Antifungal compound honokiol triggers oxidative stress responsive signalling pathway and modulates central carbon metabolism

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ABSTRACT

The fast growing evidences have shown that the plant-derived compound honokiol is a promising candidate for treating multiple human diseases, such as inflammation and cancer. However, the mode-of-action (MoA) of honokiol remains largely unclear. Here, we studied the antifungal activity of honokiol in fission yeast model, with the goal of understanding the honokiol’s mechanism of action from the molecular level. We found that honokiol can inhibit the yeast growth at a dose-dependent way. Microarray analysis showed that honokiol has wide impacts on the fission yeast transcription levels (in total, 512 genes are up-regulated, and 42 genes are down-regulated). Gene set enrichment analysis indicated that over 45% up-regulated genes belong to the core environmental stress responses category. Moreover, network analysis suggested that there are extensive gene–gene interactions amongst the co-expression gene lists, which can assemble several biofunctionally important modules. It is noteworthy that several key components of central carbon metabolism, such as glucose transporters and metabolic enzymes of glycolysis, are involved in honokiol’s MoA. The complexity of the honokiol’s MoA displayed in previous studies and this work demonstrates that multiple omics approaches and bioinformatics tools should be applied together to achieve the complete scenario of honokiol’s antifungal function.

INTRODUCTION

Although the first characterisation of honokiol from Magnolia obovata was reported in 1972 (Maruyama and Kuribara 2006), this natural product actually has been widely used in traditional medicine in China, Japan and Korea for a long time (Maruyama and Kuribara 2006; Lee et al. 2011). Honokiol started to capture attention in recent 20 years mainly because of the finding of its promising therapeutic potential to treat multiple human diseases (especially for tumour and thrombus) (Fukuyama et al. 2002; Hu et al. 2005; Arora et al. 2012). Compared with the gradually accumulated knowledge from clinical applications, nevertheless, the understanding of the honokiol’s mode-of-action (MoA) at the molecular levels still remains largely unclear.

Earlier studies indicated that honokiol can target to multiple intracellular pathways depending on the specific disease model used (Fried and Arbiser 2009). For instance, honokiol displayed clear pro-apoptotic activity against sarcoma, melanoma, leukaemia, myeloma and colon cancer cell lines, etc. (Bai et al. 2003; Battle et al. 2005; Ishitsuka et al. 2005; Chang et al. 2013). There is report of honokiol-mediated inhibition of PI3K/mTOR pathway as a promising strategy to surmount immunoresponse in glioma, breast and prostate cancers (Crane et al. 2009). Meanwhile, honokiol has a significant impact on prostacyclin metabolism. Since prostacyclin is well known for its inhibition role of platelet aggregation, above observation may explain the antithrombotic activity of honokiol (Hu et al. 2005).

The above-mentioned discoveries suggest the complexity of honokiol’s MoA. Based on our previous study of natural product resveratrol (Wang et al. 2016), here, we took advantage of a simple unicellular model, Schizosaccharomyces pombe (S. pombe), and tried to use transcriptomics and bioinformatics analysis to reduce the complexity and understand honokiol’s MoA comprehensively.

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Fission yeast is an excellent model for studying fundamental cell behaviours, such as cell growth and proliferation. At the same time, fission yeast displays cancer cell-like metabolic behaviour, called Crabtree effect (similar to the Warburg effect of cancer cell) (Piškur et al. 2006; Van de Hei den et al. 2009), means it prefers to use fermentation rather than respiration for the energy production. Because of above merits, the mechanism discovery from fission yeast system could be immigrated to clinical medicine feasibly. In addition, benefiting from many scientists’ contributions, multiple valuable bioinformatics tools and databases of fission yeast model are available, which directly facilitates the exploration of honokiol’s MoA in a systematic view (Simon and Bedalov 2004).

In the present research, with the goal of understanding the common MoA of the anti-proliferative activity of honokiol, we thus investigated the honokiol’s impacts to fission yeast both on cellular level and whole-genome transcriptional level. The experimental procedures, results and discussions are reported as follows.

**Materials and methods**

**Yeast cell culture and honokiol treatment**

Fission yeast *S. pombe* strain 972 h- was used in this study. Honokiol was purchased from Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine (Shanghai, China). The experimental procedure basically follows previous description (Wang et al. 2016). Briefly, for the drug activity experiment, a 10 ml culture of YE liquid medium (0.5% yeast extract, 3% glucose) was inoculated from single colony and was grown overnight at 30°C to the late log phase (OD$_{600}$ = 2.0–3.0). The yeast culture was next diluted to OD$_{600}$ = 0.05 and treated with a series of honokiol doses (0, 1, 2, 3, 4, 6 and 8 µg/ml) in 50 ml of fresh YE liquid culture. The optical density was measured at 600 nm at different time points (0, 4, 8, 12, 16, 20, 24, 28 and 32 h), and eventually the IC$_{50}$ value was computed based on the readout at 20 h after drug treatment.

**Cell phenotypic and FACS analysis**

Cell staining, microscopic and fluorescence-activated cell sorting (FACS) analysis were basically performed as previous described (Wang et al. 2016). The septum staining by calcofluor was conducted based on the Dr. Paul Nurse’s Lab Fission Yeast Handbook (Cortés et al. 2012). In brief, the yeast cells from late log phase culture (OD$_{600}$ = 2.0–3.0) was diluted to OD$_{600}$ = 0.05, 3 µg/ml honokiol was next added to the culture, and finally 10$^7$ cells was collected at different time points by centrifugation at 2500 rpm for 5 min. The cell pellets were washed once with cold ddH$_2$O and were re-suspended in 1 ml of cold 70% ethanol for fixation. For calcfluor staining, 30 µl of fixed cells was washed with 1 ml of water, and then mixed with 2× calcfluor stain (50 µg/ml calcfluor, 0.3 mg/ml p-phenylenediamine 50% glycerol). The samples were observed under fluorescence microscopy (DM2500, Leica). For FACS analysis, 0.3 ml of above fixed cells was washed with 50 mM sodium citrate twice. Then, RNA was firstly digested by 0.1 mg/ml RNase A at 37°C for 2 h. After that, we added propidium iodide to final concentration at 2 µg/ml. Just before processing the cells, an ultrasonic treatment of 45 s was applied to preventing the conglutination of the cells. Eventually the DNA contents were detected by FACScan (Becton Dickinson). The raw data were analysed and visualised by Flowjo software.

**Microarray analysis**

In general, the microarray analysis was performed as previous described (Wang et al. 2016). Honokiol (3 µg/ml) and vehicle control were added to YE cultured yeast at OD$_{600}$ = 0.2. Next, the cells were incubated for 4 h, harvested by centrifugation, and washed once with 25 ml of cold ddH$_2$O. The hot phenol method was used to extract the total RNA, and the RNA was purified using Qiagen RNeasy columns. The three replicated total RNA samples were sent to Shanghai Biochip Co. Ltd. for Affymetrix Yeast 2.0 microarray analysis. After passing the standard microarray quality check, the differential expressed genes were defined as (ratio >2.0, p-value <0.05).
**Bioinformatics analysis**

The gene expression data was first imported into Gene Cluster 3.0 to perform the hierarchical analysis using the centroid linkage clustering method (de Hoon et al. 2004), and the heat-map was visualised by Java Treeview 1.0. Gene Set Enrichment Analysis (GSEA) was conducted by the online tool AnGeLi (Bitton et al. 2015). The enriched gene ontology category was selected based on biological process and molecular function using list frequency >5% and p-value <0.01 as the selection criterion. The protein-protein interaction network was constructed by the online tool Pint (Pombe Interactome) based on the support vector machine and random forest algorithm (Pancaldi et al. 2012).

**Results**

**Natural product honokiol inhibits fission yeast growth**

Previous studies have shown that the natural product honokiol (3',5-di-2-progenyl-1,1'-biphenyl-2,4'-diol) structurally belongs to the polyphenol family [Figure 1(a)] (Maruyama and Kuribara 2006), and it was originally extracted from the genus Magnolia. Here, to explore the feasibility of using the fission yeast *S. pombe* as a model to study its antifungal activity, we arranged a series of honokiol concentrations (0–8 μg/ml) to treat wild-type fission yeast and quantitated the growth inhibition effect by monitoring the cell densities at
OD_{600}. The results showed that honokiol inhibits cell growth in a dose-dependent way [Figure 1(a)], with an IC_{50} value at 3 μg/ml.

**Honokiol inhibits a small group of cell’s separation mildly**

When using the IC_{50} concentration (3 μg/ml) of honokiol to challenge the yeast for 4 h, microscopic analysis showed that there is no obvious phenotypic change amongst most of the cells, although we observed elongated cell occasionally [less than 5% in the whole cell population, shown in Figure 1(b)]. We also utilised the chemical calciofluor white to probe the cell septum, as shown in Figure 1(c), the septum of several cells became noticeably thicker in contrast to the mock-treated samples, and we rarely found some cells hold more than one septum. This suggests that this group of cell’s separation is delayed. However the delaying effect is very mild (or only take effect on a small portion of the whole cell population), because there is no significant cell cycling difference between the mock and drug treated groups, based on the FACS results [Figure 1(d)].

**Honokiol-induced transcriptomic change is closely related to core environmental stress response genes**

For the purpose of understanding the molecular mechanism of honokiol, we utilised microarray analysis to identify the gene transcriptional consequences after honokiol treatment. After adding 3 μg/ml honokiol to fission yeast culture at the early time point (4 h), we characterised total 554 genes (512 genes with increased expression levels and 42 genes with decreased expression levels) whose expression levels are significantly affected by honokiol treatment (fold >2, p-value < 0.05, shown in Supplementary Table 1). We next performed the GSEA via the web-tool AnGeLi for comprehensively analysing the honokiol-modulated gene lists (Bitton et al. 2015). As shown in Table 1, honokiol treatment can significantly trigger the stress-related genes’ expression. For example, over 45% up-regulated genes (232 genes in total, \( p\)-value = 3.09 E-133) are recognised as the “Core Environmental Stress Response (CESR) Induced” category, which is largely overlapped with the secondary highest hit “Oxidative Stress Cluster4” (182 genes in total, \( p\)-value = 2.18E-102). It is worth noting that previous studies have proven that the b-ZIP transcription factor Atf1 is the key component of stress-dependent gene transcription (Zhou et al. 2012). Consistently, we found that “Atf1 activated” categories are over-represented in the statistical test (52 genes in total, \( p\)-value = 1.20E-43). We, therefore, did the combinational and comparative analysis between our results and the previous published microarray data of fission yeast responding to various environmental stresses (oxidative stress, heavy metal stress, osmotic stress, heat shock and DNA damage) (Dongrong Chen et al., 2003). As shown in Figure 2, the result of hierarchical analysis indicates that honokiol-caused transcriptomic modulation is significantly consistent with stress-caused changes, especially for \( \text{H}_2\text{O}_2\), Cd and heat-induced stresses. Compared to over 500 honokiol up-regulated genes, there are only 42 are down-regulated by the drug, and which cannot be clustered into any meaningful category. However, it is interesting to see that pyruvate

| Gene expression                          | List frequency/value | Background frequency/value | p-Value     |
|-----------------------------------------|----------------------|-----------------------------|-------------|
| Core environmental stress response induced | 45.49% (232/510)     | 7.72% (541/7006)            | 3.0887E-133 |
| Oxidative stress cluster 4              | 35.69% (182/510)     | 5.91% (414/7006)            | 2.1812E-102 |
| Stress module                          | 18.82% (96/510)      | 1.94% (136/7006)            | 5.93162E-77  |
| Chloroform module                       | 25.69% (131/510)     | 4.22% (296/7006)            | 2.15497E-71  |
| Caffeine and Rapamycin induced          | 22.35% (114/510)     | 4.34% (304/7006)            | 4.97926E-52  |
| Atf1-activated mutant                   | 10.20% (52/510)      | 0.97% (68/7006)             | 1.20199E-43  |
| Nitrogen depletion total meiotic genes  | 17.06% (87/510)      | 3.01% (211/7006)            | 7.99504E-43  |
| Late meiotic genes                      | 13.14% (67/510)      | 2.00% (140/7006)            | 1.8509E-37   |
| Continuous meiotic genes                | 9.22% (47/510)       | 0.96% (69/7006)             | 1.7246E-35   |
| Meiosis sporulation module              | 17.25% (88/510)      | 3.88% (272/7006)            | 1.37287E-33  |
| Sporulation module                      | 13.92% (71/510)      | 2.68% (188/7006)            | 1.53946E-31  |
| Induced usp102 mutant                   | 6.67% (34/510)       | 0.64% (45/7006)             | 9.20475E-28  |
| ind1 br11 br2 set1 mutant induced       | 10.00% (51/510)      | 1.84% (129/7006)            | 3.13852E-23  |
| Sty1 but not Atf1-activated genes       | 5.29% (27/510)       | 0.50% (35/7006)             | 3.24375E-22  |
decarboxylase (SPAC13A11.06) has the obviously decreased expression level, which implies a potential modulation of metabolic fluxes.

Network analysis of honokiol up-regulated gene sets indicates extensive protein–protein interactions are existed and form biofunctionally important modules

To keep dissecting the honokiol-caused transcriptomic modulation, we next constructed the protein interaction networks using the honokiol up-regulated gene sets. As shown in Figure 3, there are highly frequent protein–protein interaction events in the prediction. Interestingly, several biofunctionally important modules are emerged based on those interaction networks. The first noteworthy module is related to sugar transport and metabolism, because this module includes at least three hexose transmembrane transporters (Ght1, Ght2 and Ght4) (Heiland et al. 2000), gluconate transmembrane transporter inducer (Gti1) (Caspari 1997) and glucoside transmembrane transporter (Sut1) (Reinders and Ward 2001), which suggests the

Figure 2. Honokiol-induced transcriptomic change is closely related to core environmental stress response. IC50 value honokiol (3 µg/ml) and vehicle control were added to YE medium cultured yeast at OD600 = 0.2. Next, the cells were incubated for 4 h. Afterwards, the cells were collected and mRNA was extracted for the microarray analysis. The detailed data analysis is described in “Materials and methods” section. Basically, the data used in clustering analysis were selected by both the fold change (>2 folds) and p-value (<0.05). Honokiol-1, -2, -3 represent three biological replicates. Other stress treatment conditions are reported before (Dongrong et al. 2003). Briefly, these stress conditions include: oxidative stress (0.5 mM H2O2, treat 15 and 60 min), heavy metal stress (0.5 mM CdSO4, treat 15 and 60 min), heat stress (39°C, treat 15 and 60 min), osmotic stress (1 M sorbitol, treat 15 and 60 min) and alkylating agent (0.2%, w/v methylmethane sulfonate, treat 15 and 60 min).
glycolysis activation is a part of honokiol’s MoA. Another interesting module is related to redox balance, lipid and glycan metabolism. It includes three glutathione S-transferase genes (Gst2, Gst3 and SPCC1281.07C) (Kim et al. 2004), one NAD+/NADH kinase (SPCC24B10.02C), four mannosyltransferase (Lmt1, Lmt2, Lmt3 and Omh1) (Nakase et al. 2010), and one acyl-CoA-sterol acyltransferase (Are1). We also observed several modules which are related to signalling transduction. For example, there is a predicted transcriptional regulation complex including DNA binding factor (Tbf1 and Spt2) (Cockell et al. 2009), transcription factor TFIH complex subunit (Rad15) (Murray et al. 1992), histone acetyltransferase (Mst2) (Gómez et al. 2005) and Jmjc domain chromatin-associated protein (Epe1) (Trewick et al. 2007). There is also a potential signalling module associated with protein kinase and secondary messenger cAMP, which consists of cAMP-dependent protein kinase catalytic subunit (Pka1) (Matsuo et al. 2008), serine/threonine protein kinase Ppk18 (Beltrao et al. 2009) and a G-protein coupled receptor, Git3, which can activate the adenylate cyclase activity (Wang et al. 2005).

The honokiol’s transcriptional regulation on central carbon metabolism

We next analysed the honokiol’s impact on central carbon metabolism (CCM) to answer if its antifungal activity is related to dysregulation of crabtree effect or not. The crabtree effect-related metabolic enzyme’s expression levels were investigated. As shown in Figure 4, Fbp1 (Fructose-1,6-bisphosphatase) is strongly up-regulated by honokiol (increased to 28 folds). In glycolysis, Fbp1 is responsible for converting fructose-6-phosphate to fructose-1,6-bisphosphate, for which the reactions need ATP donating one phosphate group. However, we also observed that pyruvate kinase (Pyk1), pyruvate dehydrogenase E1 component alpha subunit (Pda1), pyruvate dehydrogenase E1 component beta subunit (Pdb1), and especially pyruvate decarboxylase (Pdc) are down-regulated obviously, suggesting that the synthesis of acetyl-CoA is impaired although its upstream metabolites, such as fructose-
1,6-bisphosphate and may be phosphoenolpyruvate, are accumulated, which becomes a waste for both carbon scaffolds and ATP molecule.

Although the production of acetyl-CoA (the precursor of TCA cycle) is slowed down, we did not find significantly changed gene expression levels in the metabolic enzymes of TCA cycle (Figure 4) and the components of mitochondrial respiration chain (Supplementary Figure 1). Above results imply that the transcriptional modulation in fermentation, rather than respiration, is part of honokiol’s MoA.

Summary of honokiol’s MoA

Based on above results, we have summarised the honokiol’s antifungal MoA. As shown in Figure 5, upon honokiol penetrating into yeast cell, it rapidly up-regulate glucose uptake by enhancing hexose transmembrane transporters’ expression. Honokiol will keep improving the converting from fructose-6-phosphate to fructose-1,6-bisphosphate by up-regulating fructose-1,6-bisphosphatase’s expression, which becomes a huge energy consuming/wasting step because meanwhile the metabolic flux of downstream glycolysis is shrunk due to the down-regulated expression of pyruvate decarboxylase. At the same time, honokiol extensively triggers the expression of CESR/oxidative stress-related genes. Many of those genes are downstream target of transcription factor Atf1, which is well-known regulator of self-adaptation for resisting the accumulated ROS. However, long-termed or high-dosed honokiol treatment may disturb the redox homeostasis eventually, which will contribute to honokiol’s cidal effect, similarly as the mechanism of many known antifungals and antibiotics.

Discussion

Despite the widely recognised pharmacological potential of the plant-derived compound honokiol, its underlying MoA still remains largely incomplete. Our studies elucidated that widespread transcriptional reprogramming and subsequent modulation of CCM are the major MoA of honokiol in response to its antifungal function in fission yeast.

Honokiol is normally extracted from Magnolia, and it chemically belongs to polyphenol, which has been well known as the capability against oxidative stress.
via its metal-chelating properties. However, sometimes things go to the opposite side, means polyphenol can also increase the generation of reactive oxygen species, such as the recent findings that red wine polyphenols and resveratrol can increase the ROS in cancer cells (Heiss et al. 2007; Sharif et al. 2010). Here, we proposed a similar model that honokiol may inhibit the cell growth also partly by stimulating the production of ROS (Figures 2 and 5 and Table 1). The strong evidences come from the microarray analysis-both GSEA and hierarchical analysis indicates that the application of honokiol can trigger a similar transcriptional signature such as the ones from environmental stress stimuli, especially for H2O2 treatment. It is worth to mention that the drug dose used for microarray analysis is sublethal (IC50 concentration), since we tried to avoid to see the dead effect. Correspondingly, there are some of the transcriptional modulations of CESR or oxidative stress responsive genes that belong to cell’s self-adaptation or detoxification program. They are mainly controlled by the well-known stress regulator Atf1 (its transcript increases three folds by honokiol treatment), because there is a large proportion overlapping between Atf1 targets and honokiol regulated genes. We, therefore, proposed that b-ZIP transcription factor Atf1 plays a key role in honokiol’s MoA as well.

Although there is significant growth inhibition effect, microscopic analysis indicated that most of cells still hold the normal phenotype. Only a small proportion of cells (less than 5% in the whole population) showed elongated cell shape and delayed sister cell separation. The GSEA suggested that some meiotic genes are dysregulated by honokiol, but it is hard to make conclusion, since over 95% cells without cell cycle defects. Although the mechanism of those “outlier cells” is not fully understood, it may be related to epigenetics modulation. However, this possibility still remains for further investigation, such as using single cell transcriptomic approach.

Recent study shows that honokiol is a promising lead to bind the peroxisome proliferator-activated receptor gamma (Atanasov et al. 2013) and stimulate basal glucose uptake in differentiated adipocytes. Interestingly, at least three glucose transmembrane
transporters (Ght1, Ght2 and Ght4) are up-regulated by honokiol treatment in fission yeast (Figure 3), which implies that the improvement of sugar uptake and potential faster glycolysis rate is a common honokiol’s MoA within both yeast and mammalian cells. We kept questioning if honokiol’s antifungal activity comes from sugar-induced cell death (SICD) or not. The transcriptional analysis of crabtree effect-related genes indicated that there is no significant regulation on mitochondrial respiration chain (Supplementary Figure 1), but in glycolysis module, fructose-1,6-bisphosphatase is strongly up-regulated while pyruvate decarboxylase is obviously down-regulated. The above-mentioned modulation caused greatly increased ATP consuming, but it eventually becomes energy and carbon scaffolds wasteful since the flux of downstream glycolysis is shrinked. These results suggested that SICD may not be the MoA of honokiol, because it normally characterised with dysregulation of mitochondrial respiration.

**Conclusion**

In summary, we elucidated that the natural product honokiol can inhibit the cell growth of fission yeast, primarily through the dysregulation of glycolysis and may be also caused by disrupting the redox homeostasis. At the same time, sublethal dose of honokiol can trigger the extensive regulation of stress responsive genes, which is controlled by the well-known transcription factor Atf1. The complexity of the honokiol’s MoA demonstrates that it is essential to apply multiple omics approaches to obtain a complete picture of its antifungal activity.

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**Disclosure statement**

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