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Molecular networking identifies an AHR-modulating benzothiazole from white button mushrooms (*Agaricus bisporus*)

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ABSTRACT

Diet-derived aryl hydrocarbon receptor (AHR) ligands have potential to maintain gut health. However, among the myriad bioactive compounds from foods, identifying novel functional ligands which would significantly impact gastrointestinal health is a challenge. In this study, a novel AHR modulator is predicted, identified, and characterized in the white button mushroom (*Agaricus bisporus*). Using a molecular networking approach, a methylated analog to benzothiazole was indicated in white button mushrooms, which was subsequently isolated and identified as 2-amino-4-methyl-benzothiazole(2A4). Cell-based AHR transcriptional assays revealed that 2-amino-4-methyl-benzothiazole possesses agonistic activity and upregulated CYP1A1 expression. This contrasts with previous findings that whole white button mushroom extract has overall antagonistic activity *in vivo*, underscoring the importance of studying the roles each chemical component plays in a whole food. The findings suggest that 2-amino-4-methyl-benzothiazole is a previously unidentified AHR modulator from white button mushroom and demonstrate that molecular networking has potential to identify novel receptor modulators from natural products.

1. Introduction

The AHR is a highly conserved ligand-based transcription factor which initiates many ligand-specific, cell-specific, environmentally influenced downstream changes in expression of a diverse set of genes (Swanson et al., 1993). The AHR was first discovered and identified based on experiments in genetically modified mouse models where TCDD exposure increased expression of cytochrome P450 genes such as *CYP1A1* and *CYP1B1* (Poland et al., 1974; Robinson et al., 1974). In the intervening years, further research has expanded the role that the AHR plays with epigenetic regulators, acting as a histone acetylation and methylation modulator (Schnekenburger et al., 2007), and the AHR has been implicated in many physiological processes such as in adaptive immunity (Lamas et al., 2018), regulation of circadian rhythm (Salminen, 2023), development of the liver (Moreno-Marín et al., 2018), lipid metabolism (Zhao et al., 2022), adipocyte differentiation (Huang et al., 2022), and the regulation of gut homeostasis (Girer et al., 2020). Therefore, its involvement in the human body is well beyond xenobiotic metabolism, and more functions are continuously being discovered. As multiple endogenous and xenobiotic molecules can alternatively activate or antagonize the AHR, the promiscuity of the AHR can be a double-edged sword: it could cause overactivation of enzyme or cellular activities, but it could also be a powerful gateway through which treatments can reach multiple targets (Shinde & McGaha, 2018).

However, identifying receptor modulating bioactive molecules from complex mixtures is a challenging enterprise. Botanical mixtures contain hundreds or thousands of constituents, and it is infeasible to purify each compound and analyze its bioactivity. The traditional approach for discovering bioactive constituents from these complex systems is a set of related techniques known as "bioassay-guided fractionation (BGF)," which relies on an iterative process of chromatographic separation and re-analysis until a single bioactive molecule is obtained (Bucar et al., 2013; Weller, 2012). While this method has resulted in the discovery of many key bioactive compounds, BGF possesses several limitations including potential loss of activity, failure to isolate a single bioactive constituent, and the omission of minor potent compounds as BGF methods tend to be biased towards more abundant peaks (Inui et al., 2012). Thus, there has been increased interest in approaches that can detect bioactive compounds earlier in the analysis workflow to prioritize discovery efforts. Approaches using chemical profiling techniques, including untargeted LC-MS metabolomics, can be integrated with bioactivity assaying to model potential bioactive compounds (Caesar

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et al., 2018; Kellogg et al., 2016). The introduction of the Global Natural Product Social molecular networking software (GNPS) (http://gnps.ucsd.edu) has facilitated the automatic spectral mining of large metabolomics samples to provide annotation (with reference to the publicly-available GNPS database spectra), as well as proposed annotations of unknown ions, based upon the fragmentation similarity to other ions in the dataset, thus expanding and accelerating identification and dereplication capabilities. Annotation can be further aided using differences in molecular formulas of features (Wang et al., 2016; Yang et al., 2013).

In the current study, we incorporated bioactive molecular networking with untargeted metabolomics to examine the white button mushroom metabolome for additional metabolites that are potential novel AHR ligands. White button mushroom extracts were assayed for their potential to activate the AHR. Then along with a library of known AHR regulators, the mushroom extracts were characterized using ultra-high-performance liquid chromatography tandem mass spectrometry (UHPLC–MS/MS) metabolomics followed by molecular networking to identify molecules with structural similarities to known AHR ligands. Using *in vitro* luciferase gene reporter assays, we confirmed the agonist activity of a previously unknown methylated amino benzothiazole compound found in white button mushrooms. We demonstrated that these advanced profiling techniques represent an improvement to discover potentially novel AHR modulators more efficiently from botanical and dietary sources. This information can then be utilized to select certain functional foods to modify AHR status within the intestinal tract for therapeutic benefit.

2. Materials and methods

2.1. General reagents/materials

All solvents and chemicals used were of reagent or spectroscopic grade, as required, and obtained from VWR (Radnor, PA, U.S.A.) or Sigma Aldrich (St. Louis, MO, U.S.A.). All AHR-active compounds (Table 1) were purchased from Sigma Aldrich and were indicated to have a purity of >97% as determined by HPLC-UV/VIS (data not shown). White button mushrooms (*Agaricus bisporous*) were obtained from Giorgio Foods (Temple, PA, U.S.A.) and freeze-dried according to Tian et al. (2019).

2.2. Liver cell culture

Recombinant cell lines HepG2 40/6 (human hepatoma cell line) and Hepa 1.1 (mouse hepatoma cell line) that respond to TCDD-like chemicals in an AHR-dependent manner, were cultured in Eagle's minimum essential medium containing 10% fetal bovine serum and 1% penicillin (100 units/mL)/streptomycin (100 μg/mL). The cells were maintained at 37 °C in an atmosphere containing 5% CO_2. The cell lines have been generously provided by the Perdew Lab and were developed as previously described (Hubbard et al., 2019).

2.3. Extraction of white button mushroom

Dried mushroom powder (35 g) was extracted with 1 L of methanol (MeOH) in an Erlenmeyer flask on an orbital shaker at 200 rpm overnight at 20 ℃. The maceration was filtered over a Buchner funnel into a new Erlenmeyer flask. The maceration was extracted overnight again a second time, then filtered again the next day. The combined extract was transferred into a round bottom flask, then evaporated by rotary evaporation (Büchi Corporation, New Castle, DE, U.S.A.).

2.4. Fractionation of white button mushroom using HPLC

White button mushroom methanol extract was reconstituted in 50 μL of methanol. The fractionation of WB mushroom was performed on a Waters 2695 HPLC system (Waters Corp., Milford, MA, U.S.A.) with a Phenomenex (Torrance, CA, U.S.A.) HPLC C18 column (4.6 × 150 mm, 5 μm particle size) and a Phenomenex C18 guard cartridge (10 × 4.0 mm, 5 μm particle size). The mobile phase solvent A was 1% formic acid in water, and solvent B was 1% formic acid in acetonitrile. The gradient program was based a previously published work (Tian et al., 2019): 0–10 min, 0–3% B in A; 10–12 min, 3–45% B in A; 12–17 min, 45–90% B in A; and 17–20 min, 90–3% B in A. The injection volume of mushroom extract was 10 μL. Simultaneous monitoring was performed at 254 nm at a flow rate of 1 mL/min. Fractions were collected every 1 min using Waters fraction collector III (Waters).

2.5. Luciferase-based reporter assay

HepG2 40/6 and Hepa 1.1 cells were cultured in 12-well plates at 100,000 cells/well and 150,000 cells/well, respectively. Cells were treated with whole white button mushroom methanol extract (5 μg/ml), the 8th minute HPLC fraction of white button mushroom extract (5 μg/ml), 2-amino-4-methyl-benzothiazole (100 μM), or 2-amino-benzothiazole (100 μM). All treatments were prepared in DMSO. After 5 h continuous incubation at 37 °C and 5% CO2, cells were lysed with 100 μL of reporter lysis buffer and kept in −80 °C for at least 10 min. Cell viability was checked using a microscope. A total of 80 μL of luciferase reporter substrate and 20 μL of lysate from each well were combined, and luciferase activity was determined using a Turner Biosystems TD-20e Luminometer (Turner BioSystems, Inc., Sunnyvale, CA, USA) and reported as relative light units (RLU).

Table 1
Representative AHR-active compounds for model development of bioactive molecular network.

Origin	Compound	Agonist/ Antagonist	Ref.
Microbial	Indole	agonist	(Heath-Pagliuso et al., 1998)
Microbial	Indole-3-acetic acid	agonist	(Heath-Pagliuso et al., 1998)
Microbial	Tryptamine	agonist	(Heath-Pagliuso et al., 1998)
Microbial	Urolithin A	antagonist	(Muku et al., 2018)
Microbial	Urolithin B	antagonist	(Muku et al., 2018)
Host	Kynurenic acid	agonist	(DiNatale et al., 2010)
Host	Kynurenine	agonist	(Mezrich et al., 2010)
Xenobiotic	α-naphthoflavone	antagonist	(Gasiewicz & Rucci, 1991)
Microbial-host metabolite	Indirubin	agonist	(Adachi et al., 2001)
Dietary	Resveratrol	antagonist	(Ciolino et al., 1999)
Dietary	Quercetin	agonist	(Ciolino et al., 1999)
Dietary	Kaempferol	antagonist	(Ciolino et al., 1999)
Dietary	Chrysin	agonist	(Ronnekleiv-Kelly et al., 2012)
Dietary	Apigenin	agonist	(Van der Heiden et al., 2009)
Dietary	Benzothiazole	agonist	(Tian et al., 2019)
Dietary	6-methylisoquinoine	antagonist	(Tian et al., 2019)

2.6. RNA isolation and real time quantitative PCR analysis

RNA was extracted from HepG2 40/6 cells using TRIzol reagent (Invitrogen, Carlsbad, CA). After extraction, the concentration of RNA was determined by NanoDrop (ND-1000, V 3.3) and its quality was confirmed by the 260/280 and 260/230 ratios. cDNA was synthesized from 1 μg of total RNA using MultiScribe reverse transcriptase (AppliedBiosystems). Quantitative real-time PCR was performed using PerfeCTa SYBR Green Supermix for iQ (Quanta Biosciences, Beverly, MA, USA) on a CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Cyp1a1 primers were used in each reaction and all results were normalized to β-*actin* mRNA.

2.7. Mass spectrometry analysis

Ultra-high Pressure (UHP) LC-MS data were acquired using an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Scientific, Waltham, MA, U.S.A.) with an electrospray ionization source coupled to a Vanquish UHPLC system (Thermo Scientific). Injections (5 μL) were separated by reverse-phase UPLC using an Acquity BEH C18 column (150 × 2.1 mm, 1.7 μm particle size (Waters)) held at 55 °C with a flow rate of 100 μL/min. The following binary solvent gradient was employed with solvent A (LC-MS grade water with 0.1% formic acid), and solvent B (LC-MS grade acetonitrile): initial isocratic composition of 97:3 (A:B) for 1.0 min, increasing linearly to 85:15 over 4 min, increasing linearly to 5:95 over 11 min, followed by an isocratic hold at 5:95 for 2 min, gradient returned to starting conditions over 0.1 min and held for 1.9 min. The positive ionization mode was utilized over a full scan of *m/z* 100–1000 with the following settings: spray voltage, 3.5 kV; IT tube temperature, 275 ℃; vaporizer temperature, 75 ℃; sheath gas flow and auxiliary gas flow, 25 and 5 units, respectively.

2.8. MS data processing & molecular networking analysis

Mass spectral data were converted to mzML format using the MSConvert program, part of the ProteoWizard platform (Chambers et al., 2012). Following file conversion, mass spectral data were uploaded to the GNPS data analysis portal in two groups, where one group was the white button mushroom extract, and the second was a library of known AHR standards (Table 1) which were run on the same mass spectrometry system. These two datasets were combined into a single molecular networking dataset using the Metabolomics-SNETS-V2 workflow (release version 30) (Wang et al., 2016). The precursor ion mass tolerance was set to 0.02 Da and a MS/MS fragment ion tolerance of 0.02 Da. A network was then created where edges were filtered to have a cosine score above 0.65 and greater than 5 matched peaks. Further, edges between two nodes were kept in the network if and only if each of the nodes appeared in each other's respective top 10 most similar nodes. The resulting networks were downloaded and imported into Cytoscape (version 3.8, Institute for Systems Biology, Seattle, WA, USA).

2.9. ^{1}H NMR analysis

^{1}H NMR spectra were acquired with a Bruker AVIII-500 (500 MHz, Bruker Corporation, Billerica, MA, USA) equipped with a triple resonance TCI single axis gradient cryoprobe running TopSpin 3.2 operating software. NMR chemical shift values were referenced to residual solvent signals for CD_3OD (δ_H 3.31 ppm). To collect ^{1}H NMR data, each sample was resuspended in CD_3OD (Cambridge Isotope Laboratories, Andover, MA, USA). Spectra were processed and analyzed with MNova 14 (Mestrelab Research S.L., Santiago de Compostela, Spain).

2.10. Statistical analysis

Values are the mean ± standard deviation (SD) or median and interquartile ranges. Graphical illustrations and statistical analysis were performed using GraphPad Prism (version 9.0, GraphPad). Statistical analyses for the *in vitro* experiments were performed using unpaired *t*-test, one-way ANOVA, and Tukey's HSD test on GraphPad.

3. Results

3.1. Bioactive molecular networking of untargeted white button mushroom metabolome

The white button mushroom was extracted in methanol and profiled via UPLC-MS/MS. To discover structural conformers from the white button mushroom that might possess AHR activity, a series of known AHR agonists and antagonists were obtained and MS^2 data for each compound acquired (Table 1). The mushroom extract data and AHR compound data were combined into a single bioactive molecular network (BMN) using the GNPS Metabolomics-SNETS-V2 workflow. The generated BMNs were imported to Cytoscape where nodes were colored based on their occurrence in the two different datasets: blue for ions present in the mushroom dataset, red for those found in the known AHR data, and purple for ions that overlapped between the two sets. The entire molecular network is given in **Figure S1** and showed a series of clusters that suggested additional compounds present in the white button mushroom that are structurally similar to AHR agonists/antagonists. Further evaluation of the network highlighted one particular subnetwork, Fig. 1, from which several benzothiazole derivatives were represented from the library of known AHR-active compounds, and suggesting another ion, similar in structure, but present only in the white button mushroom dataset. This cluster was chosen to pursue for further identification and isolation efforts.

3.2. Identification of a methylated aminobenzothiazole from white button mushrooms

The subnetwork highlighted in Fig. 1 showed a series of structurally related aminobenzothiazole derivatives, three of which were previously reported in white button mushrooms: 2-hydroxybenzothiazole (*m/z*

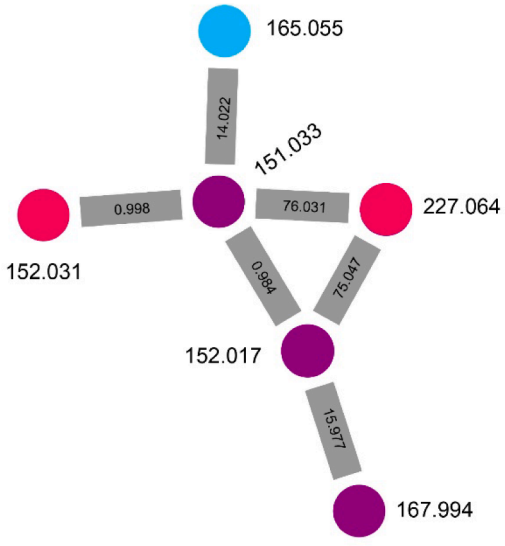

Fig. 1. Molecular networking cluster of benzothiazole derivatives from white button mushrooms. The blue dot represents the *m/z* 165 ion, which was present in the white button mushroom extract; red dots represent benzothiazole derivatives present in the AHR compound library but not present in the mushroom, and purple dots represent benzothiazole present in both the compound library as well as the mushroom. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

152.017), 2-mercaptobenzothiazole (*m/z* 167.994), and 2-aminobenzothiazole (*m/z* 151.033) (Tian et al., 2019). However, there was one ion present in the white button mushroom metabolome that was unknown to us at the time that had significant structural similarities to other aminobenzothiazoles. Based on the molecular mass difference, the unknown structure was suggested to be a methylated 2-aminobenzothiazole derivative (*m/z* 165.055, $\Delta m/z = 14$ Da). The precursor molecular signal was confirmed in the raw MS data (**Figure S2**). To annotate the ion via mass spectrometry, the MS2 spectra were compared against the four main methylation positions on the benzene ring using commercial standards.

However, the MS^2 fragmentation patterns were too similar to provide discrimination between the different methylation positions and the compounds co-eluted from the HPLC and UPLC (**Figure S4**). Therefore, to provide unambiguous structural identification, the unknown methylated aminobenzothiazole was isolated from the crude white button mushroom extract. Using the previously published protocol on HPLC fractionation of white button mushroom extract as a guide (Tian et al., 2019), 50 μg injections of the methanol extract were fractionated on a reverse-phase HPLC, and the commercial methylated aminobenzothiazoles were run to optimize fraction collection. The HPLC chromatograms of the standards and the white button mushroom extract is shown in **Figure S3**. The peaks appearing at the fraction eluting at minute 8 aligned with the peak positions of the standards' chromatograms. This eighth fraction was selected for isolation of the methylaminobenzothiazole isomer of interest. Multiple injections were concentrated together to be characterized via NMR analysis.

Structural confirmation of the aminobenzothiazole isomer was achieved via comparison of ^{1}H NMR signals in the aromatic region (δ_H 6.75–7.75 ppm, Fig. 2). A distinct pattern of 1 proton doublets (δ_H 7.42, $J = 7.8$ Hz and δ_H 7.07, $J = 7.35$ Hz) and triplet (δ_H 6.97, $J = 7.63$ Hz) matched the aromatic proton profile from 2-amino-4-methylbenzothiazole (2A4) (**Figure S5**), leading to the unambiguous assignment of the unknown *m/z* 165.055 peak.

3.3. 2-amino-4-methylbenzothiazole functions as an AHR agonist

Benzothiazoles are an important class of bicyclic heterocycles that have become increasingly attractive for their role in the synthesis of compounds with diverse biological activities, including anti-inflammatory, analgesic (Shafi et al., 2012), and anti-cancer (Chander Sharma et al., 2020). Indeed, 2-aminobenzothiazole serves as the main pharmacophore for riluzole, the only approved drug for late-stage amyotrophic lateral sclerosis (ALS) (Dharmadasa & Kiernan, 2018).

The AHR activity of methylated aminobenzothiazole from white button mushrooms was explored using AHR-responsive *in vitro* luciferase gene reporter assays performed with human (HepG2 40/6) and murine (Hepa 1.1) cell lines. The potent AHR agonist 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and CH223191, an AHR antagonist (Zhao et al., 2010), served as controls. The white button mushroom extract (WBM), crude fraction 8, and 2A4 induced AHR-mediated transcription in both cell lines (Fig. 3**A and 3B**), suggesting they may be activators of both human and mouse AHR. Testing 2A4 in the HepG2 40/6 luciferase reporter line also demonstrated dose-dependent response (Fig. 3**C**). Furthermore, the luciferase expression was significantly inhibited ($p < 0.001$) with the addition of CH223191 (Fig. 3**D**); these results further supported the hypothesis that 2A4 possesses AHR agonist activity.

AHR is one of a family of ligand-activated transcription factors that was traditionally considered a mediator of xenobiotic metabolism. As such, AHR plays a key role in regulating enzymes associated with phase I metabolism, especially transcriptional regulation of *CYP1A1* (Nebert et al., 2004; Shimada et al., 2002). In Hep G2 cells, 2A4 induced *CYP1A1* expression significantly more compared to the vehicle and was higher than the whole white button mushroom extract (WBM, $p < 0.0001$) (Fig. 4).

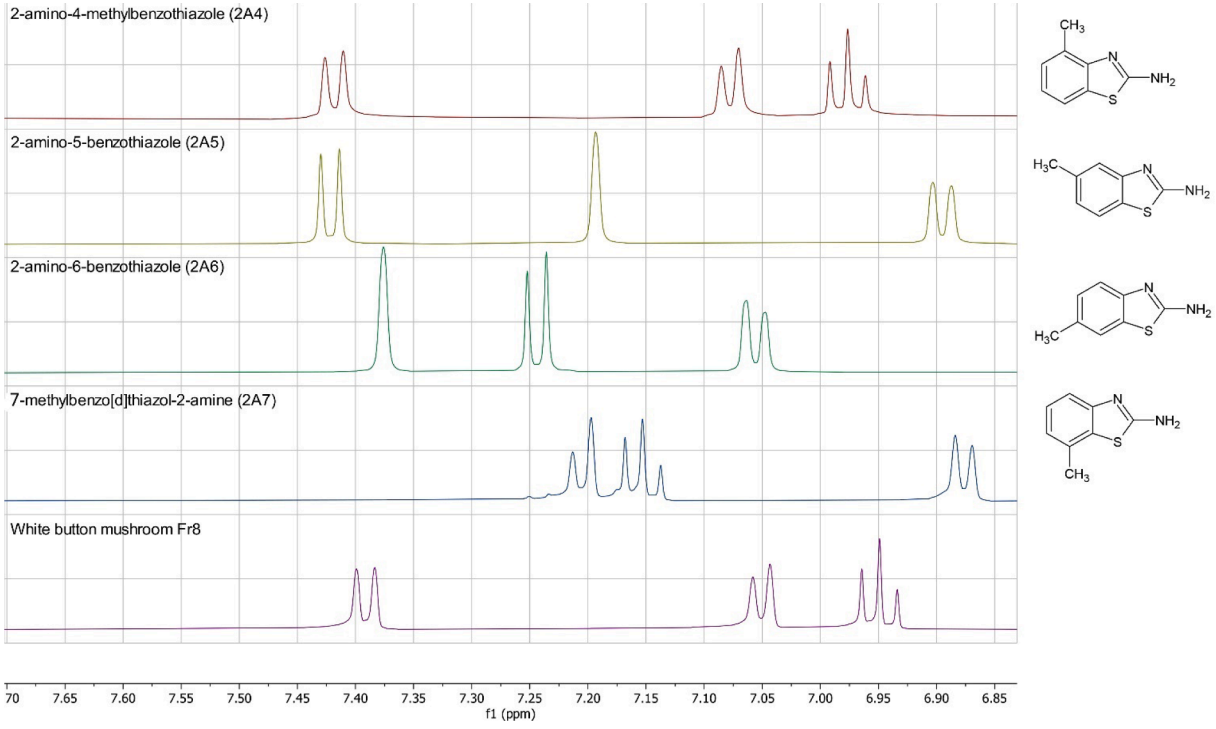

Fig. 2. Aromatic proton ^{1}H NMR spectra from methylated aminobenzothiazole derivatives (benzothiazole analogs 2-amino-4-methylbenzothiazole (2A4), 2-amino-5-methylbenzothiazole (2A5), 2-amino-6-methylbenzothiazole (2A6), 7-Methylbenzo[d]thiazol-2-amine (2A7)) compared to the isolated isomer from white button mushroom (Fr8).

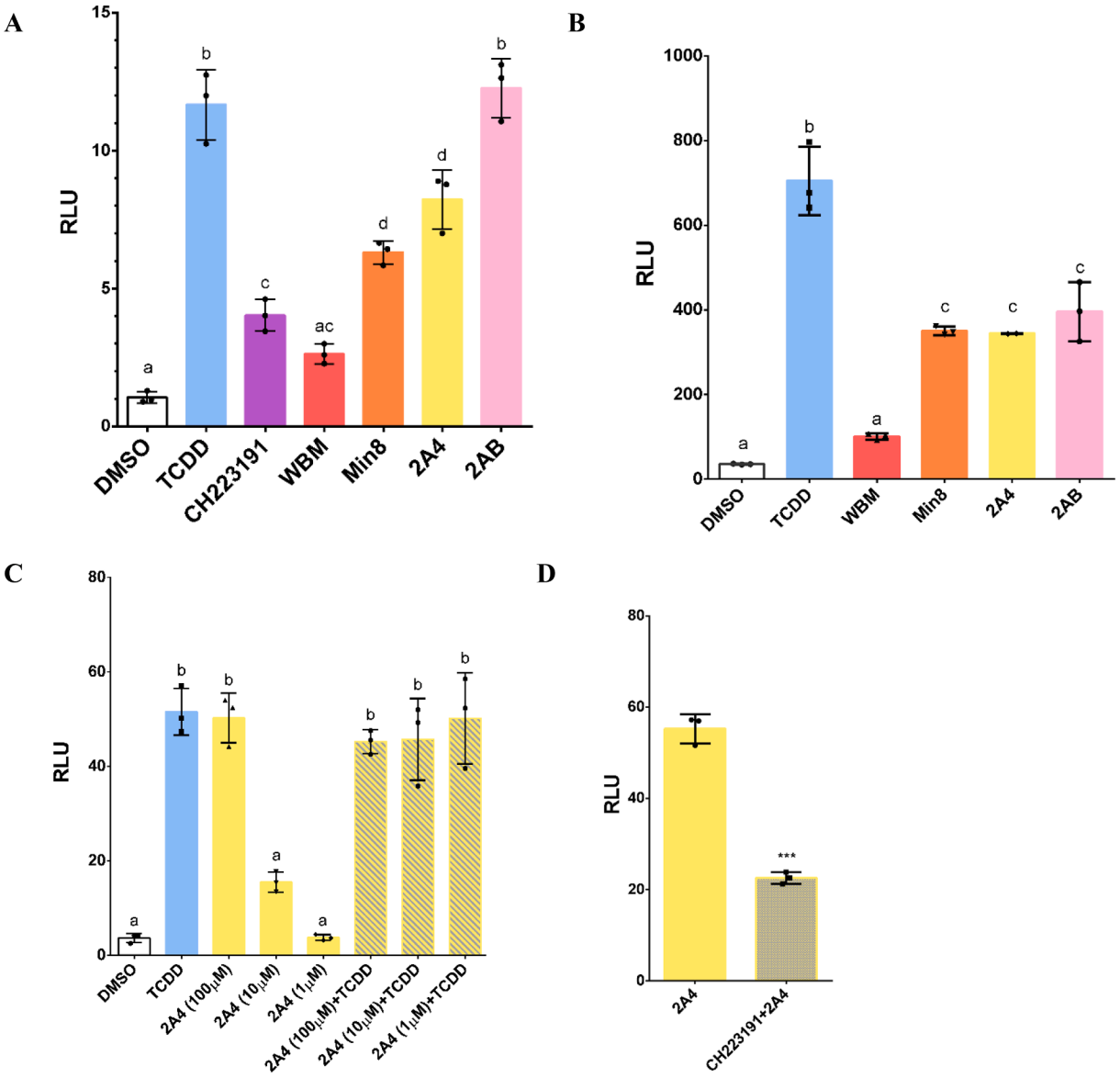

Fig. 3. AHR activity of white button mushroom (WBM) at 5 mg/ml, the crude HPLC fraction (Min8) at 5 mg/ml, and the purified 4-methyl-2-aminobenzothiazole (2A4) and 2-aminobenzothiazole (2AB) at 100 μM in human HepG2 40/6 (**A**) and mouse Hepa 1.1 (**B**) luciferase reporter assays. (**C**) 2A4 demonstrated dose-dependent luciferase activity in the human HepG2 40/6 cell assay (note: samples labeled + TCDD were analyzed as antagonists to TCDD activity). (**D**) Isolated 2A4 was treated with the known AHR antagonist CH223191 showing a reduction in activity. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) served as a positive control, the AHR antagonist CH223191 was a negative control, and DMSO was a solvent control. Values are the mean ± SD of n = 3 per group. Colored bars represent agonist activity and patterned bars represent antagonistic activity. Different letters above bars represent differences ($p < 0.05$) determined by one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test. Asterisks indicate significance measured by student's T-test. *** $p < 0.001$.

3.4. Methylated benzothiazoles evidence differing AHR activity based upon methyl position on the benzene ring

Methylated 2-amino-benzothiazoles with differing substitution patterns were tested for activity with human AHR, to probe the relationship between the position of the methyl group on the benzene ring and its resulting AHR activity. Four 2-aminobenzothiazoles with methyl groups bound to different positions around the aromatic ring were selected (Fig. 2). Structure-function relationships of the methyl amino-benzothiazoles was assessed by the *in vitro* HepG2 40/6 luciferase reporter assay, treating cells for 5 h with 100 μM of 2-amino-4-methylbenzothiazole (2A4), 2-amino-5-methylbenzothiazole (2A5), 2-amino-6-methylbenzothiazole (2A6), or 7-Methylbenzo[d]thiazol-2-amine (2A7) individually. All four analogs demonstrated significant increased AHR activity compared to the solvent control (Fig. 5**A**), and none of them reduced the activity of TCDD, suggesting a lack of antagonist activity (Fig. 5**B**). Out of the four methylated amino-benzothiazole derivatives, 2A4 and 2A7 possessed the greatest agonist activity. These two are methylated at the ortho position in relation to the thiazole moiety, whereas 2A5 and 2A6 have a *meta*-substituted benzene ring. However, the methylation position appears to reduce overall activity of the benzothiazole ring, as the unsubstituted 2-aminobenzothiazole (2AB) demonstrated the highest AHR agonist activity (Fig. 3). At 100 μM, 2AB reported a greater fluorescence than the 5 nM TCDD control, and was similar to previously reported trends (Tian et al., 2019).

4. Discussion

While nearly 50 years have passed since the discovery of the AHR (Poland et al., 1974), there remains much to understand about its

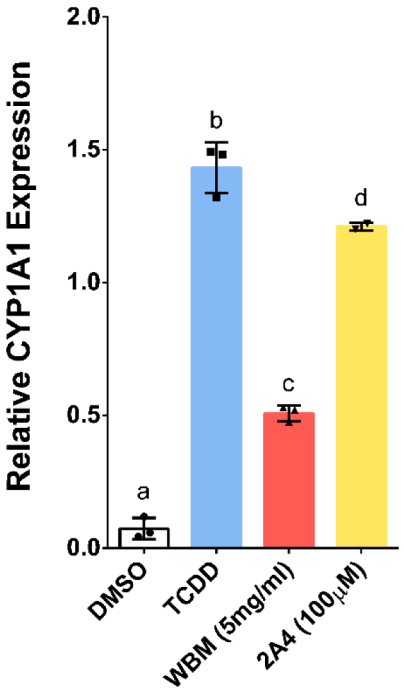

Fig. 4. Induction of CYP1A1 expression by white button mushroom (WBM) and 4-methyl-2-aminobenzothiazole (2A4) in human Hep G2 cells. Relative expression value calculated as CYP1A1/β-Actin starting quantity. TCDD served as a positive control and DMSO as solvent control. Values are the mean ± SD of n = 3 per group. Different letters above bars represent differences ($p < 0.05$) determined by one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test.

mechanisms of action, its endogenous roles within the body, with new agonist and antagonist ligands being discovered every year. Activation of the AHR has been discovered from a diverse array of food products, including coffee, corn, bell peppers, and mushrooms (Amakura et al., 2008; Jeuken et al., 2003; Tian et al., 2019; Van der Heiden et al., 2009). However, the identity of the individual phytochemicals responsible for this activity remains, mostly, unknown; this information is critical to understand how diet can be utilized to modulate AHR activity and contribute to downstream effects. The current study identified additional AHR modulating chemicals from white button mushrooms, increasing our understanding of the diversity of potential AHR modulators in white button mushrooms and building on previous studies that demonstrate how mushrooms could play a role in AHR modulation.

Dietary phytochemicals represent one of the major exogenous sources of AHR-activating ligands. Indole-3-carbinol, resveratrol, curcumin, flavonoids such as quercetin, and carotenoids such as canthaxanthin are reported to be AHR ligands (Bjeldanes et al., 1991; Goya-Jorge et al., 2021; Ito et al., 2007). Products of tryptophan metabolism by both host cell enzymes and the gut microbiota have also been identified as AHR ligands (DiNatale et al., 2010; Zelante et al., 2013). Plant based AHR ligands can be found in micro molar quantities in plasma post consumption, which is within range of the concentration of known phytochemicals that act as an agonist/antagonist (Amakura et al., 2008; Paganga & Rice-Evans, 1997). In fact, the white button mushroom (*Agaricus bisporus*) was found to contain multiple AHR antagonists, reflected in the *in vitro* data in which treatments on hepatocarcinoma and Caco2 cells revealed reduced AHR target gene expression (Tian et al., 2019). In the same study, white button mushroom also was indicated to have similar physiological effects in mouse models. Both conventional and germ-free mice showed reduced AHR target gene expression in mice who were fed white button mushrooms (Tian et al., 2019). Interestingly, the study also observed that the effect only appeared in the small intestine, possibly indicating tissue and context specific effects of diet derived ligands. It was hypothesized that AHR may play a role in maintaining homeostasis in the small intestine based on the previous publication with mice fed white button mushrooms (Varshney et al., 2013). Altogether, this demonstrates great potential for additional research in dietary derived AHR modulators for therapeutic use. Most studies conducted so far have focused on AHR activation/inactivation via dietary interventions with a limited number of foods (e.g., broccoli, mushrooms, etc.); however, the identity of individual AHR-active

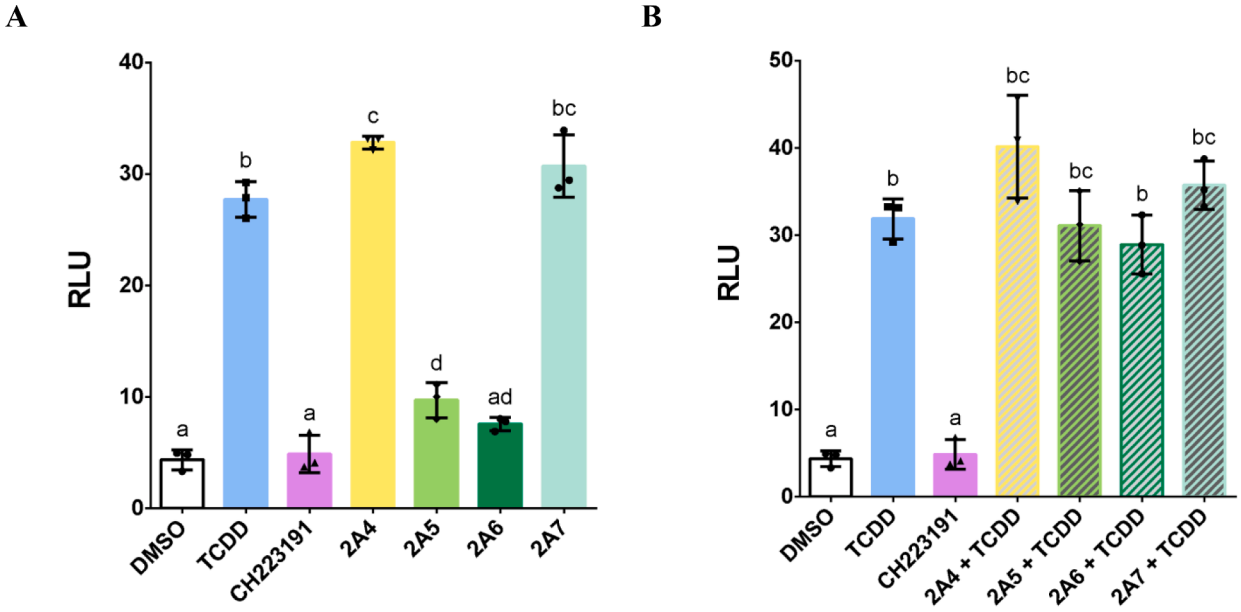

Fig. 5. AHR agonist activity (measured as relative light units, RLU) of benzothiazole analogs 2-amino-4-methylbenzothiazole (2A4), 2-amino-5-methylbenzothiazole (2A5), 2-amino-6-methylbenzothiazole (2A6), 7-Methylbenzo[d]thiazol-2-amine (2A7) (**A**). Antagonist activity of the benzothiazole analogs was also determined (**B**). Samples were tested at 100 uM in the human G2 40/6 luciferase reporter assay, with TCDD and CH223191 as positive and negative controls, respectively, and DMSO as solvent control. Values are the mean ± SD of n = 3 per group. Colored bars represent agonist activity and patterned bars represent antagonistic activity. Different letters above bars represent differences ($p < 0.05$) determined by one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test.

phytochemical compounds have, in large part, not been identified. This information is critical to understand how the diet exerts influence over AHR activation on a molecular level.

However, an inherent limitation of this approach is the reliance of the molecular networking and annotation algorithms on known ligand structures input into the databases; it is not optimized to identify entirely new structural classes or novel compounds. As demonstrated in this study, this can effectively be addressed by combining known mass spectra data and analyzing their structural similarities to predict new receptor modulators, such as the 2-amino-4-methylbenzothiazole (Fig. 1). The chemical fingerprints obtained from metabolomics studies can be integrated with bioactivity data, and by modeling the changing behavior as chemical composition varies across fractions, extracts, or taxa, we are able to highlight the most correlative and co-varying features from the metabolomics dataset. This process, known collectively as "biochemometrics" would enable heightened exploration and network building (Caesar et al., 2018; Kellogg et al., 2016), and will be the focus of future directions for further elucidating and prioritizing phytochemical compounds for AHR activation. Having confirmed the novel AHR modulator to be 2-amino-4-methylbenzothiazole (Fig. 2), and its strong *in vitro* agonistic activity (Fig. 3) as well as high levels of *CYP1A1* expression (Fig. 4), further work is needed to elucidate the effects of benzothiazole derivatives *in vivo*. Moreover, the location of the methyl group on the benzene ring of the different isomers of 2-amino-methylbenzothiazole was found to significantly affect its AHR modulating activity (Fig. 5). To this end, additional investigation is needed in the structure–activity relationship that structural modifications, even slight ones, can have on AHR activation.

In conclusion, these findings delineate an innovative networking approach to identify novel AHR ligands. The method is based on molecular network analysis, thus allowing for a more comprehensive view of possible structures that may serve as AHR regulating compounds. Additionally, by combining known AHR modulator spectra and white button mushroom derived compound spectra, this approach rapidly identified structural families which overlap between the two groups, and more importantly, highlighted structurally similar isomers which have yet to be identified as AHR ligands. By leveraging the massive amount of untargeted metabolomics data as well as powerful networking tools, it is possible to explore and identify novel modulators without the time-consuming process of bioassay guided fractionation. An additional benefit is that this approach is receptor agnostic; this method can readily be applied in exploring novel regulators for other receptors, including the liver X receptor (LXR), farnesoid X receptor (FXR), and peroxisome proliferator-activated receptors (PPARs). As more research is progressing in identifying plants with active compounds that mediate lipid metabolism pathways (Li et al., 2020), network analysis on compounds from plants could allow for dereplication of therapeutics leads.

Ethics Statement

No animal or human subjects were utliized in this study.

CRediT authorship contribution statement

Xiaoling Chen: Conceptualization, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Andrew D. Patterson:** Conceptualization, Resources, Writing – review & editing. **Gary H. Perdew:** Conceptualization, Resources, Writing – review & editing. **Iain A. Murray:** Investigation, Resources, Writing – review & editing. **Joshua J. Kellogg:** Conceptualization, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Joshua Kellogg reports financial support was provided by National Institute of Food and Agriculture. Gary Perdew reports financial support was provided by National Institute of General Medical Sciences. Gary Perdew reports financial support was provided by National Institute of Environmental Health Sciences. Andrew Patterson reports financial support was provided by National Institute of Environmental Health Sciences.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2023.105602.

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