating (Fig. 1A), partial sequencing of the inserts allowed us to reduce the number to four groups. One of them (CR1 for canthin-resistant 1) harbored a
4580 kbp genomic DNA insert with two complete coding sequences (YAPI and YML007C-A) and two truncated ones (ERG6, GIS4). YML007C-A refers to an uncharacterized ORF encoding a short putative peptide of 36 amino acids with unknown function.

On the other hand, YAPI encodes the well-characterized yeast AP1 homologue, a bZIP transcription factor [13] and we estimated that this gene would be the best candidate to focus on. G175 was transformed with a high-copy-number plasmid harboring YAPI and the transformants were plated on canthin-6-one containing medium. Colonies grew in the presence of canthin-6-one up to 30 μM concentration (Fig. 1B), leading to the conclusion that overexpression of this transcription factor renders yeast resistant to this drug.

In order to determine whether YAPI-mediated pathway might be naturally involved in the cell response to canthin-6-one, we assessed sensitivity to this drug in a YAPI deleted background. YAPI inactivation impaired growth under normal conditions (Fig. 1C), thus we could not firmly discriminate whether the slight increase of sensitivity was due to the lack of YAPI or to growth reduction.

3.2. Canthin-6-one and oxidative stress

The involvement of Yap1 in oxidative stress response has been extensively studied and documented [13,14]. Yap1 shuttles between the cytoplasm and the nucleus, and its nuclear localization is determined by its cysteine-rich domain (CRD) whose oxidized/reduced status influences the conformation of the protein [22]. Ybp1 (Yap1-binding protein) is required for oxidation of the cysteine residues of Yap1 CRD, and thus is involved in the nuclear localization of the transcription factor in response to stress [23]. In this process, Hyl1/Gpx3, a glutathione peroxidase (GPx)-like enzyme, acts as the sensor of intracellular hydroperoxide levels. In response to peroxide stress, Hyl1/Gpx3 forms a disulfide bond in the CRD of Yap1 with the help of Ybp1 [24].

The cellular localization of a GFP-tagged version of Yap1 was assessed after exposure of the yap1Δ strain to canthin-6-one (1 h after treatment with 200 μM canthin-6-one). Although a treatment of the cells with H2O2 used as an oxidative stress control led to a strong and unambiguous nuclear localization, Yap1-GFP did not accumulate in the nuclear compartment following canthin-6-one treatment (Fig. 2A). Moreover, it has been reported that YAPI expression is induced 20 min after exposure to 0.3 mM H2O2 in response to the oxidative stress [25]. Real-time qPCR was performed with RNA extracted from cells exposed to canthin-6-one (1 h after exposure to 40 μM canthin-6-one). As shown in Fig. 2B, the transcription level of YAPI was not modified. We also measured by real-time qPCR whether some of the known Yap1 targets were induced upon canthin-6-one treatment. GSH1 and TRX2 are known to be induced by Yap1 in response to oxidative stress [26,27], whereas FLR1 and YCF1 encode plasma membrane and vacuolar efflux pumps respectively that are involved in the detoxification of a wide variety of xenobiotics [15,18,28–30]. We observed that canthin-6-one exposure did not induce significantly (P > 0.05) GSH1 nor YCF1. By contrast, FLR1 and TRX2 were significantly induced, but only by a factor of 2.2 and 2.6, respectively, (Fig. 2B). Altogether this data indicate that canthin-6-one does not promote an oxidative stress comparable to that obtained by H2O2. Despite this low level of transcriptional activation of FLR1 and TRX2, we checked the effect of addition of N-acetyl-cysteine or reduced glutathione (10 mM each) to the culture medium. We observed a reduction of the sensitivity of the three control strains used throughout this study (Fig. 2C), suggesting that the redox state of the cells exposed to canthin-6-one might be affected by the drug.

Finally we examined whether YAPI mediated resistance requires Ybp1 and/or Gpx3. As depicted on Fig. 2D, deletion of any of these genes failed to abolish the effect of YAPI overexpression. Thus plasmid-borne overproduced Yap1 might be sufficient to ensure nuclear accumulation of the transcription factor thereby rendering the assistance of these factors unessential.

Fig. 4. Canthin-6-one resistance mediated by YAPI overexpression is exerted mainly by Flr1 and not by Ycf1. (A) Sensitivity of strains deleted for FLR1 or YCF1 and overexpressing YAPI was tested on medium with 30 μM or 40 μM canthin-6-one. (B) Sensitivity of flr1Δ was compared to the isogenic strain Y10000 by recording their growth in liquid medium in the presence of 40 μM canthin-6-one or in control conditions (0.2% DMSO).
3.3. Role of efflux pumps (target of Yap1) in canthin-6-one resistance

By comparison to the 20-fold increase in FLR1 transcription in the presence of benomyl \[18\], this transcript was only moderately induced upon canthin-6-one exposure (albeit significantly). We thus examined whether this efflux pump could play an active role in Yap1-mediated resistance. Ycf1 is a vacuolar efflux pump whose gene is positively regulated by Yap1 \[30\]. The transcription level of FLR1 and YCF1 was examined by real-time qPCR in a strain overproducing the plasmid-borne YAP1 [31] in the case of diazaborine resistance mediated by Yap1, FLR1 inactivation completely abolished this effect, demonstrating that this drug: H+ antiporter of the plasma membrane was indeed acting downstream of YAP1 to mediate resistance to the drug \[Fig. 4A\]. However, we observed that sensitivity to canthin-6-one was not increased in the FLR1-deleted strain as compared to the control Y10000 \[Fig. 4B\] showing that the basal production of this protein is not sufficient to counteract the effects of canthin-6-one. This observation indicates that this efflux pump is not naturally involved in adaptation of wild-type yeast to reduce the toxicity of this drug or alternatively that additional efflux pump(s) could be involved in the extrusion of canthin-6-one.

We then checked the effect of FLR1 overexpression both in a wild-type background and in a YAPI-deleted background. As depicted in \Fig. 5\, plasmid-borne overproduction of Flr1 led to canthin-6-one tolerance, up to 30 μM. This effect was fully dependent on Yap1 since it was totally abolished in the YAPI-deleted strain \[Fig. 5\]. A similar observation was reported by Jungwirth et al. \[31\] in the case of diazaborine resistance mediated by the Flr1 efflux pump, showing that the transcription factor Yap1 is essential for full activation of FLR1 transcription \[19\].

3.4. Summary

This study did not provide novel clues about the mechanism of action of the alkaloid canthin-6-one. Although FLR1 and TRX2, two well characterized Yap1 target genes, were moderately but significantly induced upon treatment, our data show that this drug does not generate a significant oxidative stress. However, considering that addition of N-acetyl-cysteine or reduced glutathione decreased sensitivity, the effect of this drug on the redox state of the cells would deserve further investigation. Given that deletion of YAPI resulted in a slight increase of sensitivity, a role of this transcription factor in the intrinsic resistance to canthin-6-one is not totally excluded. Additionally we demonstrated that the MFS-type transporter Flr1 is able to reduce the sensitivity to this drug when overproduced via a higher gene dosage, and that Flr1-mediated tolerance to the drug is strictly dependent on the transcription factor Yap1. Thus although the Yap1-Flr1 pair is not naturally involved in yeast tolerance to canthin-6-one, this study demonstrates that their overexpression can lead to resistance to the chemical stress generated by this drug.

References


\[6\] Ferreira, M.E. et al. (2007) Effects of canthin-6-one alkaloids from Zanthoxylum chiloperone on Trypanosoma cruzi-infected mice. J. Ethnopharmacol. 109 (2), 258–263.


\[8\] de Souza Almeida, E.S. et al. (2011) Pharmacological mechanisms underlying the anti-ulcer activity of methanol extract and canthin-6-one of Sinuha ferruginea A. St-Hil in animal models. J. Ethnopharmacol. 134 (3), 630–636.


