RESEARCH ARTICLE

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2019, **10**, 985Fungal natural alkaloid schizocommunin activates
the aryl hydrocarbon receptor pathwayRoxana Filip,^a Tyler A. Shaw,^a Atsushi Nishida^b and John Paul Pezacki^{✉*}

Fungi, including mushrooms and mycelia, are a rich source for natural products with medicinal properties. In some cases, they can lead to opportunistic infections in humans and other mammals. In 1994, the first case of bronchopulmonary mycosis caused by the *Schizophyllum commune* fungus was described. Culture of the isolated specimen led to the extraction of an alkaloid compound, schizocommunin, which was more recently synthesised for biological characterization. Herein we describe schizocommunin and one of its analogues as cytotoxic against human hepatoma cells at low micromolar concentrations. Schizocommunin is shown to be a potent activator of the aryl hydrocarbon receptor (AhR) gene battery, more specifically increasing expression of the CYP1A1, CYP1B1 and UGT1A genes in human liver and lung cells. A luciferase reporter assay further confirms induction of transcription by these compounds at the xenobiotic response element. This study improves our understanding of the interaction between this fungal metabolite and xenobiotic detoxifying mechanisms in the body, and points to schizocommunin as a putative mediator of the allergic response and a useful molecule for the study of the AhR pathway.

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Introduction

The *Schizophyllum commune* fungus is an edible basidiomycete species found throughout the globe mostly as a saprobic tree pathogen.¹ It is one of the only species of fungi that can retract by movement. It has also attracted attention for medically relevant properties including immunomodulatory functions.² Interestingly, in 1989, a specimen was found to cause allergic bronchopulmonary mycosis (ABPM) by growing in the upper lobe bronchus of a patient at the Chiba University Hospital in Japan.³ The first isolation of the associated natural product schizocommunin was performed from this specimen and it was characterized as a cytotoxic pigment with potential anticancer activity against murine lymphoma.⁴ Recently, the structure of the molecule was revised (Fig. 1) and a synthetic approach was developed to produce amounts amenable to biological characterization. It was also shown to be antiproliferative against a cervical cancer cell line.⁵ However, the effects of this fungal molecule on cell biology have not yet been clearly established.

The aryl hydrocarbon receptor (AhR) is a highly conserved ligand-activated transcription factor which responds to chemical stimuli to activate adaptive responses of detoxification, homeostasis and immunity.⁶ Despite recent advancements regarding the endogenous activation of AhR, its best charac-

terized role is in the xenobiotic response. AhR was first discovered as the main target of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the most infamous of the dioxins. Normal activation of the receptor leads to the expression of oxidizing enzymes as a defensive mechanism against environmental toxins. However, over-activation was proven highly toxic, as exposure to TCDD has been shown to cause chloracne, cancer and teratogenicity.^{7,8} Inactivated AhR is cytosolic and complexed with Hsp90, XAP2 and p23 (Fig. 1). Upon ligand penetration in the cell and binding to AhR, the receptor translocates to the nucleus where the ligand-receptor complex dissociates and subsequently binds the Arnt nuclear protein. This complex then binds AhR responsive elements (AHRE), also referred to as xenobiotic-responsive elements (XREs), upstream of target genes and induces expression of AhR battery genes.⁹ The best known examples of AhR activated genes are the cytochromes CYP1A1, CYP1A2 and CYP1B1, aldehyde dehydrogenase 3 (ALDH3A1), the UDP glucuronosyltransferase UGT1A and glutathione S-transferase alpha 1 (GSTA1).^{10,11}

In an effort to uncover the mechanism of action of schizocommunin and its synthetic analogue, we have performed genomic profiling and functional assays. Herein, we report that schizocommunin is an exogenous activator of the aryl hydrocarbon receptor. Treatments with the compound as well as with a synthetic analogue showed toxicity against cultured Huh7 human hepatoma cells in the low micromolar range. Transcriptomic analysis showed an increase in the expression of CYP1A1, as well as other genes in

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Fig. 1 A) Structure of schizocommunin and B) a synthetic analogue of schizocommunin. C) AhR transduction pathway. Introduction of the ligand in the cell results in translocation of the receptor complex to the nucleus and activation of the AhR battery genes (see text for details).

the AhR pathway with sub-toxic treatments of liver and lung cells. Transcription activation through the xenobiotic response element (XRE) is confirmed using a luciferase reporter assay (Fig. 4). Activation of the aryl hydrocarbon receptor by schizocommunin may be one of the mechanisms through which the *Schizophyllum commune* fungus was able to elicit an allergic response in the bronchopulmonary mycosis case.

Results and discussion

Schizocommunin exhibits toxicity towards Huh7 cells

Uehata *et al.* have previously reported low-micromolar toxicity of schizocommunin and its analogue against HeLa human cervical cancer cells.⁵ Similarly, we observed a dose-dependent decrease in the metabolic activity of Huh7 cells using an oxidoreductase MTT assay (Fig. 2). Treatment with the schizocommunin analogue exhibited an EC_{50} value of 9.6 μ M, indicative of increased cytotoxicity when compared with an EC_{50} of 31.1 μ M for the fungal compound. Interestingly, a

tautomerizing hydroxy analogue of this compound in its keto form did not exhibit cytotoxicity in the previously mentioned study on Hela cells.⁵

Schizocommunin activates the expression of genes in the AhR battery

After determining the EC_{50} of each compound, concentrations of 10 μ M for schizocommunin and 0.5 μ M for the analogue were chosen for further experiments to avoid cytotoxicity while generating maximal cellular effects. A microarray screen was first performed in Huh7 liver cells to assess the effects of the compounds on cellular transcriptome. This allowed the identification of genes differentially regulated at the RNA level, including messenger RNAs and small non-coding RNAs. Fig. 3A shows a heat-map representation of the fold changes of major gene categories. Notably, schizocommunin highly upregulated the expression of cytochrome (CYP) genes. Expression of CYP1A1 and CYP1B1 enzymes was strongly upregulated (10.5 and 8.6-fold

Fig. 2 Cytotoxicity curves for A) schizocommunin and B) the schizocommunin analogue. Cells were treated with compound at concentrations of 0.012 μ M to 200 μ M for 24 hours after which the cell metabolic activity was quantified via MTT assay.

(Huh7) and lung cells (A549), although to a generally lower extent in the latter. CYP1A2 basal expression was too low to accurately quantify. Expression levels of GSTA1 were not noticeably affected in the liver cells and could not be accurately detected in the lung cells. Interestingly, while ALDH3A1 did not change in expression with analogue treatment in the liver cells, we observed a decrease in its levels with schizocommunitin to 0.65-fold expression, but also noted a

Fig. 4 Treatments with schizocommunin and its analogue activate the XRE response element in a dose-dependent manner. Cells were transfected with a XRE-luciferase reporter plasmid and treated for 24 hours with the indicated concentrations of the compound. FICZ is a known activator of the AhR pathway. Signal was normalised over co-transfected Renilla luciferase plasmid and DMSO vehicle control.

significant increase in the lung cells. This points to some variability in the effects of AhR activation in different tissues. The overall lower levels of gene activation by the analogue compound could be due to the lower concentration used in the treatment but could also be reflective of a reduced affinity of the analogue compound for the aryl hydrocarbon receptor. Additionally, the levels of the receptor itself (AhR) and its interacting protein (XAP2) did not change, pointing to a classic xenobiotic ligand activation of AhR. This was confirmed through a xenobiotic response element reporter assay where luciferase expression was indicative of transcriptional activation by induction of the AhR pathway (Fig. 4). Schizocommunin and its analogue activate the receptor to a lesser degree than the potent AhR agonist FICZ; however, they show dose-dependent induction up to 17-fold with 10 μM schizocommunin. Activation of AhR by these compounds is not surprising as a diverse array of polycyclic aromatic hydrocarbon have been shown to affect the pathway.⁹

This study has shed some light on the biological effects of the compounds, but it has also opened an array of further investigative avenues. Notably, activation of the AhR has been linked to modulation of immune and allergic responses. AhR expression is high in lungs, liver and a number of immune cells such as Th17 and Treg CD4 T cells as well as activated dendritic cells and macrophages.¹² TCDD exposure has been shown to be immunosuppressive;¹³ however, the effects of AhR activation have been reported to be ligand specific. Indeed, in a mouse model of autoimmune encephalomyelitis (EAE), while activation of the receptor by TCDD reduced severity of the disease, activation by another ligand, 6-formylindolo[3,2-*b*]carbazole (FICZ), prevented Treg differentiation and worsened the disorder by modulating Th17 cells.^{14,15} Additionally, induction of AhR has been linked to mast cell activation and IgE response by both endogenous and exogenous ligands.¹⁶ For example, kynurenine, a metabolite of tryptophan was shown to enhance mast cell degranulation in a AhR-dependent manner¹⁷ while, from a xenobiotic perspective, treatments with FICZ lead to IL-17 and IL-6 pro-

inflammatory cytokine production in human and murine mast cells.¹⁸ More recently, tryptophan metabolites have also been shown to mediate CD1d-dependent intestinal inflammation *via* AhR in response to oxazole exposure.¹⁹ Furthermore, AhR has been shown to modulate the recruitment of inflammatory leukocytes in response to bacterial virulence factors.²⁰ Therefore, the activation of the pathway by schizocommunin may lead to various direct and indirect immunological effects which may explain the allergic symptoms experienced by the ABPM patient, such as increased serum IgE levels.³

The AhR receptor has different established functions depending on the cellular context. While induction of AhR is a putative mechanism for the development of ABPM, activation of the pathway has also been linked to apoptosis in several cell types. AhR ligands have been shown to cause cell death in colorectal cancer,²¹ primary melanocytes²² and while TCDD is known to prevent apoptosis in some instances, it was shown to activate it in a large number of lineages.²³ It is therefore possible that schizocommunin and its analogue exert some of their cytotoxic activity through AhR.

Potent AhR ligands are useful tools for investigating this detoxifying pathway, as well as signal transduction pathways involving AhR that are medically important. We have shown that schizocommunin provides strong upregulation of AhR battery genes, indicating its potential as an AhR modulating small molecule. Future studies should aim to investigate the relationship between these small molecules and the effects of aryl-hydrocarbon receptor induction in the contexts of immunity and cancer.

Materials and methods

Cell culture and treatments

Human hepatoma (Huh7) or human lung carcinoma (A549) cells were grown at 37 °C in DMEM (Gibco, Life Technologies) with 10% FBS (Wisent) and 10 mM non-essential amino acids. One day prior to treatment, cells were seeded at 2.75×10^5 cells per well in a 6-well plate. The following day, schizocommunin or its analogue were added to final concentrations of 10 μM or 0.5 μM respectively and 0.1% final DMSO. Mock treatment consisted of DMSO only. Cells were incubated with the compounds for 24 hours, after which they were washed with PBS and lysed for RNA isolation. The Huh7 cell line was a gift from Prof. P.G. Schultz (TSRI, USA). Other cell lines were obtained from ATCC.

RT-qPCR

RNA isolation was performed using the RNeasy (Qiagen) isolation kit as per manufacturer's protocol. Reverse transcription was performed using the iScript cDNA Synthesis Kit (Bio-Rad) using 500 ng of RNA as per manufacturer's protocol. Quantitative PCR (qPCR) was performed using SYBR Green Supermix (Bio-Rad) as per manufacturer's protocol on the CFX Real-Time PCR Detection System (Bio-Rad) with the following primer sequences (5'–3'): CYP1A1 fwd TCGGCCACG GAGTTTCTTC, CYP1A1 rev GGTCAGCATGTGCCCAATCA;

CYP1B1 fwd AAGTTCTTGAGGCACTGCGAA, CYP1B1 rev GGCCGGTACGTTCTCCAAAT; ALDH3A1 fwd TGTTCTCCAGC AACGACAAGG, ALDH3A1 rev AGGGCAGAGAGTGCAAGGT; UGT1A fwd TTGTCTGGCTGTTCCCACTTA, UGT1A rev GGTC CGTCAGCATGACATCA; GSTA1 fwd CTGCCCCGTATGTCCAC CTG, GSTA1 rev TCAAAGGCAGGGAAGTAGCG; AhR fwd CAAATCCTTCCAAGCGGCATA, AhR rev CGCTGAGCCTAAGA ACTGAAAG; XAP2 fwd GAAGGGGAGATTGCCCAAGTTC, XAP2 rev CGATGTTGCGGAGACTCTTGG. 18S rRNA was used for normalisation and expression fold changes relative to mock treatments were calculated using the $2^{-\Delta\Delta Ct}$ method. Significance assessed with two-tailed, unpaired student's *t*-test where $p < 0.05$ was considered significant.

MTT cytotoxicity assays

Huh7 cells were cultured and treated as described. One day before treatment, cells were seeded at 10 000 cells per well in a 96 well plate. The next day, cells were treated with a serial dilution of schizocommunin or its analogue for final concentrations between 0.012 and 200 μ M and 2% final DMSO. Cells were incubated with the compounds for 24 hours after which they were washed with PBS and incubated with 50 μ L MTT reagent (2.5 mg mL⁻¹ 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (Sigma-Aldrich) in PBS) for 3 hours. Excess reagent was removed, and crystals were dissolved in 50 μ L DMSO. Absorbance was read at 562 nm using a SpectraMax i3 plate reader (Molecular Devices). Raw values were normalised relative to mock treatment.

Microarray analysis

Huh7 cells were cultured and treated as described. RNA isolation was performed using the RNeasy (Qiagen) isolation kit as per manufacturer's protocol. Expression profiling was performed at the TCAG Facilities of the Centre for Applied Genomics of the Hospital for Sick Children using Affymetrix Human Gene ST.2.0 arrays. Analysis was performed using the Affymetrix Expression Console and Transcriptome Analysis Console. Data is available at NCBI Gene Expression Omnibus, accession number GSE129836.

XRE-reporter assay

Huh7 cells were cultured as described. One day before transfection, cells were seeded at 20 000 cells per well in a 24-well plate. The next day, cells were simultaneously transfected with 50 ng per well pGL4-Renilla and 200 ng per well pGL4.43[luc2P/XRE/Hygro] (Promega) using 1.25 μ L per μ g of DNA Lipofectamine 2000 (Invitrogen, ThermoFisher). The next day, cells were treated with schizocommunin, its analogue or FICZ to final concentrations of 0.1, 0.5, 5 or 10 μ M respectively and 1% final DMSO and incubated for 24 hours. Cells were washed with PBS and lysed in passive lysis buffer (Promega). Luciferase assay was performed in technical triplicates in a 96 well-plate as previously described.²⁴

Conflicts of interest

The authors have no conflicts of interest to declare.

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