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Fusaric acid induces a notochord malformation in zebrafish via copper chelation

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Abstract Over a thousand extracts were tested for phenotypic effects in developing zebrafish embryos to identify bioactive molecules produced by endophytic fungi. One extract isolated from *Fusarium* sp., a widely distributed fungal genus found in soil and often associated with plants, induced an undulated notochord in developing zebrafish embryos. The active compound was isolated and identified as fusaric acid. Previous literature has shown this phenotype to be associated with copper chelation from the active site of lysyl oxidase, but the ability of fusaric acid to bind copper ions has not been well described. Isothermal titration calorimetry revealed that fusaric acid is a modest copper chelator with a binding constant of 4.4×10^5 M^{-1}. These results shed light on the toxicity of fusaric acid and the potential teratogenic effects of consuming plants infected with *Fusarium* sp.

Keywords Copper chelation · Endophytic fungi · Fusaric acid · Natural products · Zebrafish notochord

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Introduction

Regulating safe levels of mycotoxin contamination in food is a worldwide concern (EFSA 2012; Gnonlonfin et al. 2013). Crops for consumption may harbor endophytic fungi or endophytes, which live symbiotically within host plant tissue and can produce harmful compounds including mycotoxins (Finch et al. 2013). Alkaloids produced by certain fungi have been found to cause toxic syndromes in cattle that have consumed contaminated grasses (Porter 1993). Prominent examples are citrinin, a nephrotoxin produced by the fungal plant pathogens *Aspergillus* sp., *Penicillium* sp., *Monascus* sp. and the water mold *Pythium ultimum,* and aflatoxin produced by *Aspergillus flavus* and *Aspergillus parasiticus* (Endo and Kuroda 1976; Flajs and Peraica 2009; Abrar et al. 2013). A more thorough understanding of bioactive fungal natural products could help shape food and agriculture guidelines to protect consumers from mycotoxins.

To study the effects of fungal endophytic compounds on vertebrate development, we used an in vivo zebrafish embryo assay, as whole organism assays are

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advantageous for bioactive compound discovery (Murphey and Zon 2006; Crawford et al. 2011). The zebrafish (*Danio rerio*) is an excellent model organism for studying development because zebrafish share many genetic and morphological characteristics with humans (Santoriello and Zon 2012; Howe et al. 2013). Its transparent embryo allows for the observation of development in real time through a light microscope, facilitating relatively high-throughput screens for bioactive compounds in an approach known as forward chemical genetics (Lokey 2003).

As part of a research course at Yale University we screened a library of over one thousand extracts derived from endophytic fungi for phenotypic effects in developing zebrafish embryos. Here we report that fusaric acid causes an undulated notochord in zebrafish development as a result of copper chelation.

Materials and methods

Zebrafish embryos

All zebrafish used in this study were of the TLF strain. Fish were handled in accordance with protocols approved by Yale University Institutional Animal Care and Use Committee (Assurance number A3230-01). Embryos were incubated at 28.5 °C in E3 solution (salinated water) prior to treatment and staged according to Kimmel et al. (1995).

Zebrafish assays

Zebrafish were mated overnight, and fertilized embryos were collected at about 1 hpf (hours post fertilization). For fungal extract screening at 4 and 10 hpf, 3 embryos were incubated in duplicate in 50 µL of 0.5 % extract in 96-well flat-bottom plates. All other assays were done in triplicate with 12 embryos per well in 12-well flat bottom plates with 500 ul E3. Plates were covered to prevent evaporation. Embryos were screened for developmental phenotypes 24 hpf. 0.5 % DMSO served as a vehicle control for screening assays. 1 % and below DMSO was not observed to induce non-wildtype phenotypes in developing zebrafish. Commercial reagents used for embryo treatments were fusaric acid (Acros Organics), 2-mercaptopyridine-N-oxide (Acros Organics) and 3-aminopropionitrile fumarate (β-aminopropionitrile) (Acros Organics).

Extract purification

1022 dried experimental extracts were obtained from a Yale-specific extract library. Briefly, extracts were prepared by growing endophytic fungal isolates for 14–35 days in potato dextrose liquid media (Becton–Dickinson), followed by filtration to remove fungal cell mass and sequential 1:1 volume liquid extraction in the organic solvents dichloromethane and ethyl acetate. Extracts or suspensions of fusaric acid (99 % purity from Acros Organics) were dried and resuspended in dimethysulfoxide (DMSO) for screening.

High-performance liquid chromatography (HPLC) separation was performed on a Luna 5u C18(2) 100A 250 × 21.1 mm column (Phenomenex) using acetonitrile:H_2O as the mobile phase. All HPLC fractions were dried and suspended in DMSO to approximately 1 mg/mL for further testing. A range of HPLC fraction concentrations were tested for phenotypic effects in zebrafish. Crystals of the bioactive natural product were grown in methanol at 25 °C from an HPLC fraction. Crystals appeared within 1 week and were determined to be fusaric acid by small molecule crystallography and nuclear magnetic resonance (NMR) spectroscopy.

Crystallography

Low-temperature diffraction data (ω-scans) were collected on a Rigaku MicroMax-007HF diffractometer coupled to a Saturn994 + CCD detector with Cu Kα ($\lambda = 1.54178$ Å) for structure determination. All structures were solved by direct methods using SHELXS (Sheldrick 1990) and refined against F2 on all data by full-matrix least squares with SHELXL-97 (Sheldrick 2008) using established refinement techniques (Muller 2009). All non-hydrogen atoms were refined anisotropically. All hydrogen atoms were included in the model and refined using a riding model. The isotropic displacement parameters of all hydrogen atoms were fixed to 1.2 times the U value of the atoms to which they are linked (1.5 times for methyl groups). All disorders were refined with the help of similarity restraints on the 1,2- and 1,3-distances and displacement parameters as well as rigid bond restraints for anisotropic displacement parameters.

Fusaric acid crystallizes in the monoclinic space group P21/n with two molecules in the asymmetric

unit, which appear to differ by the position of a hydrogen atom. The crystal did not diffract past 1 Å, and it was necessary to truncate the data. Information for structure determination has been deposited in the Organic and organometallic compounds: Cambridge Crystallographic Data Centre with accession number 935978.

Spectroscopy

A compound with an m/z of 179.095 by liquid chromatography-mass spectrometry (LCMS) was assigned a molecular formula of $C_{10}H_{13}NO_2$. The structure was elucidated by spectral analyses, including ^{1}H and ^{13}C-NMR 1D and 2D-NMR techniques ($^{1}H,^{1}H$-COSY, $^{1}H,^{13}C$-HSQC, and $^{1}H,^{13}C$-HMBC data not shown). Analysis of ^{1}H NMR suggested the presence of three aliphatic methylene groups at chemical shifts of 1.22, 1.73 and 2.82 ppm, a methyl group at 0.97 ppm and three aromatic protons 8.23, 8.43 and 8.62 ppm. From the carbon data, five aromatic carbons between 126 and 149 ppm and a carbonyl carbon at 165.21 ppm were identified allowing for the discernment of substructures that were assigned as fusaric acid. NMR experiments were performed on Varian Inova 500 triple resonance probes spectrometers at 20 °C with samples prepared in $CDCl_3$.

Isothermal titration calorimetry

A MicroCal ITC200 instrument (GE Healthcare) was used to quantitatively measure the copper-binding ability ($CuCl_2$ dihydrate from Acros Organics) of fusaric acid (Acros Organics) in triplicate. Distilled water was used as the solvent due to interactions between copper and several other buffering agents that were tested.

Experiments were run by titrating 2.5 mM copper (II) chloride ($CuCl_2$) into 0.5 mM fusaric acid (99 % purity from Acros Organics) at 25 °C. The initial $CuCl_2$ injection was 0.4 µL for 0.8 s with a delay of 150 s, followed by 25 injections of 1.5 µL for 3.0 s with 180 s delays. A total of 375.4 µL of $CuCl_2$ was injected. Origin software (Version 5.0, MicroCal, Northampton, MA) was used to quantify titration heats. Results were adjusted to account for heat released by dilution of $CuCl_2$ into water. The corrected data were plotted as a function of molar ratio and fit with a one binding site model.

Results and discussion

Using zebrafish embryos, we screened 1022 fungal endophyte-derived natural products produced as part of a Yale University class effort from 2007 to 2011. When incubated with developing zebrafish as described in the Methods section, 849 of the natural products induced a wildtype phenotype, 101 led to general necrosis within 24 h, and 72 displayed non-wildtype phenotypes. One ethyl acetate extract from a *Fusarium* sp., isolate E4712A, induced an undulated notochord (see Fig. 1). The notochord is a rod-shaped structure ventral to the neural tube that runs along the rostral-caudal axis. The notochord forms during early embryogenesis in all chordates and provides a structural support for the trunk and tail of the embryo. The notochord secretes and is coated by an extracellular matrix (ECM) including collagen. The notochord cells develop vacuoles that swell due to hydrostatic pressure. The ECM prevents radial expansion of the notochord, thus the swelling causes the notochord to elongate through a process called directed dilation. If the ECM is compromised, then the swelling of the vacuoles causes the notochord to undulate (Stemple 2005). Since the notochord contributes to the vertebral column, an undulated notochord would cause abnormal curvature of the spine (Ellis et al. 2013). Based on the specificity of the notochord phenotype and the importance of notochord in embryonic development, we set out to identify the compound responsible for the observed activity.

The active compound was purified by HPLC, and its structure was determined by NMR, mass spectrometry, and x-ray crystallography to be fusaric (5-butylpicolinic) acid (see Fig. 3). Fusaric acid, originally isolated from *Fusarium heterosporum,* is a well-studied mycotoxin that induces vascular relaxation and inhibits dopamine-β-hydroxylase, a copper-dependent enzyme needed for synthesis of the neurotransmitter norepinephrine (Hidaka 1971; Asano and Hidaka 1977; Dove 2004). Studies have reported changes in brain chemistry in animals fed *Fusarium*-contaminated grains and their offspring, verifying that fusaric acid is an orally active toxin with gestational and lactational transmission (Porter et al. 1996; Swamy et al. 2004). It has also been shown to reduce manic symptoms in patients with Wilson's disease, a disease involving copper accumulation in tissues, and has antimicrobial properties (Hidaka et al. 1969; Curzon 1977; Pandey et al. 1981).

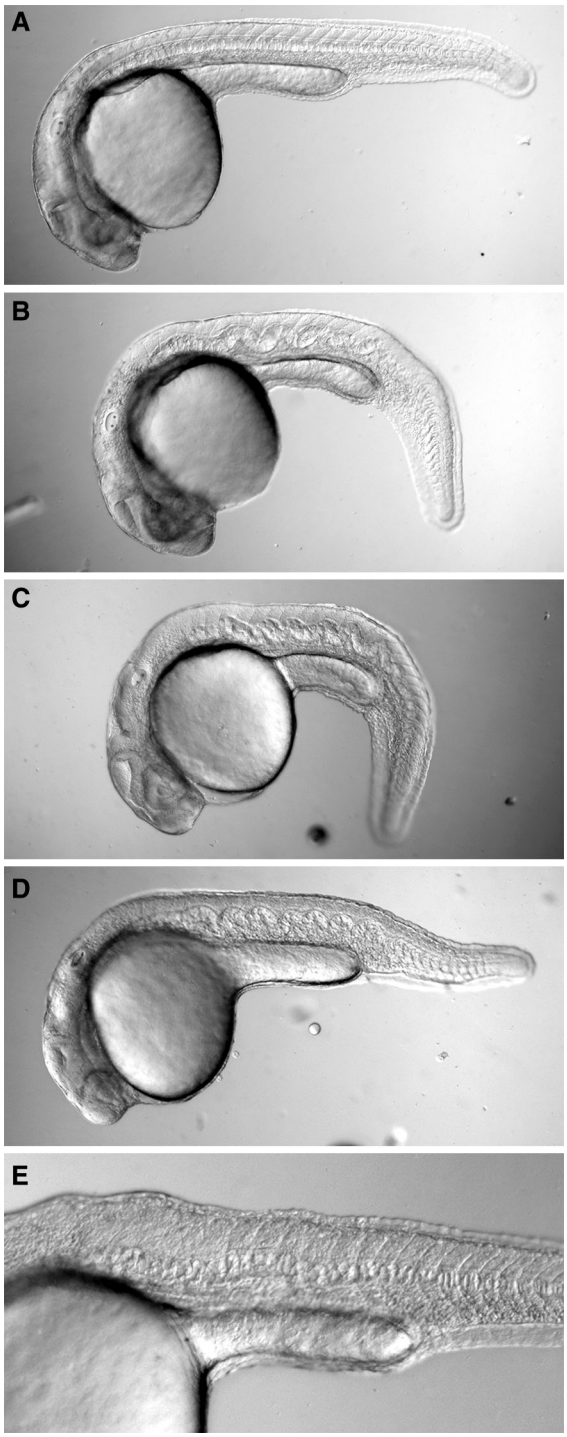

Fig. 1 Treatment of zebrafish embryos with fusaric acid causes an undulated notochord phenotype. **a** Wildtype control, **b** 10 nM β-aminopropionitrile-treated embryo, **c** 500 nM 2-mercaptopyridine-N-oxide-treated embryo, **d** 550 μM fusaric acid-treated embryo, **e** 110 μM fusaric acid-treated embryo. Embryos shown were drug-treated 10 hpf and images were recorded 26 hpf

Few reports have measured fusaric acid concentrations in food although it has been found in corn-based foods, wheat, barley, other cereal grains and animal feeds worldwide (Placinta et al. 1999). A recent study of mycotoxins in corn and wheat silage reported that fusaric acid had the highest prevalence and concentration compared to 22 other common mycotoxins (Shimshoni et al. 2013). Some governments and agencies regulate *Fusarium* sp. mycotoxins including deoxynivalenol, trichothecenes, zearalenone and fumosins in food for human and animal consumption (Park and Troxell 2002; van Egmond et al. 2007). Fusaric acid, however, is not monitored or regulated.

To verify that fusaric acid was responsible for the observed developmental phenotype, we used commercial fusaric acid with 98 % purity and observed the same phenotypic effect caused by E4712A extracts. A wide range of concentrations was tested at 4 and 10 h post fertilization (hpf), and it was determined that 110–600 μM fusaric acid induced the undulated notochord phenotype (Fig. 1). General necrosis or death in the shield stage was observed at concentrations above 600 μM and all embryos developed undulated notochords when treated with 400–600 μM fusaric acid at 4 or 10 hpf. Embryos treated at 4 hpf were more sensitive to low concentrations of fusaric acid compared to embryos treated at 10 hpf. Specifically, 24 % of embryos treated with 110 μM fusaric acid at 4 hpf developed a weak undulated notochord phenotype compared to 10 % when treated at 10 hpf with the same concentration.

Previous research has connected an undulated notochord phenotype with the disruption of lysyl oxidase through mutation and interactions with copper-chelating compounds (Tilton et al. 2006; Gansner et al. 2007). Lysyl oxidase is a copper-dependent enzyme that cross-links collagen and elastin by catalyzing allysine formation (Siegel et al. 1970). Precisely timed exposures of zebrafish embryos to the copper-chelating compound, β-aminopropionitrile, causes malformations of notochord macro and microstructures that are observed in lysyl oxidase gene knockdown and copper deficient embryos. Similarly, 2-mercaptopyridine-N-oxide inhibits lysyl oxidase in zebrafish extracts. (Anderson et al. 2007; Gansner et al. 2007). We hypothesized that fusaric acid, a known weak metal ion chelator, induces the same malformation by binding copper, thereby inhibiting lysyl oxidase. We provide evidence that fusaric acid

treatment phenocopies the undulated notochord caused by 2-mercaptopyridine-N-oxide and β-amino-propionitrile treatment (Fig. 1).

In 1973, Hidaka et al. used changes in absorbance measurements of copper-oxide compounds to determine the K_D of Cu^{2+}-fusaric acid complex to be 12.2 μM (Hidaka et al. 1973). To explore this organo-metallic interaction, we performed isothermal titration calorimetry (ITC) to measure small molecule interactions (Turnbull and Daranas 2003). Using ITC, we determined the binding constant (K_A) of the Cu^{2+}-fusaric acid complex to be 4.4×10^5 $M^{-1} \pm 1.1 \times 10^5$ and the dissociation constant (K_D) to be on the order of 2.3 μM (Fig. 2). These data revealed a binding event with a stoichiometry (N) of 0.5, which supports crystal structures showing two fusaric acid molecules coordinate one metal ion (Fig. 3) (He et al. 2007). Thermodynamic parameters are given in Table 1.

It has been reported that copper-fusaric acid complexes are biologically relevant as they upregulate fusaric acid production by *Fusarium* sp (Pan et al. 2010). Given that copper chelators are known to induce an undulated notochord phenotype, we infer that the binding of copper by fusaric acid induces this phenotype despite weak affinity measured in vitro (Tilton et al. 2006).

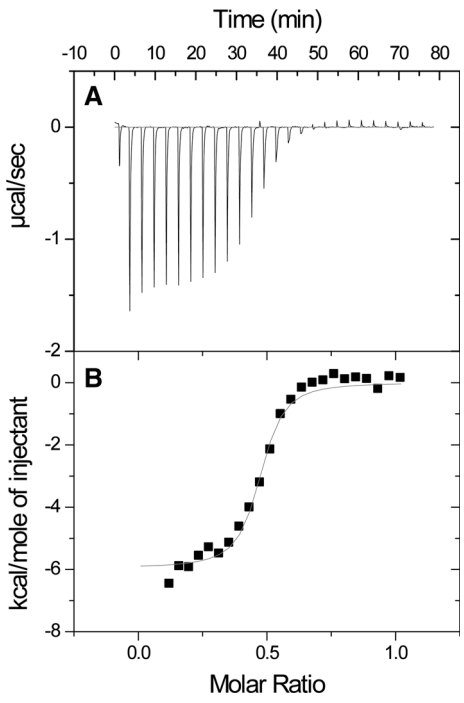

Fig. 2 ITC titrations and data analysis. **a** Titration of $CuCl_2$ into fusaric acid. **b** Integration of the $CuCl_2$-fusaric titration data corrected by subtraction of the $CuCl_2$-water titration and fit with nonlinear regression

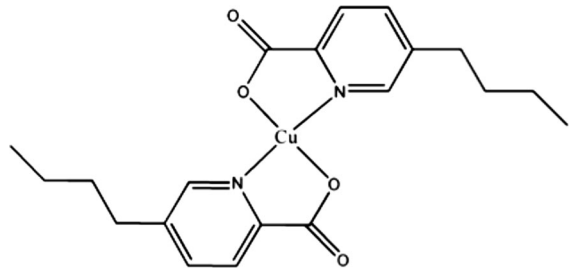

Fig. 3 Structure of fusaric acid and copper ion binding. In a previously reported crystal structure, two fusaric acid molecules coordinate one copper ion (He et al. 2007)

Table 1 ITC data thermodynamic parameters

Parameter	Value
N	0.46 ± 0.01
K_A	4.37E5 ± 1.09E5
ΔH	−5951 ± 134.8
ΔS	5.84

Collectively these results show that (1) screening developmental phenotypes using zebrafish is an effective means of characterizing bioactive natural products, and (2) fusaric acid is a mycotoxin that causes significant malformations in developing zebrafish through a proposed mechanism of copper chelation from the active site of lysyl oxidase. Due to its role in collagen and elastin crosslinking, lysyl oxidase is necessary for normal respiratory functioning, wound healing, and ovulation (Fushida-Takemura et al. 1996; Maki et al. 2005; Papachroni et al. 2010). Inhibition of lysyl oxidase has been shown to promote the development of emphysema, a lung disease in which lung tissue is destroyed (Kountz et al. 1932; Soskel et al. 1984; Gao et al. 2005). The teratogenic effects of fusaric acid and the possibility of general lysyl oxidase inhibition should be considered in efforts to regulate safe levels of mycotoxins in food.

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microorganisms that produced the extracts tested here. Endophytes were isolated from plants collected with permission of the Ministerio del Ambiente of Ecuador.

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