Toxicology Reports 14 (2025) 101883

Contents lists available at ScienceDirect

Toxicology Reports

journal homepage: www.elsevier.com/locate/toxrep

Exploring the biological impact of bacteria-derived indole compounds on human cell health: Cytotoxicity and cell proliferation across six cell lines

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ARTICLE INFO

Handling Editor: Dr.Lawrence Lash

Keywords:
Indole derivatives
Cytotoxicity
Gut microbiota
Tryptophan metabolism
AhR activation

ABSTRACT

Over the past two decades, research has increasingly focused on the interactions between diet, gut microbiota, and host organisms. Recent evidence suggests that tryptophan, an essential amino acid, can be metabolized by gut microbiota into indoles, which have significant biological effects. However, most research is limited to indole and its liver metabolite, indoxyl sulfate. This study examines the cytotoxic effects of five indole derivatives — indole-3-carboxylic acid (I3CA), indole-3-aldehyde (I3A), indole-3-acetic acid (IAA), indole-3-propionic acid (IPA), and 3-methylindole (skatole, 3-MI) — on six human cell lines: adipose-derived mesenchymal stem cells (MSC), hepatocellular carcinoma (HepG2), liver progenitor cells (HepaRG), colorectal carcinoma cells (Caco-2), breast cancer cells (T47D), and lung fibroblast (MRC-5). Results show no sensitivity to indole itself across cell lines. MRC-5 was sensitive to all other compounds (EC50 0.52–49.8 μM). MSCs responded to IPA, I3CA, I3A, and 3-MI (EC50 0.33–1.87 μM), while HepaRG cells were affected by IAA, I3CA, I3A, and 3-MI (EC50 1.98–66.4 μM). T47D cells were sensitive to IPA and IAA, and Caco-2 cells only to IAA (EC50 2.02, 1.68, 0.52 μM, respectively). HepG2 cells showed no change in viability. AhR activation in HepG2-AhR-Lucia cells was triggered by all derivatives, particularly I3A, IPA, and I3CA. Growth experiments revealed I3CA decreased Caco-2 proliferation while increasing T47D proliferation. The findings suggest indole derivatives are generally non-cytotoxic to carcinomas but may adversely affect stem cells, with effects varying across cell lines.

1. Introduction

Tryptophan (Trp) is an essential amino acid obtained from diet, but once in the gut, it can be metabolized by microbes into over 25 metabolites [1]. Most of the Trp is processed through the kynurenine pathway, but it can also be transformed into serotonin, tryptamine, and a series of indole derivatives [1,2]. While the enzymatic production mechanisms of these indoles are well studied [3,2,1,4], the biomolecular and cellular interactions with these indole derivatives remain elusive.

Indoxyl sulfate is well documented for its toxic effects as a uremic bioproduct [4], and indole-3-carbinol has been studied as it can be obtained from cruciferous vegetation [2]. However, the biological roles and effects of other indole derivatives, such as indole, indole-3-carboxaldehyde (I3A), indole-3-carboxylic acid (I3CA), indole-3-propionic acid (IPA), indole-3-acetic acid (IAA), and 3-methylindole (3-MI), remain poorly understood. Illustrated in Fig. 1, these compounds maintain the basic indole bicyclic structure but differ in the functional groups attached to the third carbon.

Over 85 species of both gram-positive and gram-negative bacteria possess genes that express tryptophanase A (TNA), an enzyme responsible for metabolizing tryptophan into indole derivatives [5]. The phylum Firmicutes encompasses many bacterial families containing the TNA gene. Withing that phylum, the *Clostridium* species is particularly well-documented in their ability to convert tryptophan into a range of indole derivatives including: indole-3-acetic acid (IAA), indole-3-propionic acid (IPA), indole-3-aldehyde (I3A), indole-3-acetamide (IAM), indole-3-acetaldehyde (IAAld), indole-3-pyruvic acid (IPyA), indole lactic acid (ILA), 3-methylindole

List of Abbreviations: 3-MI, skatole, 3-methylindole; AhR, aryl hydrocarbon receptor; Caco-2, colorectal carcinoma cells; HepaRG, liver progenitor cells; HepG2, liver carcinoma cells; HeG2-AhR-Lucia, liver carcinoma cells transfected with AhR-luciferase gene; I3A, indole-3-carboxaldehyde; IAA, indole-3-acetic acid; I3CA, indole-3-carboxylic acid; I3P, indole-3-propionic acid; MRC-5, lung fibroblasts; MSC, mesenchymal stem cells; T47D, ductal breast carcinoma cells.

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https://doi.org/10.1016/j.toxrep.2024.101883
Received 4 September 2024; Received in revised form 18 December 2024; Accepted 23 December 2024
Available online 24 December 2024
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(3-MI), and indole. Other key phyla in Trp metabolism include Bacteroidetes, Actinobacteria, and Proteobacteria [6-8,4].

While the mechanisms by which bacteria metabolize indole into these various derivatives remain unclear, research suggests that diet significantly impacts microbial Trp metabolism [6,8]. Studies have demonstrated that indoles influence the diversity of the microbiome [4, 8], contribute to biofilm and cellular barrier formation [4,5], and play roles in cellular communication and proliferation [5,8,4].

Moreover, prebiotic and probiotic supplementation has been shown to enhance tryptophan metabolism, while a reduction in tryptophan-metabolizing bacteria is associated with colorectal cancer [6]. However, most studies investigating the effects of indoles focus on a single indole compound and are conducted in vivo, indicating a need for broader research into the role of these metabolites in human health [4].

Many indole derivatives serve as the precursor for macromolecules designed for cancer treatment [9-11]. However, the stability of these drugs in practice and the effects of their metabolites in the human body are not well characterized. Additionally, it is unclear if the anticancer properties are derived from the indole metabolite or the other attachments. Although only a tiny percentage of Trp is converted to indole derivatives, the metabolic balance of indole metabolites could significantly impact gut and liver health and the progression or inhibition of carcinomas [1]. Literature suggests that indoles and derivatives are protective, but the distinction between healthy and cytotoxic concentrations remains ambiguous [2,12].

In this study, six cell lines were selected: Caco-2 (colorectal adenocarcinoma), HepaRG (hepatic progenitor), HepG2 (hepatocellular carcinoma), MRC-5 (lung fibroblast), MSC (mesenchymal stem cells), and T47D (ductal carcinoma). Given that indole compounds are produced in the gut, colon cells are expected to encounter the highest concentrations. Caco-2 cells were used to model colon tissue for cytotoxicity and proliferation assays and to test the indoles' possible anticancer properties. Previous studies indicate that in Caco-2 cells, IPA is protective against radiation [13], 3-MI decreases cell viability [14], I3CA has no effect on cell viability, and indole can induce toxic effects at 5 mM [15]. However, no studies were found investigating the proliferation rates of Caco-2 cells with indole derivative exposure. This study aims to address this knowledge gap. Indoles produced in the gut are believed to have a strong relationship with the liver in managing and restoring hepatotoxic effects [2]. HepaRG and HepG2 cells were selected to model liver tissue and liver carcinoma cells, respectively, to compare the effects on stem-like cells versus carcinomas and to assess hepatotoxicity. While indole derivative hepatic toxicity/protection is commonly modeled with mice there are a limited number of studies using human liver cell lines to assess and compare cytotoxicity. We aim to shed light onto this understudied topic.

Furthermore, the HepG2-AhR Lucia™ cell line was employed to investigate the degree of AhR activation to the six indole derivatives, given the known relationship between indoles and AhR. Although there is a known relationship between AhR and indole-based compounds, no studies were found comparing the potency of AhR activation across indole derivatives. In this work, we seek to address this disparity. Historically, indole toxicity, such as 3-MI, has been documented in respiratory contexts [16]. MRC-5 cells were used to determine if similar toxicity is observed with other indole forms. Lastly, T47D cells were included to explore the anticancer effects of indole derivatives further. MFC7 (murine breast cancer), and MDA-MB-231 (triple negative human breast cancer) are commonly studied in indole-based cancer treatment research, but little is known about the indole derivative affects. Furthermore, T47D is a human cell line similar to MFC7, but it is unclear if there are differences in breast cancer behavior across species.

In vitro cell systems may be important for evaluating the toxicity and mechanisms of action of bacterial indoles because they provide a controlled and manipulable environment to study these compounds at the cellular and molecular levels. These systems allow researchers to isolate the effects of indoles on specific cell types, enabling detailed

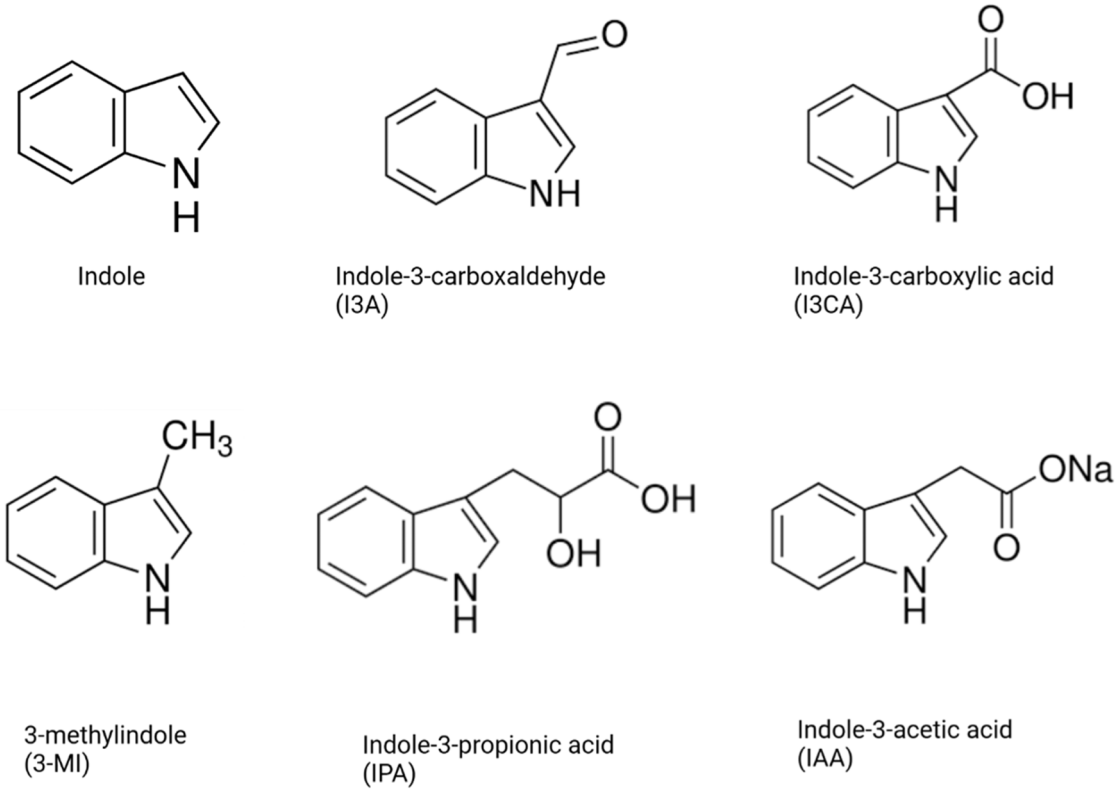

Fig. 1. Chemical structure of indole and derivatives. The chemical structures of the indole derivatives studied in this research: indole-3-carboxylic acid (I3CA), indole-3-aldehyde (I3A), indole-3-acetic acid (IAA), indole-3-propionic acid (IPA), and 3-methylindole (skatole, 3-MI).

investigation into how these compounds interact with cellular pathways, affect gene expression, and induce cytotoxicity. Additionally, *in vitro* assays facilitate high-throughput screening, allowing for the efficient evaluation of multiple indole compounds or concentrations, which accelerates the toxicity profiling and mechanistic understanding necessary for developing therapeutic strategies or assessing health risks.

This study aimed to systematically investigate the effects of six indole-based compounds - indole-3-acetic acid (IAA), indole-3-aldehyde (I3A), indole-3-carboxylic acid (I3CA), indole-3-propionic acid (IPA), indole, and 3-methylindole (3-MI) - on a diverse range of human cell lines, including Caco-2 (colorectal carcinoma), HepaRG (liver progenitor), HepG2 (hepatocellular carcinoma), HepG2-AhR Lucia™ (a gene-reporter cell line for aryl hydrocarbon receptor activity monitoring), MRC-5 (lung fibroblast), and T47D (breast carcinoma). The study focused on evaluating the cytotoxicity, aryl hydrocarbon receptor (AhR) activation, and proliferation effects of these compounds. Although some studies have explored the use of CaCo-2 cells with indole derivatives such as IPA, 3-MI, and IAA, the available data remains limited. This study aims to elucidate the effects of a wide range of indole derivatives on cytotoxicity and cell proliferation across various concentrations and cell lines. Notably, this is the first known study to employ MSC, T47D, HepG2, and HepaRG cell lines to model the effects of indole derivatives on stem-like cell, breast cancer, and liver toxicity. Furthermore, no prior research was found investigating the effects of IPA or I3CA on MRC-5 cells.

By conducting viability assays, the research sought to determine the concentration-dependent cytotoxicity of these indole derivatives, providing a comprehensive understanding of their toxicological profiles. Additionally, the study investigated the activation of AhR by these compounds using HepG2-AhR Lucia™ cells to understand their role in modulating detoxification pathways and other AhR-regulated processes. Furthermore, the study aimed to explore the impact of indole derivatives on cell proliferation across different cell lines to understand their effects on cancerous versus non-cancerous cells. This involved growth and proliferation assays to identify whether these compounds promote or inhibit cell growth. By comparing the effects across six distinct cell types, the research aimed to identify patterns of sensitivity and resistance, offering insights into how structural differences in indole compounds influence their biological activity.

Finally, the study sought to assess the broader implications of its findings for human health, particularly concerning dietary indoles and their metabolites produced by gut microbiota. These findings provide a foundation for evaluating health risks and potential benefits associated with indole exposure, supporting both risk assessments and further studies on the health effects of these compounds. Given that indole derivatives influence gut health, immunity, and cellular processes associated with disease prevention, this research may impact dietary recommendations on a global scale. Understanding the role of diet and probiotics in shaping gut microbiota and tryptophan metabolism could lead to more effective, preventive dietary strategies for maintaining gut health. Additionally, the study's insights into cytotoxicity and proliferation in various human cell types, including cancerous and stem cells, contribute valuable knowledge to toxicology, pharmacology, and cancer research. These findings may help guide the development of therapeutic interventions that harness the unique properties of indole derivatives, advancing safer and more effective approaches to cancer treatment and gut health management worldwide.

2. Material and methods

2.1. Chemicals and reagents

Leibovitz's L-15, William's E Medium, Glutamax™, Eagle's minimum essential media, L-glutamine, penicillin G, streptomycin, phosphate-buffered saline (PBS), and trypsin-EDTA were obtained from Gibco Life Technologies (ThermoFisher Scientific, Waltham, MA). Sterile RPMI culture medium, Triton™ X-100, and sodium heparin were obtained from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS) was obtained from Atlas Biologicals (Fort Collins, CO). Deionized water (DI water) was obtained using a Milli-Q water purification system (Millipore, Billerica, MA). Standards for the indole derivatives (indole-3-carboxaldehyde, indole-3-propionic acid, indole, 3-methylindole, indole-3-acetic acid, indole-3-carboxylic acid) were purchased from TCI Chemicals (Tokyo, Japan). (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) was obtained from Invitrogen (Carlsbad, CA).

2.2. Cell culture and exposures

All cell lines were grown at 37 °C, 5 % CO_2, and positive humidity, and all growth media were supplemented with 2 mM L-glutamine, 50 U/mL penicillin G, and 50 µg/mL streptomycin. HepG2 and MRC-5 were cultured in Eagle's Minimum Essential Medium (EMEM), 10 % fetal bovine serum (FBS), while Caco-2 utilized EMEM with 20 % FBS. T47D was grown in RPMI-1640, 0.2 units/mL human insulin, and 10 % FBS (Table 1). Adipose-derived mesenchymal stem cells (MSCs) were obtained from ATCC and cultured in mesenchymal stem cell basal media supplemented with 2 % FBS. HepaRG cells were cultured in Williams E, 2.2 g/L sodium bicarbonate, 2 mM Glutamax, 10 % FBS, 5 µg/mL human insulin, 50 µM hydrocortisone hemisuccinate for approximately 4 weeks or until 90–100 % confluent. Then, cells were differentiated with growth media supplemented with 1.7 % dimethyl sulfoxide (DMSO).

Previous studies have reported that using a serum-supplemented medium during *in vitro* chemical compound exposure may mitigate toxicity by reducing cellular uptake [23]. As a result, the growth medium was replaced with FBS-free media before administering the selected indole metabolite or solvent control medium. As demonstrated by Solan et al. [23], cells can endure for 72 h without FBS despite FBS containing growth factors and other elements crucial for cell attachment, expansion, maintenance, and proliferation [24]. The experiments were performed with biological (96 well plates) and technical (wells)

Table 1
Human cell lines used in this study (ATCC: American Type Culture Collection. EMEM: Eagle's minimum essential medium. FBS: fetal bovine serum).

Cell line	Cell type	Provider	Culture Media	Ref.
Caco−2	Enterocytes	ATCC	EMEM + 20 % FBS	Fogh et al., [17]
HepG2	Hepatocytes	ATCC	EMEM + 10 % FBS	Arzumanian et al., [18]
HepaRG	Hepatocytes	BioPredic International	William's E Medium + 2 mM Glutamax + 10 % FBS + 5 µg/mL insulin + 50 µM hydrocortisone hemisuccinate	Gripon et al., [19]
MSC	Mesenchymal stem cells	ATCC	Mesenchymal stem cell basal medium + 2 % FBS	Li et al., [20]
MRC−5	Epithelial	ATCC	EMEM + 10 % FBS	Jacobs et al. [21]
T47D	Epithelial	ATCC	RPMI−1640 medium + 0.2 units/mL insulin + 10 % FBS	Judge, Chatterton [22]
HepG2-LUCIA™	HepG2 reporter gene cells for AhR activity	InvivoGen	EMEM + 10 % FBS + 1X non-essential amino acids medium + 100 µg/mL Normocin + 100 µg/mL Zeocin	Gao et al., [3]

replicates. Three to four biological with four technical replicates were used to determine cytotoxicity. Indole exposure concentrations were assessed from a very low human serum-relevant concentration (10^{-12} M) to a high non-human-relevant concentration (10^{-4} M). The ten concentrations tested were 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} M. Following dosing, plates were returned to the incubator for a 24 h exposure period.

2.3. Cytotoxicity

Cytotoxicity was assessed with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Cells were seeded onto 96 well plates and allowed to reach 60–70 % confluency. In which cells were dosed with indole compounds for 24 hours. At the 20-hour exposure mark, MTT was applied to the cells and incubated for 4 hours. Then, the MTT-exposure media solution was removed, and formazan crystals that formed during MTT mitochondrial metabolism were dissolved with a 1:1 DMSO-ethanol solution. The color change between metabolized and unmetabolized MTT was quantified by measuring optical density (OD) at 595 nm with a spectrophotometer (BioTek SynergyTM H1, Winooski, VT, USA) and correcting for background noise at 650 nm.

2.4. Aryl hydrocarbon receptor activation

HepG2-AhR Lucia™ reporter cells (InvivoGen, San Diego, CA) were used to evaluate the AhR activation of indole metabolites. Cells were grown and maintained according to the manufacturer's instructions in Eagle's minimal essential medium, 10 % FBS, 1X non-essential amino acids medium, 100 μg/mL Normocin™ (InvivoGen), and 100 μg/mL Zeocin (Invivogen). The cells were incubated in a humidified atmosphere at 37 °C and 5 % CO_2. The AhR activity was measured using the luciferase reporter assay method according to the manufacturer's instructions. Briefly, to determine agonistic activity, 20 μL of the compound of interest was added per well in duplicates, and 2×10^4 cells in 180 μL were added to a 96-well flat-bottom plate. 2,3,7,8-Tetrachlorodibenzodioxin (TCDD), benzo[*a*]pyrene (BaP), and β-naphthoflavone (BNF) were used as positive controls for agonistic activity. The cells were incubated at 37 °C and 5 % CO_2 in a humidified atmosphere. After 24 h, 20 μL supernatants were transferred into a white 96-well plate, followed by the addition of 50 μL QUANTI-Luc (InvivoGen). The luminescence was measured on a Varioskan LUX Multimode Microplate Reader (ThermoFisher).

2.5. Cell proliferation and migration

Cell proliferation and migration with indole and derivatives exposure were assessed in T47D and Caco-2 cell lines with modified methods described in Zheng et al. [25]. Cells were seeded into a 48-well plate at 100,000 cells/mL and allowed 24 h to stabilize or until reaching 90–100 % confluency. Upon reaching 90–100 % confluency, the monolayer was gently disrupted by carefully dragging a pipette tip vertically to create a cell-free space, ensuring that neighboring cells remained attached. Then, the growth media was replaced with exposure media supplemented with 0.1, 1, or 10 μM indole or indole derivative. Cytochalasin D (1 mM) was used to inhibit cell proliferation and served as a positive control. Growth was recorded using an inverted phase-contrast automated microscope Lionheart FX (BioTek, Winooski, VT) at 30 min intervals for 24 h and quantified using Gen 5 version 3 software (BioTek).

2.6. Indole derivatives quantification in cell media

Indole derivatives were analyzed by high-performance liquid chromatography (HPLC) coupled with an RS fluorescence detector (UltiMate 3000, Thermo Fisher Scientific, Waltham, MA) following a modified method from Tuomola et al. [26] and [27]. An AcclaimTM VANQUISH C-18 column (2.2 μm, 150 ×2.1 mm; ThermoFisher Scientific, Waltham, MA) was used for chromatographic separation. The binary gradient system consisted of eluent A (50 mM potassium dihydrogen phosphate in water adjusted to pH 3.3 with phosphoric acid) and eluent B (700 mL eluent A + 300 mL acetonitrile). Gradient elution was performed according to the following elution program: 0–2 min, 80 % A, 20 % B; 2–10 min, 25 % A, 75 % B; 10–10.1 min, 0 % A, 100 % B; 10.1–15 min, 0 % A, 100 % B; 15–15.1 min, 80 % A, 20 % B; 15.1–20 min, 80 A, 20 % B. The gradient applied was linear; the flow rate was 0.5 mL/min. The chromatography was performed at 30 °C. The native fluorescence of indolic compounds was monitored by using an excitation wavelength of 270 nm and an emission wavelength of 350 nm. Indole derivatives were identified by co-chromatography with authentic standard compounds and quantified by integrating the area under the peaks.

2.7. Statistical analysis

Before conducting statistical analysis, all data underwent preprocessing to ensure adherence to the homoscedasticity and normality assumptions requisite for parametric tests. Statistical analyses and EC_{50} calculations were executed utilizing GraphPad Prism version 9 (GraphPad Software, San Diego, CA). Cytotoxicity data were fitted to a sigmoidal curve and a non-linear logistic model. EC_{50} values were derived from the average cytotoxicity data of three independent experimental trials, along with their corresponding errors. The EC_{50} values are presented alongside 95 % confidence intervals (95 % CI). The mean $LogEC_{50}$ values of two distinct groups were juxtaposed utilizing an independent *t* test, with significance set at a *p* value of 0.05.

3. Results

3.1. Indole derivatives quantification in cell media

After 24 h of incubation, the quantification of indole metabolites in the cell media showed no significant differences between the nominal and actual concentrations. Most indole metabolites closely matched their nominal concentrations, with recovery rates ranging from 83.9 ± 9.6 % to 103.6 ± 12.1 %. These values indicate only minor discrepancies that were not statistically significant. Notably, indole had a recovery rate exceeding 100 %, which suggests slight variability, possibly due to measurement inaccuracies or experimental conditions.

3.2. Cytotoxicity

The EC_{50} values for indole exposure across all cell lines were consistently above 100 μM, indicating a lack of sensitivity to this

Table 2
Cytotoxic evaluation (EC_{50}) in μM following 24 h exposures of several indole metabolites on different cell lines: enterocytes (Caco-2), hepatocarcinoma cells (HepG2), hepatocytes (HepaRG), lung fibroblasts (MRC-5), mesenchymal stem cells (MSC), and breast epithelial (T47D). Data are presented as average ± standard deviation (n = 8–16). IPA: Indole-3-propionic acid. IAA: Indole-3-acetic acid. I3CA: Indole-3-carboxylic acid. I3A: Indole-3-carboxaldehyde. 3-MI: 3-Methylindole.

Cell line	IPA	IAA	I3CA	I3A	3-MI	Indole
Caco−2	> 100	0.52 ± 0.12	> 100	> 100	> 100	> 100
HepG2	> 100	> 100	> 100	> 100	> 100	> 100
HepaRG	> 100	2.21 ± 0.32	53.0 ± 12.1	1.98 ± 0.73	66.4 ± 21.8	> 100
MRC−5	0.91 ± 0.10	2.55 ± 1.95	0.52 ± 0.08	49.8 ± 14.5	21.9 ± 8.06	> 100
MSC	0.40 ± 0.14	> 100	0.64 ± 0.31	1.87 ± 0.83	0.33 ± 0.10	> 100
T47D	2.02 ± 0.13	1.68 ± 0.46	> 100	> 100	> 100	> 100

compound (Table 2).

When analyzing different cell lines with other indole derivatives, HepG2 cells showed no noticeable sensitivity to any of the tested ones. In Caco-2 cells, IAA was the only derivative that demonstrated detectable toxicity, with an EC_{50} value of 0.52 ± 0.12 μM. Breast cancer T47D cells exhibited moderate sensitivity to IPA and IAA, with EC_{50} values of 2.02 ± 0.13 μM and 1.68 ± 0.46 μM, respectively. No toxic responses were observed in T47D cells at concentrations of 100 μM or lower for the other indole-based compounds. In HepaRG cells, there was no sensitivity to IPA. However, I3A and IAA showed the highest toxicity in these cells, with EC_{50} values of 1.98 ± 0.73 μM and 2.21 ± 0.32 μM, respectively. Additionally, 3-MI and I3CA induced toxic responses in HepaRG cells, but only at much higher concentrations of 66.4 ± 21.8 μM and 53.0 ± 12.1 μM, which are considered non-relevant. Lung fibroblasts (MRC-5 cells) showed decreased viability when exposed to all indole derivatives, with a particular sensitivity to I3CA and IPA, which had EC_{50} values of 0.52 ± 0.08 μM and 0.91 ± 0.10 μM, respectively. IAA also showed toxicity in MRC-5 cells, with an EC_{50} value of 2.55 ± 1.95 μM. Meanwhile, 3-MI and I3A caused toxicity only at higher concentrations of 21.9 ± 8.06 μM and 49.8 ± 14.5 μM, respectively. Although mesenchymal stem cells (MSC) did not show toxicity to IAA, they were more sensitive to other indole derivatives compared to the other cell lines. MSC cells were most affected by 3-MI (EC_{50} = 0.33 ± 0.10 μM) and IPA (EC_{50} = 0.40 ± 0.14 μM), followed by I3CA (EC_{50} = 0.64 ± 0.31 μM) and I3A (EC_{50} = 1.87 ± 0.83 μM) (Table 2).

In summary, most cell lines showed varying degrees of sensitivity to different indole derivatives, with HepG2 cells being the least sensitive and MSC cells and MRC-5 cells being the most sensitive, particularly to 3-MI, IPA, and I3CA, while indole itself had no significant toxic effects at concentrations below 100 μM.

3.3. Aryl hydrocarbon receptor (AhR) activation

The HepG2-AhR Lucia™ reporter cells (InvivoGen) were used to assess the activation of the human aryl hydrocarbon receptor (AhR) by indole and its derivatives. Tetrachlorodibenzodioxin (TCDD), benzo[*a*]pyrene (BaP), and β-naphthoflavone (BNF) were employed as positive controls for agonistic activity. TCDD is known as a potent AhR activator, with EC_{50} values as low as 0.0003 nM depending on the cell line [28]. In our study, we observed an EC_{50} ranging from 0.0003 to 0.0023 μM. BaP and BNF served as partial agonists for AhR, showing activation with EC_{50} values of 3.21 μM (2.60–4.00 μM) and 5.51 μM (4.06–7.45 μM), respectively. Notably, I3A demonstrated greater efficacy than BaP and BNF in activating AhR in HepG2-AhR Lucia™ cells, with an EC_{50} of 0.04 μM (0.03–0.05 μM). Following I3A, IPA and I3CA also showed effective AhR activation, with EC_{50} values of 0.11 μM (0.10–0.13 μM) and 0.88 μM (0.80–0.95 μM), respectively (Table 3).

Table 3
Aryl hydrocarbon receptor (AhR) activation by indole metabolites (IPA: indole-3-propionic acid; IAA: indole-3-acetic acid; I3CA: indole-3-carboxylic acid; I3A: indole-3-carbaldehyde; 3-MI: 3-methylindole; Indole: indole) using the HepG2-Lucia™ AhR gene-reporter cells. Data obtained from positive controls are also shown (TCDD: 2,3,7,8-tetrachlorodibenzo-p-dioxin; BaP: benzo[*a*]pyrene; BNF: β-naphthoflavone). Data are presented as EC_{50} and average (95 % Confidence Interval) (n = 12–18).

Metabolite	EC_{50} (μM)
Indoles	
IPA	0.11 (0.10–0.13)
IAA	1.37 (1.13–1.67)
I3CA	0.88 (0.80–0.95)
I3A	0.04 (0.03–0.05)
3-MI	1.40 (0.79–1.79)
Indole	32.09 (27.48–37.41)
Positive Controls	
TCDD	0.0018 (0.0003–0.0023)
BaP	3.21 (2.60–4.00)
BNF	5.51 (4.06–7.45)

Indole exhibited the weakest activation of the AhR among the compounds tested, requiring the highest concentration to achieve effective activation, with an EC_{50} of 32.09 μM, classifying it as a weak AhR ligand (Table 3). Interestingly, this activation occurs at relatively low concentrations of indole, which are not cytotoxic to the cells. This finding suggests that while indole is a less potent activator of AhR, its ability to engage the receptor without inducing cytotoxic effects may point to a nuanced role in modulating AhR activity. This characteristic could be particularly significant in understanding the physiological relevance of indole in cellular contexts where mild activation of AhR is beneficial or desired without accompanying toxicity.

3.4. Cell proliferation and migration

In Caco-2 cells, there were no significant differences in growth and proliferation at any tested concentrations of IAA or IPA (Figs. 2 and 4). However, the data for IAA were not particularly relevant, as most cells died due to the high cytotoxicity of the metabolite in this cell line. High concentrations (10 μM) of indole, I3CA, I3A, and 3-MI caused a significant reduction in cell proliferation rates compared to cells exposed only to the media ($p < 0.05$) (Fig. 2A, 2C, 2D, and 2F). Notably, even at a lower concentration of 1 μM, I3A, and 3-MI significantly reduced cell proliferation ($p < 0.05$), with a proliferation speed of 71.3 ± 5.1 % and 68.2 ± 7.4 %, respectively, compared to control (100 %). Among all tested compounds, 3-MI was the most toxic, showing a significant decrease in proliferation at concentrations as low as 0.1 μM (Fig. 2F). This suggests that 3-MI has a strong inhibitory effect on cell growth, even at very low concentrations, highlighting its potential potency as an antiproliferative agent in Caco-2 cells.

Breast cancer T47D cells exhibited varied responses when exposed to indole and its derivatives (Figs. 3 and 5). At a concentration of 10 μM, indole, IAA, IPA, and 3-MI significantly reduced cell proliferation rates compared to the control group exposed only to the media ($p < 0.05$). Specifically, the reduction in proliferation was highly significant for IAA ($p = 0.001$) and 3-MI ($p = 0.0003$) at this concentration. Intriguingly, at the same concentration of 10 μM, both I3CA, and I3A significantly increased the rate of cell proliferation compared to the control, with proliferation rates reaching 145.2 ± 8.0 % for I3CA and 139.7 ± 9.3 % for I3A (Fig. 3C and 3D). These findings suggest that while some indole derivatives may inhibit cell growth, others, like I3CA and I3A, may stimulate cell proliferation, indicating a complex interaction between these compounds and breast cancer cell biology.

4. Discussion

Indole metabolites, which are primarily produced by gut bacteria through the metabolism of tryptophan, play a significant role in human health due to their diverse biological activities in human cells [4]. These metabolites, including indole and its derivatives, have been shown to interact with various cellular pathways, influencing processes such as cell proliferation, apoptosis, and immune response. Understanding the effects of indole metabolites is particularly relevant in the context of the gut microbiome's impact on health and disease, as these compounds can modulate the function of intestinal epithelial cells and potentially affect the development and progression of cancers, metabolic disorders, and inflammatory conditions. As research continues to unravel the complex interactions between indole metabolites and human cells, these compounds may offer new insights into therapeutic targets and strategies for maintaining health and treating disease.

In this study, the use of six cell lines, rather than a lower number, in indole toxicity studies is relevant for achieving a comprehensive evaluation of the compound's effects across a diverse range of cellular environments. Each cell line represents a distinct tissue origin, metabolic profile, and receptor expression pattern, which collectively influence

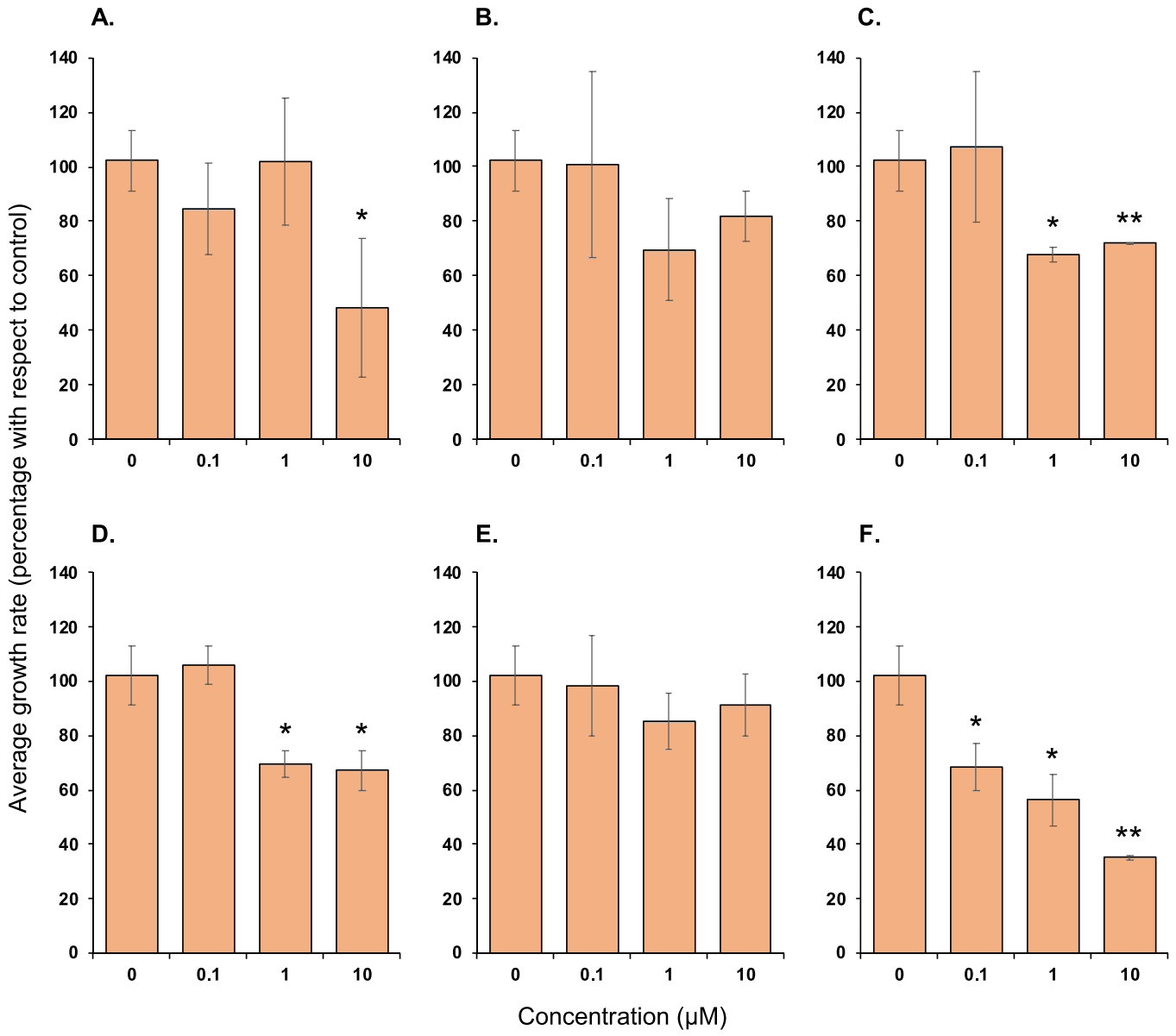

Fig. 2. Analysis of proliferation and migration of Caco-2 enterocyte cells exposed to indole (**A**), indole-3-acetic acid (IAA) (**B**), indole-3-carboxylic acid (I3CA) (**C**), indole-3-aldehyde (I3A) (**D**), indole-3-propionic acid (IPA) (**E**), and 3-methylindole (3-MI) (**F**). Data are presented as average ± standard deviation (n = 8–16) and calculated as a proliferation rate with respect to control (media exposure without indole metabolites). Significant differences with control are shown as * ($p < 0.05$) and ** ($p < 0.01$) (One-way ANOVA, Dunnett's test).

cellular responses to indole exposure. Expanding the number of cell lines enhances the ability to detect tissue-specific toxicological responses and identify selective sensitivities that might otherwise be missed with fewer models. This approach also improves the robustness and reliability of the data, increasing the likelihood of uncovering subtle or rare mechanisms of action. Additionally, utilizing a broader panel of cell lines enhances the translational relevance of the findings, providing a more representative assessment of the complexity of multicellular organisms and enabling more accurate risk evaluations for human health.

In this study, Caco-2 cells showed sensitivity only to IAA. Because indole and its derivatives are naturally present in the gut, Caco-2 cells might have developed some inherent resistance to the cytotoxic effects of IPA, I3CA, I3A, 3-MI, and indole. Our findings are consistent with previous research showing that neither IPA nor indole caused toxicity or altered proliferation rates in Caco-2 cells [13,29]. Although Kurata et al. [14] reported cytotoxicity and apoptosis in Caco-2 cells exposed to 3-MI, the doses used were ten times higher than those in our study.

To our knowledge, this study is the first to investigate the cytotoxic effects of I3CA on Caco-2 cells. Although I3CA did not demonstrate cytotoxicity at concentrations below 100 μM, a significant reduction in cell proliferation was observed at concentrations of 1 μM and above. In comparison, a structurally similar compound, I3A, was studied by Liu et al. [30], who reported an 89.6 % reduction in cell viability at 160 μM in Caco-2 cells. In the current study, no reduction in cell viability was observed with 100 μM of I3A, but there was a dose-dependent decrease in cell proliferation with increasing concentrations of I3A. The structural similarity between I3CA and I3A (Fig. 1) might explain their comparable effects on cell proliferation, but further investigation is needed to clarify these findings. In contrast, Caco-2 cells were most sensitive to IAA. Tomii et al. [31] reported that exposure to 500–1000 μM of IAA reduced Caco-2 cell count without causing cytotoxicity, as measured by the lactate dehydrogenase (LDH) assay. However, their study used a 72-hour exposure period, unlike the 24-hour exposure period in this investigation, which may suggest a potential recovery of cell viability

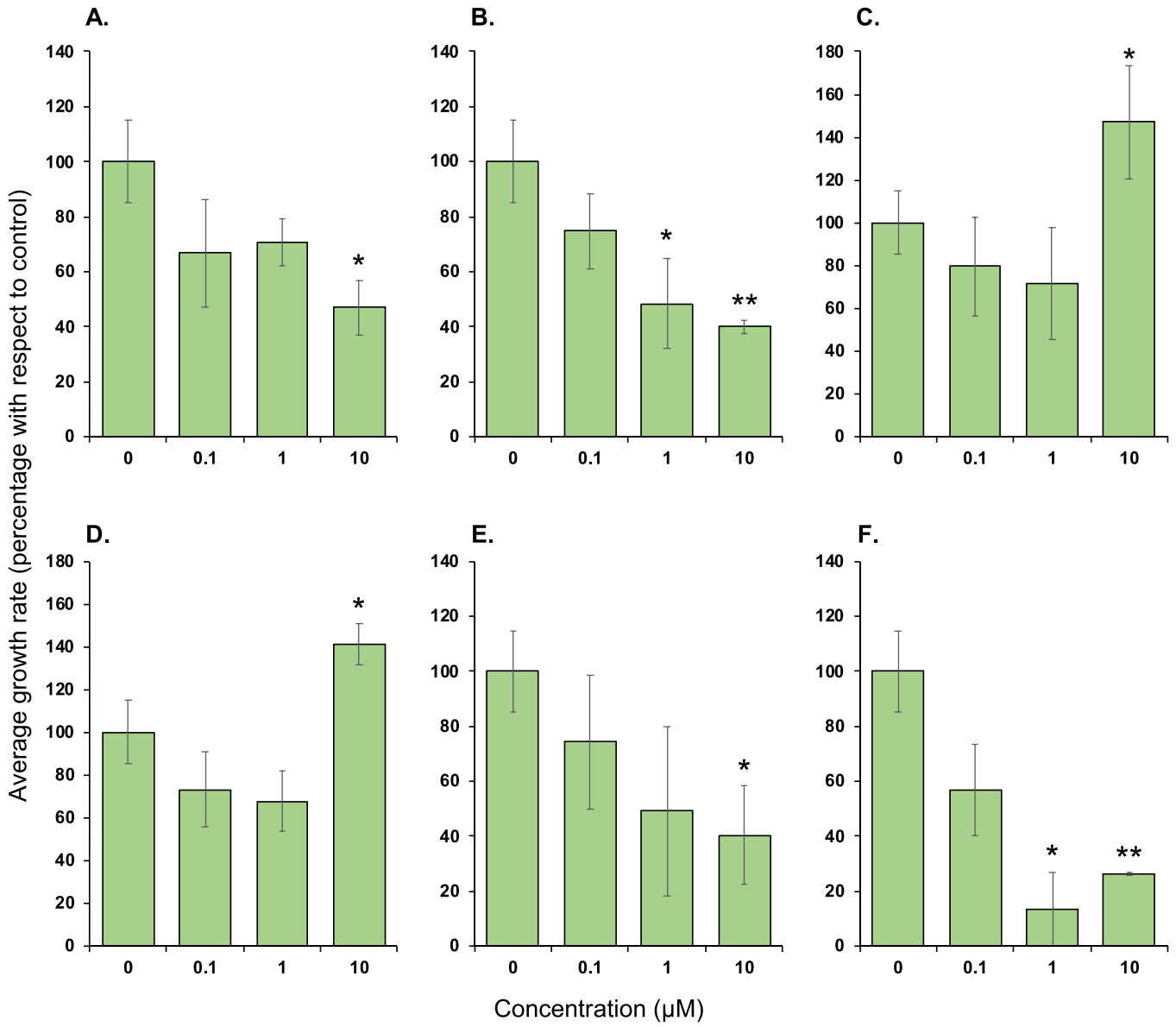

Fig. 3. Analysis of proliferation and migration of T47D breast cancer cells exposed to indole (**A**), indole-3-acetic acid (IAA) (**B**), indole-3-carboxylic acid (I3CA) (**C**), indole-3-aldehyde (I3A) (**D**), indole-3-propionic acid (IPA) (**E**), and 3-methylindole (3-MI) (**F**). Data are presented as average ± standard deviation (n = 8–16) and calculated as a proliferation rate with respect to control (media exposure without indole metabolites). Significant differences with control are shown as * ($p < 0.05$) and ** ($p < 0.01$) (One-way ANOVA, Dunnett's test).

following a shorter exposure duration. Despite the difference in exposure times, both studies demonstrated that IAA inhibited Caco-2 cell proliferation. Tomii et al. [31] proposed that IAA binds to toll-like receptor 4 (TLR4), leading to increased expression of extracellular signal-regulated kinase (ERK) and *c*-jun *N*-terminal kinase (JNK), which subsequently reduces cell proliferation. These findings suggest that IAA may possess anti-proliferative properties that could be beneficial in the treatment of colorectal adenocarcinomas. However, further research is required to validate these potential therapeutic effects.

Indoles generally improve intestinal barrier function in Caco-2 cells; for example, indole-3-aldehyde and indole have been shown to restore transepithelial electrical resistance, which is an indicator of epithelial tight junction integrity [15,32]. Indole-3-propionic acid (IPA) at a concentration of 500 μM has been found to activate extracellular ERK and inhibit JNK in Caco-2 cells, suggesting an increase in cell proliferation [29]. This proliferative effect was not observed in our study, likely because the highest concentration tested was 100 μM. Strong evidence indicates that metabolites such as indole-3-aldehyde, indole-3-acetate, and indole-3-acetic acid (IAA) enhance CYP1A1 and CYP1B1 activity via interactions with the aryl hydrocarbon receptor (AhR) [33,34,32]. Additionally, in Caco-2 cells, IAA and indole-3-aldehyde (I3A) have been shown to reduce levels of pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNFα), interleukin-6 (IL-6), and interleukin-1 beta (IL-1β) [30,33]. These findings highlight the mechanisms by which indole derivatives exert their biological effects and emphasize their potential therapeutic applications.

Conversely, studies using animal models have demonstrated that indoles and their derivatives can have protective and restorative effects on gut health and inflammation. In mouse models, indole-3-propionic acid (IPA) has been shown to restore colon length, villi structure, and goblet cell numbers following radiation exposure [13]. Indole-3-acetic acid (IAA) has been found to reduce inflammatory responses by decreasing the expression of cytokines such as tumor necrosis factor-alpha (TNFα), interleukin-6 (IL-6), interleukin-7 alpha (IL-7α), and interleukin-23 (IL-23), and also provides protection against ankylosing spondylitis [35]. In pigs, exposure to I3A increased the weight of

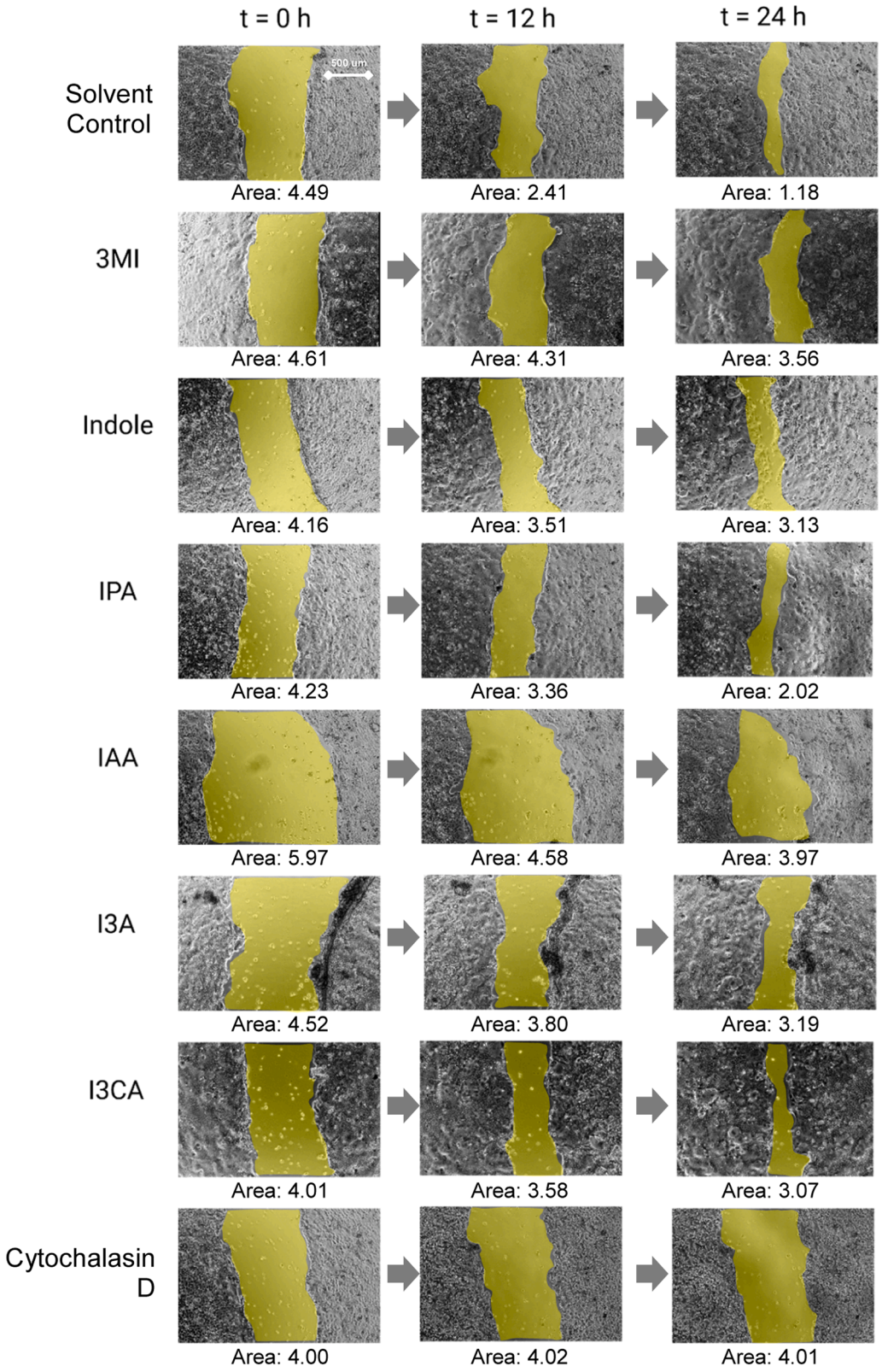

Fig. 4. Microscopic images of Caco-2 cell proliferation and migration under indole exposure at 10 μM. Indole-3-acetic acid: IAA. Indole-3-carboxylic acid: I3CA. Indole-3-aldehyde: I3A. Indole-3-propionic acid: IPA. 3-Methylindole: 3-MI. (**F**). Data are presented as average (n = 8–16) and calculated as the area empty of cells in mm^2. Solvent control: media exposure without indole metabolites. Cytochalasin D (1 mM) was used as a total blocker of cell proliferation (positive control).

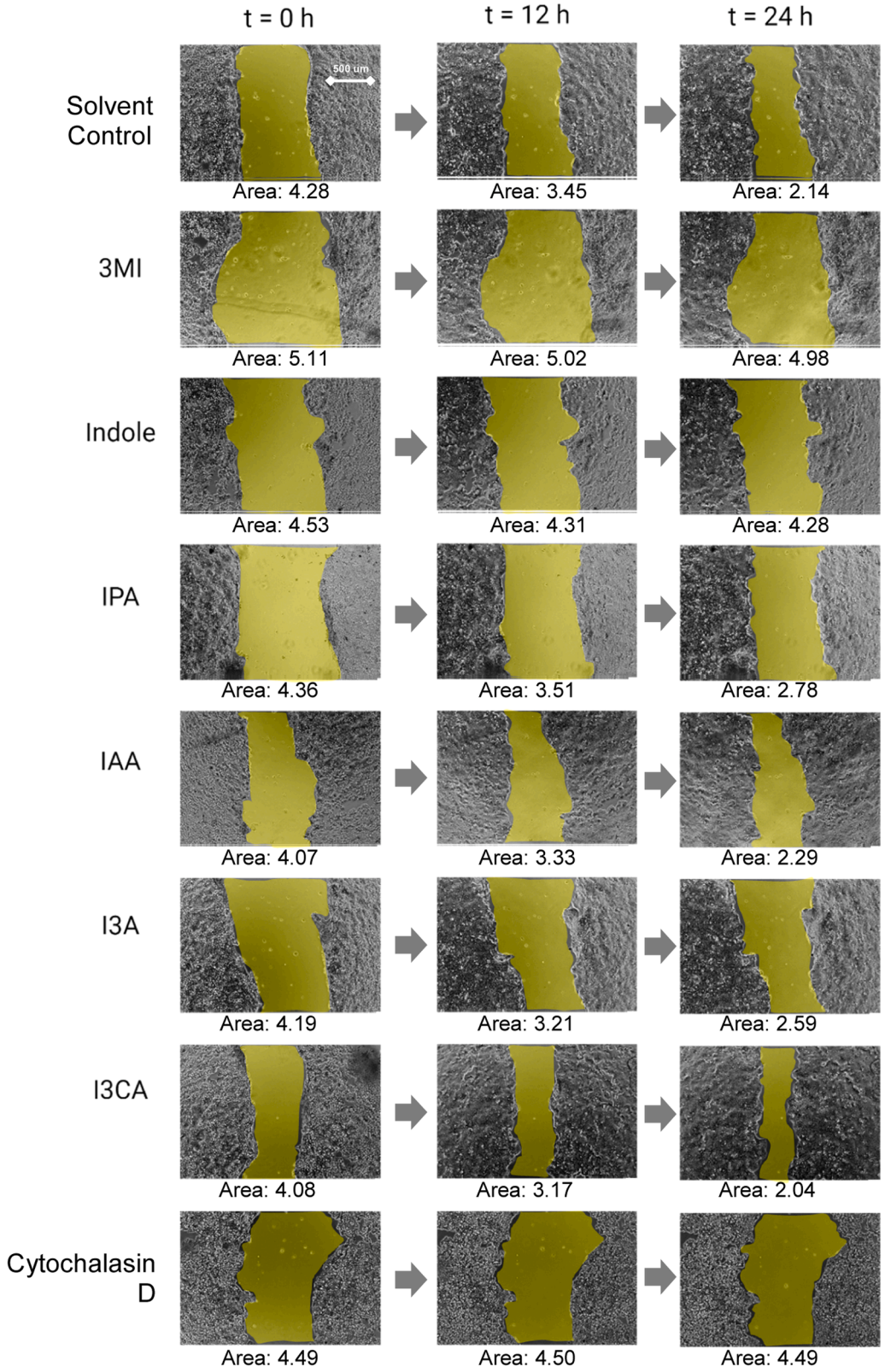

Fig. 5. Microscopic images of T47D cell proliferation and migration under indole exposure at 10 μM. Indole-3-acetic acid: IAA. Indole-3-carboxylic acid: I3CA. Indole-3-aldehyde: I3A. Indole-3-propionic acid: IPA. 3-Methylindole: 3-MI. (**F**). Data are presented as average (n = 8–16) and calculated as the area empty of cells in mm^2. Solvent control: media exposure without indole metabolites. Cytochalasin D (1 mM) was used as a total blocker of cell proliferation (positive control).

the ileum, jejunum, and colon, enhanced jejunal proliferation, and upregulated AhR expression [36].

While the beneficial effects of indoles on the colon are well-documented, it is also important to consider their impact on other organs, particularly the liver, which is critical for the metabolism of various compounds. After being produced in the gut, indoles and their derivatives are often transported to the liver via the gut-liver axis, where they can have various physiological effects. In this study, HepaRG cells showed the highest sensitivity to I3A, followed by IAA, I3CA, and 3-MI, while exposure to IPA and indole at concentrations up to 100 μM did not impact cell viability. In contrast, HepG2 cells did not display sensitivity to any of the tested compounds, suggesting that the response to indoles may vary significantly depending on the specific cell type and derivative. This variation in sensitivity emphasizes the need for further investigation into the mechanisms by which indoles interact with liver cells and their potential effects on liver function and health.

HepaRG cells have been shown to express higher levels of cytochrome P450 (CYP) enzymes compared to both HepG2 cells and primary hepatocytes [37]. Since activation of the AhR is known to increase CYP enzyme expression, it is possible that HepaRG cells overexpress these enzymes, leading to greater cytotoxicity than seen in HepG2 cells. However, excessive AhR activation can also promote hepatocarcinogenesis by causing overexpression of CYP enzymes [38]. In both the cytotoxicity and AhR activation assays, I3A exhibited the lowest EC50 value, indicating high toxicity and receptor activation. This overexpression of CYP enzymes might explain the hepatotoxicity observed in this study. Further investigation into the differential toxicity of I3A in hepatic progenitors versus carcinoma cells, as well as its impact on CYP activation, is needed to better understand these findings.

Indole compounds are known to activate AhR, which is involved in regulating interleukin production [39]. Our findings using HepG2-Lucia-AhR cells show that AhR activation is most sensitive to I3A, followed by I3CA, IPA, IAA, 3-MI, and indole. In mice, activation of AhR by IAA reduces the production of cytokines such as TNFα, IL1β, and MCP1 and also inhibits macrophage migration from the bone marrow [40]. Additionally, IPA, IAA, I3CA, and 3-MI have been shown to activate AhR, which subsequently induces the expression of CYP1A1, CYP1B1, and NF-kB in liver tissues [41,42]. 3-MI acts as a partial AhR agonist, and HepaRG cells have been found to be more sensitive to 3-MI than HepG2 cells and primary cultures [41,43]. Moreover, 4-MI and 6-MI strongly induce the expression of CYP1A genes at levels comparable to TCDD, while 1-MI and 3-MI induce CYP1A genes to about 50 % of the level of TCDD in HepaRG cells [44].

Interestingly, in murine models, these compounds have shown protective and restorative effects against hepatotoxicity. For example, mice treated with I3A exhibited reduced toxic effects, such as the activation of IL-22, reduced oxidative stress, and less weight gain when exposed to acetaminophen and 3,5-diethoxycarbonyl-1,4-dihydrocollidine [45,46]. IAA has been shown to lower blood glucose, triglyceride, and cholesterol levels in mice on a high-fat diet, as well as decrease lipid peroxidation [45,47]. I3CA helps restore gut-liver function and reduces the population of pro-inflammatory bacteria while interacting with NF-kB pathways and regulating CYP1A1 and CYP1B1 activity [45,48]. Although HepaRG cells showed no sensitivity to IPA, IPA has been found to enhance toxic effects in mice when combined with carbon tetrachloride (CCl_4) [49].

Another important organ affected by indoles and their metabolic pathways is the lungs. Historically, research on indoles has often focused on respiratory exposure, as occupational exposure to indoles and their derivatives can occur through inhalation of fecal particulate matter. Among the six compounds tested, MRC-5 lung fibroblasts showed the greatest sensitivity to I3CA, followed by IPA, IAA, 3-MI, I3A, and indole. In contrast, V79–4 Chinese hamster fibroblasts exhibited no toxicity at concentrations of 300 μM with the structurally similar compound indole-6-carboxaldehyde [50]. Additionally, V79 379 A cells (Chinese hamster lung fibroblast-like cells) did not show a cytotoxic response after a 2-hour exposure to 0.1 mM IAA; however, the combination of IAA with peroxidase increased cytotoxicity to levels comparable to positive controls and resulted in increased DNA damage [51]. Yap et al. [52] found no cytotoxic effects at 30 μg/mL IAA in MRC-5 cells, although the duration of exposure was not specified. Prolonged exposure to IAA metabolites in MRC-5 cells could potentially increase toxicity, and there may be proteomic differences between hamsters and human lung fibroblasts that influence their response to indole exposure.

Although 3-MI exposure resulted in the second-lowest cytotoxic response in MRC-5 cells, oral administration of 3-MI to goats (0.16 g/kg body weight) caused severe pulmonary edema, denuded bronchioles, and necrotic alveolar cells within 4–24 h [53]. Similarly, in rats injected with 3-MI, there was necrosis of type I epithelial cells, hypertrophy and hyperplasia of type II epithelial cells, and cytoplasmic blebbing in the alveolar septa observed 24–96 h after injection [16]. Recent studies have also demonstrated that indole, 1-MI, 2-MI, 3-MI, and 2-phenylindole reduce cell viability in MRC-5 cell lines in a dose-dependent manner [54]. While 3-MI was not the most cytotoxic indole derivative in this study, it shows clear evidence of toxicity to lung tissues even without direct inhalation exposure.

Complex indole derivatives, such as the indole-3-acrylic acid ester and indole-3-carboxylic acid ester of melampomagnolide-B, showed great potential in anticancer research [55]. However, their stability in the body is still uncertain. To explore the effects of simpler indole derivatives and their potential anticancer properties, we examined their impact on breast cancer T47D cells. In this study, I3CA did not cause cytotoxicity but significantly increased the proliferation of T47D cells. Further research is needed to understand the interactions between I3CA and breast cancer cell proliferation.

This study was the first to examine the effects of 3-MI, I3A, IPA, and IAA on T47D breast cancer cells. While 3-MI and I3A showed no toxicity, IAA produced mixed results; it reduced cell viability but slightly increased proliferation at a concentration of 10 μM, though this increase was not statistically significant. In contrast, IAA at 100 μM did not show cytotoxicity in MCF-7 cells, another luminal A breast cancer cell line [56]. Understanding the mechanisms behind IAA's effects on T47D and MCF-7 cells is crucial for drawing definitive conclusions. In line with previous research showing that IPA reduces carcinoma proliferation, inhibits metastasis, and induces apoptosis in murine models and MCF-7 and MDA-MB-231 cells [57,58], this study also observed a decrease in T47D cell viability with IPA exposure.

To our knowledge, this study was the first to explore the cytotoxic effects of indole derivatives on adipose-derived mesenchymal stem cells (MSCs). MSCs were sensitive to all tested indole derivatives except for IAA and indole. Among the compounds, 3-MI caused the greatest reduction in cell viability, followed by IPA, I3CA, and I3A. In contrast, IAA has been shown to be protective and restorative in dental pulp stem cells from children, shielding them from oxidative stress and apoptosis induced by H_2O_2 [59]. On the other hand, indoxyl sulfate has been found to inhibit proliferation, induce senescence, and decrease cell viability in MSCs derived from the human umbilical cord (hUCMSCs) [60,61]. When hUCMSCs express indoleamine-2,3-dioxygenase (IDO), they produce kynurenic acid (KYNA), which activates the AhR to increase the expression of TSG-6, an anti-inflammatory enzyme. However, kynurenine and other metabolites from the tryptophan metabolism pathway can be toxic to MSCs, promoting senescence and suppressing autophagy via the AhR/p62 and p21/p53 pathway [62,63]. The indole derivatives tested in this study might exhibit similar behaviors, but further research is needed to clarify these effects.

Since Caco-2 is a carcinoma-based cell line, we recommended further research of these effects on non-carcinoma colorectal cell lines such as CCD-841-CoN, FHC, or primary cultures. This will help determine if the anti-proliferation effects of indole derivatives are specific to colorectal carcinoma cells or affect non-cancer cells as well. Furthermore, while it is well known that many indole metabolites are transformed into to uremic toxins such as indoxyl sulfate, the effects of other indole

derivatives are on kidney related toxicity is poorly understood [8]. Recently it was found that in contrast to indoxyl sulfate, I3A is protective in mice against cisplatin, a chemotherapeutic drug, induced nephrotoxicity. Additional research into the effects into of Trp derived indole compounds on nephrotoxicity and uremic toxicity is recommended.

In vivo studies typically show that indole derivatives have protective effects, but the findings of this study highlight inconsistencies between animal-based *in vivo* models and human cell-based *in vitro* models. One possible explanation is that *in vivo* models capture inter-organ communication that helps reduce toxicity, which may not be accurately reflected in *in vitro* studies. Alternatively, human cells might express different proteins in response to indole derivative exposure, indicating species-specific toxicity. Further research is necessary to clarify these possibilities. Given the significant variability in how different indole derivatives affect various cell lines, more studies are needed to understand their impact on human healthfully.

Lastly, the use of *in vitro* systems is relevant in toxicology and mechanism-of-action studies, despite their limitations and challenges in direct comparability to *in vivo* models. While *in vitro* systems lack the integrated physiological context of *in vivo* models, they are instrumental in identifying specific pathways of toxicity, receptor interactions, and dose-response relationships that are foundational for understanding broader biological effects. Acknowledging their limitations, such as the absence of systemic interactions, metabolism, or immune responses present in living organisms, is essential for interpreting *in vitro* findings appropriately. However, these in vitro systems remain valuable tools for reducing reliance on animal testing and facilitating high-throughput screening to generate initial toxicity profiles. When combined with complementary in vivo or computational approaches, *in vitro* studies bridge critical gaps in toxicology research, ensuring a more comprehensive understanding of chemical impacts while addressing both scientific and ethical considerations.

5. Conclusions

In conclusion, this study demonstrates that indole itself is non-toxic to any of the six cell lines tested at a concentration of 100 μM, whereas its derivatives exhibit varying degrees of cytotoxicity depending on the cell type. Caco-2 cells are sensitive exclusively to IAA, while HepG2 cells are resistant to the cytotoxic effects of all derivatives. In contrast, HepaRG cells are responsive to all derivatives except IPA, and MRC-5 cells show sensitivity to all indole derivatives, with EC_{50} values ranging from 0.52 to 49.8 μM, especially for I3CA and IPA. Mesenchymal stem cells (MSC) display sensitivity to all derivatives except IAA, and T47D cells are specifically sensitive to IPA and IAA. In HepG2-AhR Lucia™ cells, I3A, IPA, and I3CA function as strong agonists of the aryl hydrocarbon receptor, while IAA and 3-MI behave as partial agonists, comparable to BaP and BNF. Additionally, I3CA reduces proliferation in Caco-2 cells but increases proliferation in T47D cells, indicating diverse and context-dependent responses across different cell types. The findings suggest that indole derivatives are more cytotoxic to stem-like cells than to carcinoma cells, as evidenced by the heightened sensitivity of MRC-5 and MSC cells. The lower sensitivity of carcinoma cells implies that the base structure of indole derivatives may not directly contribute to anticancer properties in indole-based treatments. The differential sensitivities observed between HepG2 and HepaRG cells could be due to enhanced AhR and CYP enzyme activation in HepaRG cells. Furthermore, while IAA exhibits cytotoxic effects in T47D and Caco-2 cells, the data indicate that I3CA plays a more significant role in modulating proliferation in these cell lines.

Further research is needed to elucidate the mechanisms underlying these differential responses and to explore the therapeutic potential of indole derivatives in cancer treatment. These findings indicate that indole toxicity varies significantly between *in vitro* and *in vivo* models, underscoring the need for further comparative studies between these systems. The observed differences in response to indole exposure may stem from complex systemic communication in living organisms or from interspecies metabolic variations. If these results accurately reflect human indole toxicity, further investigation is warranted into the stability of indole-based cancer therapeutics and their interactions with non-target cells, to ensure that stem-like cells remain unharmed during cancer treatment. Additionally, this study demonstrates that indole derivatives act as potent activators of the aryl hydrocarbon receptor (AhR). However, it remains unclear whether these indole derivatives function as natural ligands or xenobiotic activators of AhR. Further research into the mechanisms between indole-based AhR activation and downstream pathways could help clarify these uncertainties. This study addresses several knowledge gaps regarding indole toxicity, including assessments of cytotoxicity in novel cell lines and unexpected proliferation outcomes in carcinoma models. However, extensive research is still required to fully understand the impact and mechanisms of these endogenous compounds.

CRediT authorship contribution statement

Ramon Lavado: Writing – review & editing, Visualization, Validation, Supervision, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Maria Teresa Fernandez-Luna:** Writing – review & editing, Resources, Project administration, Methodology, Investigation, Formal analysis. **Alisha Janiga-MacNelly:** Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis. **Ava E. Roat:** Writing – original draft, Methodology, Investigation. **Maddison Vrazel:** Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported in part by funds from the ONE-URC University Research Committee Awards from the Vice Provost for Research and the C. Gus Glasscock, Jr. Endowed Fund of Excellence in Environmental Sciences at Baylor University.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.toxrep.2024.101883.

Data availability

Data will be made available on request.

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