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RESEARCH ARTICLE

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Comparative analysis of diisononyl phthalate and di(isononyl) cyclohexane-1,2 dicarboxylate plasticizers in regulation of lipid metabolism in 3T3-L1 cells

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Abstract

Diisononyl phthalate (DINP) and di(isononyl)cyclohexane-1,2-dicarboxylate (DINCH) are plasticizers introduced to replace previously used phthalate plasticizers in polymeric products. Exposure to DINP and DINCH has been shown to impact lipid metabolism. However, there are limited studies that address the mechanisms of toxicity of these two plasticizers. Here, a comparative toxicity analysis has been performed to evaluate the impacts of DINP and DINCH on 3T3-L1 cells. The preadipocyte 3T3-L1 cells were exposed to 1, 10, and 100 μM of DINP or DINCH for 10 days and assessed for lipid accumulation, gene expression, and protein analysis. Lipid staining showed that higher concentrations of DINP and DINCH can induce adipogenesis. The gene expression analysis demonstrated that both DINP and DINCH could alter the expression of lipid-related genes involved in adipogenesis. DINP and DINCH upregulated *Pparγ*, *Pparα*, *C/EBPα Fabp4*, and *Fabp5*, while both compounds significantly downregulated *Fasn* and *Gata2*. Protein analysis showed that both DINP and DINCH repressed the expression of FASN. Additionally, we analyzed an independent transcriptome dataset encompassing temporal data on lipid differentiation within 3T3-L1 cells. Subsequently, we derived a gene set that accurately portrays significant pathways involved in lipid differentiation, which we subsequently subjected to experimental validation through quantitative polymerase chain reaction. In addition, we extended our analysis to encompass a thorough assessment of the expression profiles of this identical gene set across 40 discrete transcriptome datasets that have linked to diverse pathological conditions to foreseen any potential association with DINP and DINCH exposure. Comparative analysis indicated that DINP could be more effective in regulating lipid metabolism.

KEYWORDS
disease, fatty acid, gene expression, lipid homeostasis, obesity

Ceyhun Bereketoglu and Isabel Häggblom contributed equally to this study.

1 | INTRODUCTION

Phthalates are chemical additives used as plasticizers to provide elasticity, flexibility, and durability to polymeric compounds.[1–4] The worldwide production of phthalates has reached approximately 6–8 million tons per year, while Western Europe alone is estimated to use about 1 million tons per year.[5,6] Since phthalates are not covalently bound to products, they can easily leach out and contaminate the environment.[7] Phthalates and their metabolites have been widely detected in urine samples of the population from the United States, Europe, and Canada.[8–11] Several studies have shown that phthalates cause various health problems, such as carcinogenesis, cardiotoxicity, hepatotoxicity, nephrotoxicity, neurotoxicity, and reprotoxicity.[12–16] As a result, the use of several phthalates has been restricted or banned in several products,[17–19] which led the industry to search for environmentally safe plasticizers.

Diisononyl phthalate (DINP) is a large molecular weight phthalate which is primarily used in the production of polymers and commercial plastic products.[20] DINP has become one of the major phthalates utilized in indoor[21] and outdoor products.[22] Maximum reported concentrations of DINP are 2100 and 15 500 μg/g in floor and multi-surface dusts, respectively.[23] DINP has also been detected in human urine samples,[24] and studies have indicated that DINP has adverse effects on different animals. A study performed on zebrafish showed that DINP could potentially affect the skeletal muscle composition and the endocannabinoid system in the liver and brain.[25] In another study, it was also reported that DINP activates NF-kB, Akt/mTOR, and induces Th2-mediated cytokines (IL-4 and IL-5) production.[26] Epidemiological studies further indicated that the primary metabolite of DINP, monoisononyl phthalate, has a significant correlation with serum levels of tyrosine and the incidence of insulin resistance.[27] DINP was found to alter adipocyte activity and increase lipid accumulation in 3T3-L1 preadipocytes.[28]

DINCH is used as a plasticizer in polyvinyl chloride plastic products, such as children's toys, medical devices, and food packaging.[29] DINCH was approved to be used in various food contact products by the European Food Safety Authorities.[30] DINCH covers about 70% of the plasticizer market, and several studies have indicated that DINCH and its metabolites reached its detection level in urine samples in different European countries.[31–35] In 2017, the measured median value of DINCH in German rivers was 117 ng/g dry weight (dw), which is a 28 times higher value than the one measured in the mid-2000s. Another measurement conducted in 2017 likewise indicated a level of DINCH above all quantificational limits (4.2 ng/g dw).[36]

Several studies have investigated the negative impacts of DINCH, and controversial results were obtained.[37–42] In a study on rats, DINCH did not show any adverse effect on behavior, organ weight, and serum chemistry.[37] However, in another study, rats gavaged with 30 and 300 mg/kg/day of DINCH showed a higher incidence of hemorrhagic testes in the offspring.[41] In an in vitro study using MA-10 Leydig cells, DINCH showed a decrease in steroid production indicating a negative effect on reproductive system.[43] Several other in vitro studies have also shown an induced cytotoxicity in kidney[44] and fibroblast cells,[45] and oxidative stress in liver cells.[44] A research group demonstrated that DINCH affects the expression of rat liver genes,[39] impairs Leydig cell function, liver metabolic capacity,[40] and disrupts fat storage in adipocytes that leads to obesity.[38] It was also reported that DINCH metabolites M2NCH, MINCH, OH-MINCH, oxo-MINCH, and cx-MINCH could affect ERα, ERβ, AR, PPARα, and PPARγ receptors in HEK293 cell line.[42] In another study using Sprague Dawley rats, a gestational and lactational exposure to DINCH altered the lipid metabolism through decreasing and/or increasing the expression of lipid-related processes.[46] In our previous study, we showed that DINCH could cause hatching delay, result in a slight lipid accumulation, and alter the expression of genes involved in lipid transport, cholesterol biosynthesis, and homeostasis in zebrafish.[47] In the present study, DINP was selected since it has been listed in water and environmental pollutants by European Protection Agency.[48] DINCH was involved in this study, because it is produced by catalytic hydrogenation of the aromatic ring of DINP and it becomes one of the most dominant used alternative plasticizer worldwide.[49] The controversial or no negative effect results on DINCH are primarily from the industry that produces it,[37] and thus, there is a need to better demonstrate and understand the effects of DINCH at the molecular level.

2 | MATERIALS AND METHODS

2.1 | Chemicals

DINP (CAS No: 28553-12-0) and DINCH (CAS No: 166412-78-8) were purchased from Sigma-Aldrich (St Louis, MO). The physical chemical properties of DINP are as follows: Vapor pressure [Pa] at 25°C is 5.17×10^{-7}; solubility in water [mg/L] at 25°C is 1.74×10^{-5}; and logKow at 25°C is 9.52. The physical chemical properties of DINCH are as follows: Vapor pressure [Pa] at 25°C is 1.28×10^{-4}; solubility in water [mg/L] at 25°C is 8.8×10^{-6}; and logKow at 25°C is 10. The molecular formulas are given in Figure 1A. DMSO (Sigma) was used to prepare the stock solutions. Rosiglitazone (Rosi) was purchased from Sigma, and it was used as a positive control. Rosi is a thiazolidinedione (TZD) antihyperglycemic agent.[50] It is an agonist for the peroxisome proliferator-activated receptor gamma (*Pparγ*) transcriptional factor involved in the lipid differentiation. Rosi improves insulin action by activating *Pparγ* that is largely expressed in adipose tissue.[50] Rosi has a molecular weight of 357.4 g/mol and solubility of 10.45 mg/L in water at 25°C.[51,52]

2.2 | Cell culture and differentiation

The preadipocyte cell line (3T3-L1) was purchased from ATCC and cultured in basal media (BM) containing Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin at 37°C with a 5% CO_2 atmosphere. Cells were plated in a 24-well plate with 500 μL BM and a density of

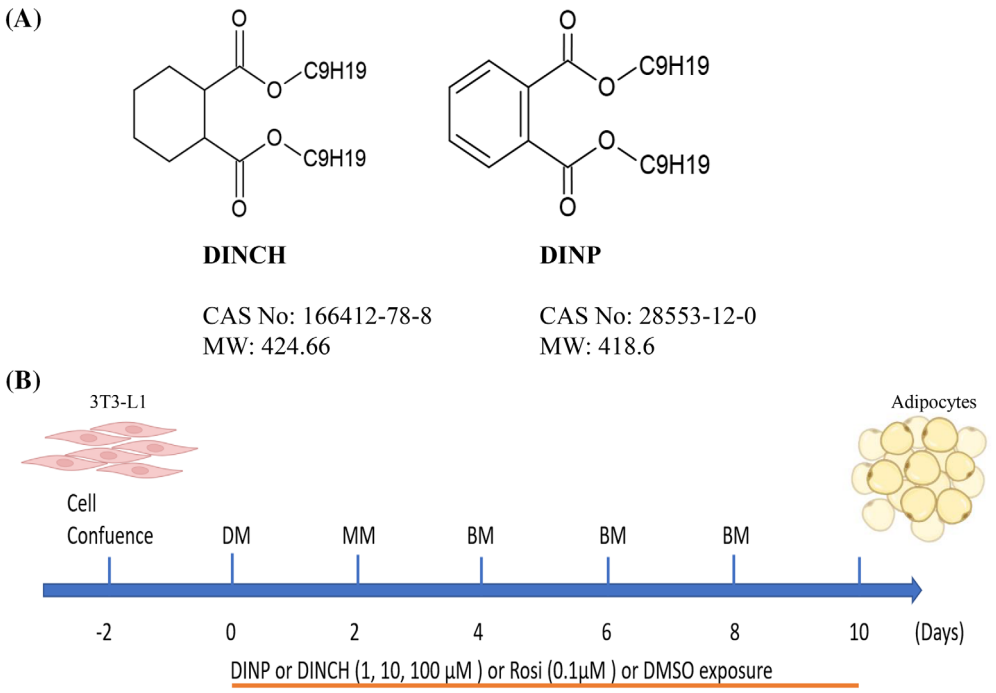

FIGURE 1 The chemical structure of the plasticizers and timeline of the cell culture exposure. (A) The non-phthalate 1,2-cyclohexane dicarboxylic acid diisononyl ester (DINCH) and diisononyl phthalate (DINP) with their CAS number (no) and molecular weight (MW). (B) Differentiation medium (DM), basal medium (BM), and maintenance medium (MM).

5×10^4 cells/well. Cells were allowed to grow to a 100% confluence. After reaching confluence, cells were promoted to differentiation, and DINP, DINCH, or DMSO (control) was added to media to obtain the final assay concentrations of 1, 10, or 100 µM for each compound (Day 0). The final volume of DMSO in the media was kept at 0.1%. The differentiation media (DM) contained BM supplemented with 500 mM IBMX (Sigma), 5 mM dexamethasone (Sigma), and 10 mg/mL insulin (Sigma).[28] Cells were kept in DM for 48 hours, and then, the media was changed with maintenance media (MM) (containing BM and insulin 10 mg/mL) (Day 2). After 2 days, the media was changed with BM (Day 4) and was further renewed every 2 days until the end of exposure (Day 10). DINCH and DINP were replenished during media change. The experiments were performed in triplicates. The timeline of the exposure is given in Figure 1B.

2.3 | Lipid staining

Oil red O (Sigma) stock solution (0.5%) was prepared by dissolving 0.2 g ORO in 40 mL of 100% isopropanol. Using the stock solution, a working solution of ORO (0.3%) was then prepared in distilled water with a ratio of 3:2. Following exposure, media was discarded and the cells were washed twice with 500 µL PBS. For cell fixation, 4% paraformaldehyde was added to each well and incubated for 10 min at room temperature. Thereafter, 0.1% triton ×100 (Sigma) was added to each well for 5–10 min to increase the permeability of the cells. The working solution of ORO was added to each well, and the plates were incubated for 1.5 h.[28] The solution was then discarded, and the cells were washed twice with PBS. Images were taken under the microscope, BX51 (Olympus) using 20× magnification.

2.4 | RNA extraction and quantitative real-time PCR

Gene expression analysis was performed using three independent replicates which were used for each exposure condition and the control. For cell lysis, 350 µL of lysis buffer (Macherey-Nagel; Item number: 740961) was added to each well and the plate was rocked for 5–10 min. RNA samples were obtained using RNA extraction kit (Macherey-Nagel; Item number: 740955.50) according to the manufacturer's instructions. The quality and quantity of the RNA samples were determined with a DS-11 spectrophotometer (DeNovix). The cDNA synthesis was performed using the cDNA synthesis kit (PCR Biosystems; Cat No: PB30.11–10). Quantitative real-time PCR (qPCR) was carried out using SYBR green (PCR Biosystems; Cat No: PB20.11-20) on a CFX maestro (Bio-Rad) thermocycler system. The qPCR conditions were as follows: an initial denaturation step at 95°C for 2 min and 40 cycles of 95°C for 5 s and 60°C for 30 s. Data normalization was carried out using *glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*), and fold change was determined using ΔΔCt method.[53] The list of primer used is given in Table 1.

2.5 | Elucidation of lipid differentiation gene signatures in 3T3-L1 cell line

Gene expression profile within accession number GSE129957[54] was obtained from Gene Expression Omnibus (GEO) database.[55] In experimental design of original study, 3T3-L1 cells were collected from three stages of lipid differentiation (pre-, 4-day differentiated, and 8-day differentiated adipocytes) and RNA sequencing was

TABLE 1 Primers used in the qPCR analysis.

Symbol	Gene name	Primer sequence
Fasn	*Fatty acid synthase*	F: GGAGGTGGTGATAGCCGGTAT R: TGGGTAATCCATAGAGCCCAG
Fabp4	*Fatty acid-binding protein 4*	F: AAGGTGAAGAGCATCATAACCCT R: TCACGCCTTTCATAACACATTCC
Fabp5	*Fatty acid-binding protein 5*	F: TGTTGTTGCCATCACACGTA R: AGGATCTCGAAGGGAAGTGG
Pparγ	*Peroxisome proliferator-activated receptor gamma*	F: GGAAGACCACTCGCATTCCTT R: GTAATCAGCAACCATTGGGTCA
Pparα	*Peroxisome proliferator-activated receptor alpha*	F: ATGCCAGTACTGCCGTTTTCA R: GGGCCTTGACCTTGTTCATGT
Gata2	*GATA-binding factor 2*	F: CACCCCGCCGTATTGAATG R: CCTGCGAGTCGAGATGGTTG
Actβ	*Beta-actin*	F: ATGGAGGGGAATACAGCCC R: TTCTTTGCAGCTCCTTCGTT
B2M	*Beta-2-microglobulin*	F: TTCTGGTGCTTGTCTCACTGA R: CAGTATGTTCGGCTTCCCATTC
Eef1	*Elongation factor 1*	F: TGTCAGTCATCGCCCATGTG R: CATCCTTGCGAGTGTCAGTGA
Gapdh	*Glyceraldehyde 3-phosphate dehydrogenase*	F: AGGTCGGTGTGAACGGATTTG R: GGGGTCGTTGATGGCAACA
Tbp	*TATA-binding protein*	F: ACCGTGAATCTTGGCTGTAAAC R: GCAGCAAATCGCTTGGGATTA
Maf1	*Maf1 homolog, a negative regulator of RNA polymerase III a*	F: TGGGAAGGGAGATTCTTGTG R: GCACCGACACTAAACAACCC
Acc1	*Acetyl-CoA carboxylase*	F: ACAGTGGAGCTAGAATTGGAC R: ACTTCCCGACCAAGGACTTTG
Llp	*Lipoprotein lipase*	F: TTGCCCTAAGGACCCCTGAA R: TTGAAGTGGCAGTTAGACACAG
Srebp1	*Sterol regulatory element-binding protein 1*	F: AGGTGTATTTGCTGGCTT R: CTGTGTGTGTTTCTGGATATACG
C/EBPα	*CCAAT-enhancer-binding protein alpha*	F: GCGGGAACGCAACAACATC R: GTCACTGGTCAACTCCAGCAC

performed.[54] In the current study, the expression of gene set consisting of 13 genes that are associated with lipid process and pathways was chosen and visualized based on z-score. We employed the heatmap.2 function under the gplots package of R software (version 3.1.3). Gene set analysis was performed via ConsensusPathDB,[56] and enriched pathways were determined due to threshold (p-value <.005).[57]

2.6 | Evaluation of lipid differentiation gene set expressions in various diseases

First, lipid metabolism associated diseases were listed due to the evidence of lipid alterations in the disease state. To distinguish lipid metabolism-associated disease gene expression datasets, GEO database[55] was searched and datasets were collected if a dataset includes both diseased and healthy samples without any manipulation or treatment (drug, mutation, siRNA, etc.) and with at least three samples in each group. Transcriptome data from 101 microarray datasets comprising 44 different diseases and 29 tissues were obtained. Differentially expressed genes (DEGs) were analyzed according to the previously published pipeline,[58,59] and DEGs were considered significant due to the criteria of p-value <.05. Selected lipid gene set was searched in each DEG list and associated with selected diseases.

2.7 | Statistical analysis

All statistical analyses was performed using the GraphPad Prism software version 8 (GraphPad software). Student's t-test was used to compare differences between two groups, and one-way ANOVA (Dunnett's post-test) was used for comparison of multiple groups. The differences between groups were considered statistically significant if the p-value was <.05 (*p < .05; **p < .01; ***p ≤ .001; and ****p < .001).

2.8 | Western blot analysis

The cells were washed with PBS, and then, RIPA lysis buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 50 mM tris, pH 8) containing protease inhibitor (Sigma) was added to the cells. The cells were kept at cold condition during the lysis process. The cell lysate from each sample was transferred to their respective tube and centrifuged at 4° for 15 min. The supernatant was then mixed with loading buffer (Laemmli sample buffer) and denatured at 95° for 5 min. The samples were put on ice and then loaded into 12% polyacrylamide gel (Invitrogen). The gel was removed and transferred to a polyvinylidene fluoride membrane using the iBlot (Invitrogen). Following the transfer, the PVDF membranes were incubated in 5% milk for blocking, and then, primary antibodies were added and the PVDF membrane was incubated overnight at 4°C. The PVDF membranes were washed with TBST next morning, and the secondary antibodies (1:5000 dilution in TBST) were added and incubated for 1 h at room temperature. Then, the PVDF membranes were washed with TBST for about half an hour with a buffer change every 5 min. For detection, chemiluminescent substrate (Thermo Fisher Scientific) was added to the PVDF membranes and incubated for 5 mins. The membranes were visualized using the Odyssey imaging system (LI-COR Biosciences).

3 | RESULTS

3.1 | DINP and DINCH increase the lipid content

Oil red staining was performed to determine whether DINP and DINCH alter lipid metabolism in 3T3-L1 cells. The results showed that DINP and DINCH increase the lipid content in a dose-dependent manner, as the intensity of ORO stain increased in the cells upon exposure to both compounds (Figure 2C–I). The higher dose (100 μM) of DINP and DINCH demonstrated a similar staining pattern with the positive control Rosi (Figure 2B,F,I). At 100 μM exposure group, DINP resulted in higher lipid accumulation compared with DINCH (Figure 2F,I). However, in the lower concentrations, there was no apparent difference in the lipid content between the compounds.

3.2 | DINP and DINCH alter lipid metabolism genes

Housekeeping genes are the gold standard in normalizing mRNA fractions to get reliable and accurate gene expression results. Although there are several candidates of housekeeping genes, determining a stable one across control and treatments is very important. Multiple housekeeping genes, including *actin beta*

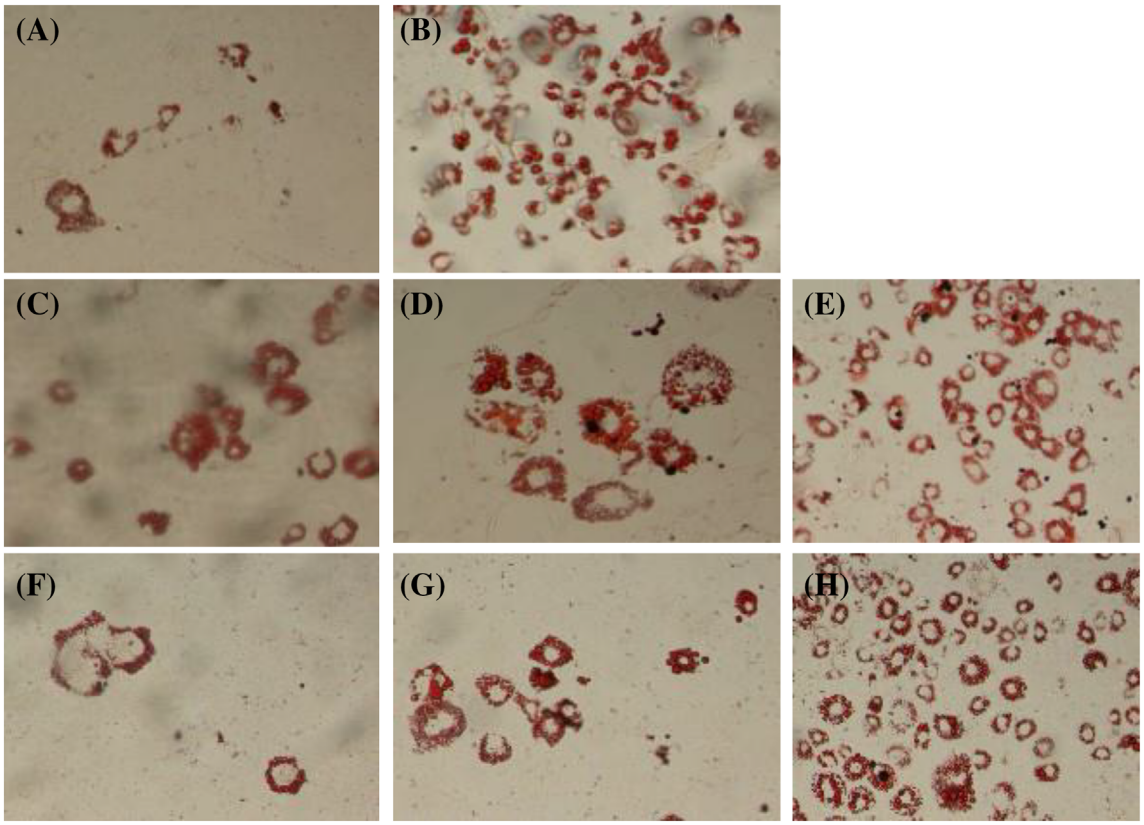

FIGURE 2 DINCH and DINP increase lipid accumulation in 3T3-L1 cells. Oil red O staining of 3T3-L1 cells at 20× magnification treated with DMSO, Rosi, DINCH, and DINP at different concentrations for 10 days of exposure. (A) Control containing DMSO, (B) positive control containing Rosi, (C) DINCH at 1 μM, (D) DINCH at 10 μM, (E) DINCH at 100 μM, (F) DINP at 1 μM, (G) DINP at 10 μM, and (H) DINP at 100 μM.

(*Actb*), TATA-box-binding protein (*Tbp*), eukaryotic translation elongation factor 1 alpha 2 (*Eef1*), *beta-2-microglobulin* (*B2M*), and *Gapdh*, were analyzed using NormFinder and geNorm algorithms. *B2M*, *Actb*, and *Eef1* showed inconsistent Ct values and lower stability. *Gapdh* and *Tbp* showed higher stability, and both algorithms suggested that *Tbp* was the most stable gene (Figure S1). Since there was not a big difference between *Tbp* and *Gapdh* (Figure S1), *Gapdh* was used as the reference gene.

Rosi significantly induced all the analyzed genes except *sterol regulatory element-binding protein 1* (*Srebp1*) (Figure S2). The data indicate that Rosi could be used as an efficient positive control in the study.

To investigate the molecular mechanisms behind the lipid accumulation, the expression of lipid-related genes was evaluated. An increased expression of *fatty acid-binding protein 5* (*Fabp5*) was observed only by 100 μM dose of both compounds with a higher effect in DINP (Figure 3A), while *Fabp4* was significantly induced by

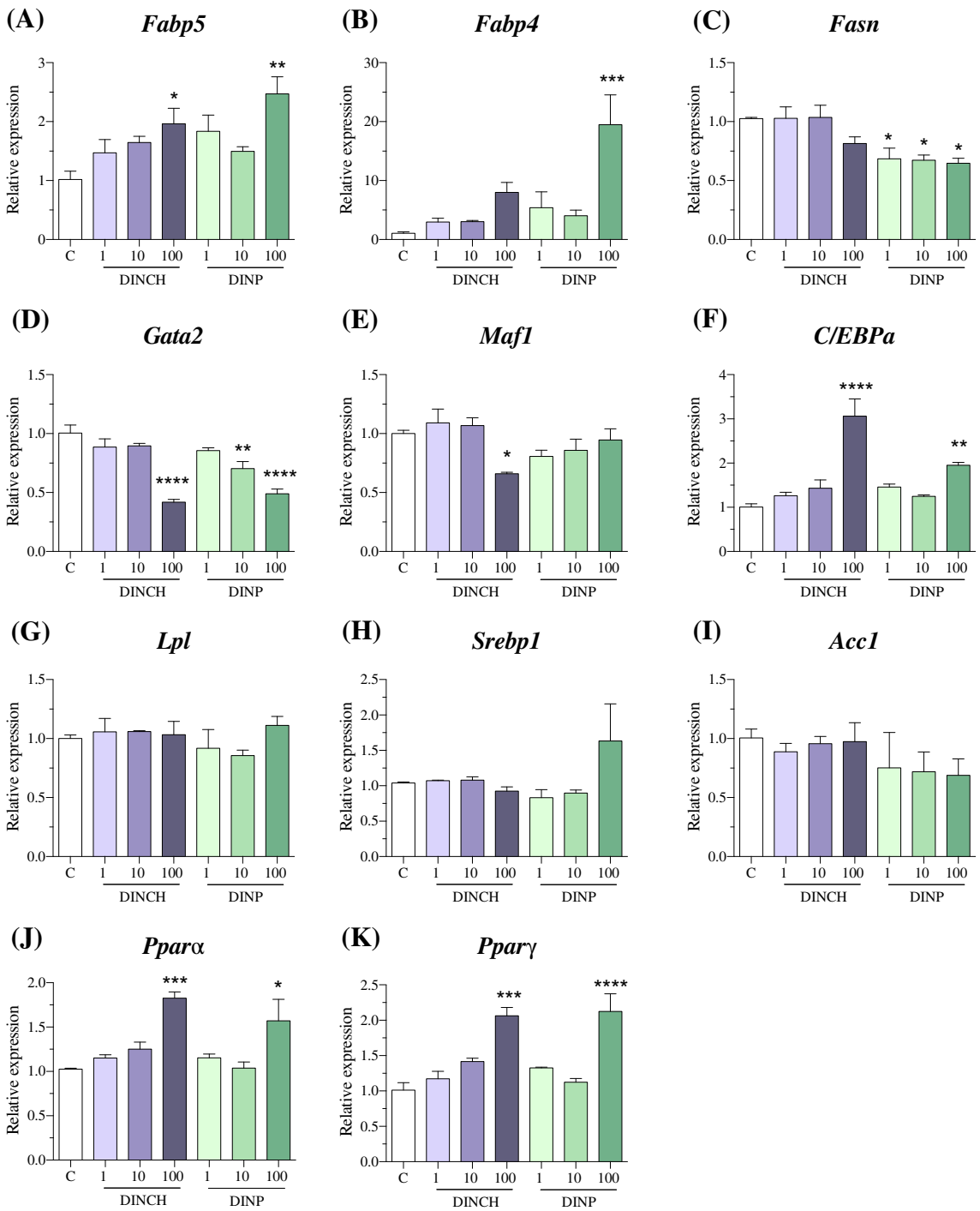

FIGURE 3 DINCH and DINP alter lipid metabolism genes. DINP and DINCH at the concentrations of 1, 10, and 100 μM were exposed to cells, and qPCR analysis was performed for different genes. Expression was compared against the differentiated solvent control (C). *$p < .05$; **$p < .01$; ***$p \leq .001$; and ****$p < .001$. One-way ANOVA followed by Dunnett's post-test. Error bars represent mean ± SEM, $n = 3$.

only 100 μM dose of DINP (Figure 3B). *Fatty acid synthase* (*Fasn*) was significantly downregulated by all the doses of DINP (Figure 3C). Interestingly, *Fasn* did not show any significant change at any doses of DINCH (Figure 3C). *Gata-binding protein 2* (*Gata2*) was significantly repressed only by 100 μM of DINCH, while exposure to 10 and 100 μM of DINP resulted in decreased expression of this gene (Figure 3D). In the meantime, *MAF1 homolog, negative regulator of RNA polymerase III* (*Maf1*), was downregulated by 100 μM dose of DINCH(Figure 3E), while *CCAAT-enhancer-binding protein alpha (C/EBPα)* was induced by 100 μM dose of both compounds (Figure 3F). The expression of other genes, including *lipoprotein lipase* (*Lpl*), *Srebp1*, and *acetyl-CoA carboxylase alpha* (*Acc1*), did not show any significant change at any exposure condition of the compounds (Figure 3G–I). Peroxisome proliferator-activated receptor genes, *Pparα* and *Pparγ*, were further analyzed, and the expression of both genes was found to be significantly induced in response to 100 μM of DINP and DINCH (Figure 3J,K).

Gene expression profiles of lipid differentiation in 3T3-L1 have been shown by high-throughput RNA sequencing.[54] By taking advantage of this

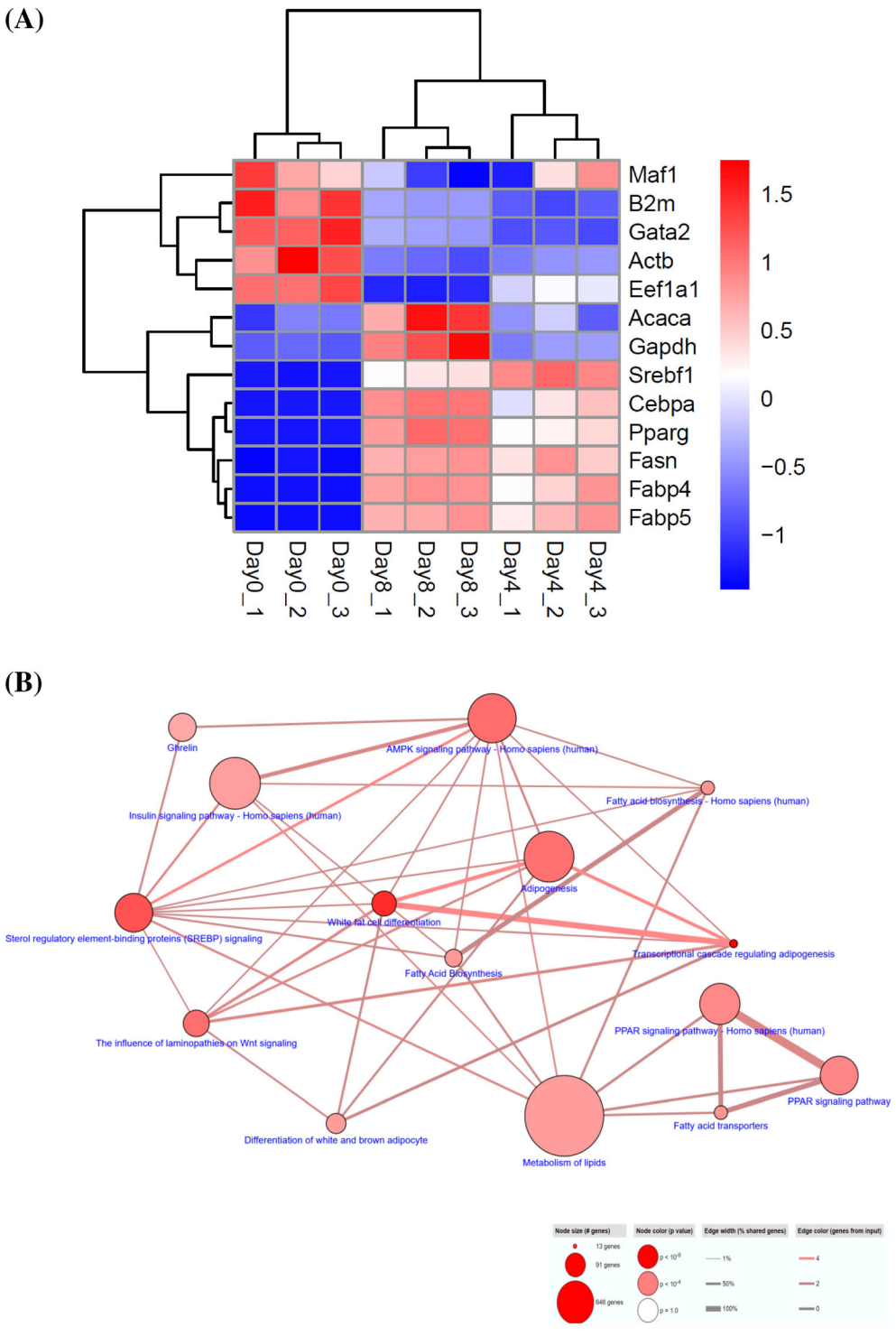

FIGURE 4 Transcriptome data illustrating the temporal expression profiles of lipid gene signatures across various stages of 3T3-L1 adipocyte differentiation, accompanied by pathway enrichment results. (A) Utilizing an independent RNAseq dataset (GSE129957), differentiation stages of selected gene signatures are found indicating a strong shift lipid progression after fourth day while housekeeping genes such as GADPH and ACTB had more mild progression at mRNA expression level. (B) Enrichment analysis unveiled significant associations with key biological processes and pathways encompassing metabolism of lipids, fatty acid biosynthesis, adipogenesis, ghrelin, transcriptional cascade regulating adipogenesis, AMPK signaling pathway, insulin signaling pathway, and PPAR signaling pathway collectively highlighting lipid-specific metabolic processes and crucial signaling pathways.

RNAseq data, comprehensive systems biology analysis was performed to further confirm the experimental findings. We found a lipid-specific gene set that are significantly altered from Day 0 to Day 8 (Figure 4A). Over-representation analysis of lipid gene set denoted in this study highlighted lipid-specific pathways comprising metabolism of lipids, differentiation of white and brown adipocyte, fatty acid biosynthesis, adipogenesis, and insulin signaling pathway (Figure 4B). While we focus on determining lipid process-related genes, we also checked known housekeeping gene expressions before the experimental validation by qPCR. According to RNAseq analysis, *Gata2*, *B2M*, *Actb*, *Eef1a1*, and *Maf1* were found as downregulated, while *Acc1*, *Srebp1*, *Fasn*, *Gapdh*, *Fabp5*, *Pparγ*, *C/EBPα*, and *Fabp4* were found as upregulated during adipocyte differentiation.

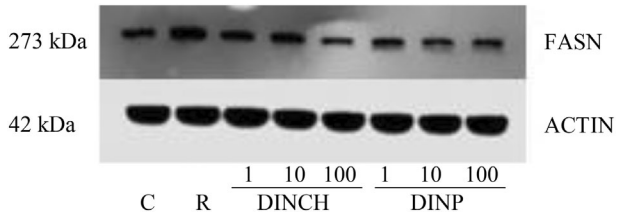

FIGURE 5 DINCH and DINP alter FASN protein expression in the 3T3-L1 cell line. Protein analysis on DINCH and DINP exposed cells (1, 10, and 100 μM) with the primary antibody FASN. Actin was used as a loading control.

The comparative analysis of gene expression profiles pertaining during lipid progression in cell line model[54] and those subjected to DINP and DINCH treatments in this study has similar expression patterns of key regulatory genes such as *Pparγ*, *Fabp5*, *Fabp4*, *C/EBPα*, and *Gata2*.

3.3 | DINP and DINCH repressed the expression of FASN protein level

To examine changes in protein expression upon exposure to DINP and DINCH, Western blot analysis for FASN, FABP4, SREBP1, and ACC1 was performed. Beta-actin was used as the loading control. The evaluation of the data was performed qualitatively. The expression level of FASN was downregulated in response to all doses of DINP and DINCH (Figure 5). This result was consistent with the gene expression pattern of *Fasn* although *Fasn* was found upregulated during adipocyte differentiation in 3T3-L1 cell line. Interestingly, no result was obtained for SREBP1, FABP4, and ACC1 at different optimization parameters.

3.4 | Pathway and disease similarity analysis

Pathway analysis was performed to better determine DINP and DINCH toxicity. Figure 6 enhances understanding the mechanism

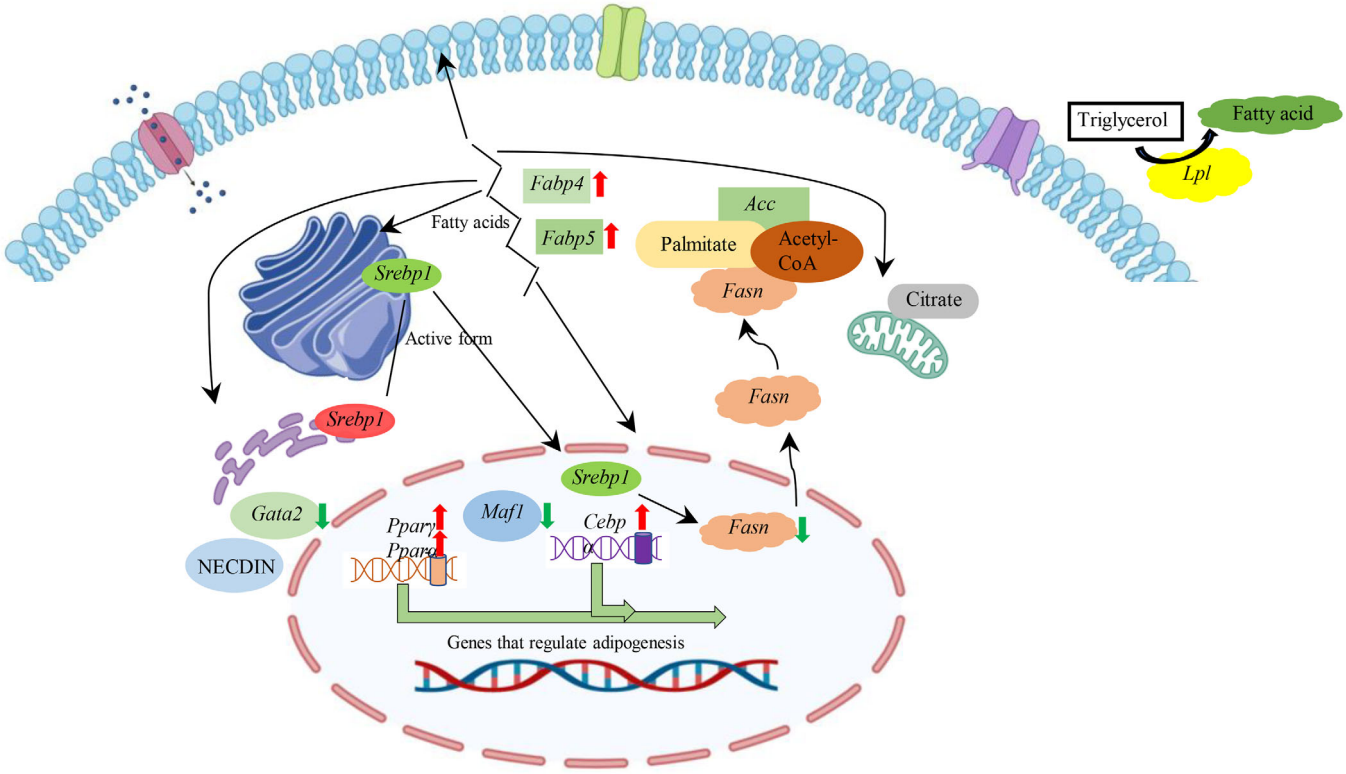

FIGURE 6 Pathway overview of the differentially expressed genes in response to DINP and DINCH. The affected genes upon exposure to DINP and DINCH are shown as upregulated (↑) and downregulated (↓). DINP and DINCH induced the expression of *Fabp4* and *Fabp5* which are involved in transporting fatty acids to organelles including endoplasmic reticulum, Golgi apparatus, nucleus, mitochondria, and cell membrane. This induced the expression of genes associated with adipose differentiation such as *Gata2*, *Pparα*, *Pparγ*, and *C/EBPα*. Meanwhile, DINP and DINCH further downregulated the transcription of *Maf1* in the nucleus to reduce the levels of MAF1 intracellular and increase lipid formation.

behind the toxicity of both compounds. Accordingly, it was observed that DINP and DINCH altered lipid metabolism via inducing fatty acid biosynthesis as *Fabp4* and *Fabp5* genes were significantly overexpressed. Other genes associated with lipid uptake and/or transport were not affected by both compounds. This could trigger adipose differentiation, as the expression of several genes associated with this process including *Gata2*, *Pparγ*, *Pparα*, and *C/EBPα* was significantly altered (Figure 6).

Altered lipid metabolism is associated with many diseases from cancers[60] to neurodegenerative disorders.[61] Since there are concerns about the negative impacts of plasticizers on lipid metabolism and endocrine system, we aimed at determining diseases that have common DEGs with DINP- and DINCH-treated 3T3-L1 cells. Not surprisingly, the expression of housekeeping genes including *ACTB*, *EEF1A1*, *B2M*, and *GAPDH* was observed in several diseases. Except housekeeping genes, *GATA2*, a transcription factor, showed expression in 30 diseases. On contrary, *MAF1* was shown to be associated with 12 diseases, including obesity, endometriosis, various cancers, and neurodegenerative diseases (Figure 7).

4 | DISCUSSION

The extensive use of phthalates has resulted in their accumulation in the environment leading to detrimental effects on animals and humans.[7] Recent epidemiological studies have associated phthalates and their metabolites with several diseases, including carcinogenesis, neurotoxicity, reprotoxicity, hepatotoxicity, and nephrotocixity.[12–16] Several phthalates have also been shown to affect lipid metabolism.[62,63] These findings have led the regulatory bodies to reduce or ban the use of several phthalates, resulting in increasing demand for environmentally safe alternative compounds. DINP and DINCH are such alternative plasticizers introduced into the market. Although there are some data indicating the toxicity of DINP, there is a lack of risk assessment on DINCH. Hence, it is of great importance to determine whether these plasticizers are safe for animal and human health. In the present study, we performed a comparative toxicity study to assess the impacts of DINP and DINCH on lipid metabolism using 3T3-L1 cells.

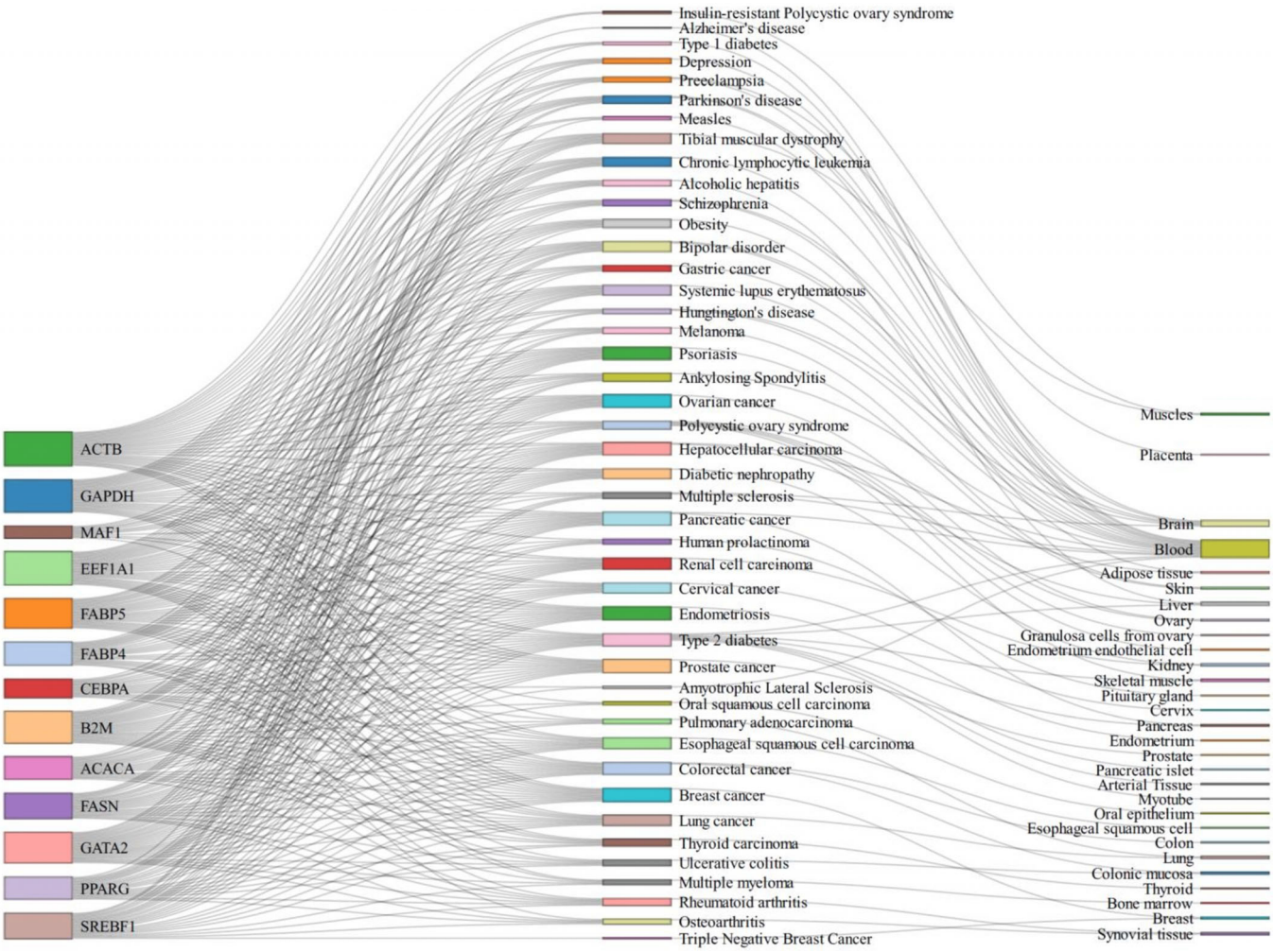

FIGURE 7 Differential expression of selected lipid gene signatures in multi-factorial disease and tissues. In addition to housekeeping genes such as GADPH and ACTB, selected genes are also determined in diseases from cancers to neurodegenerative diseases similar to altered expression of the metabolic changes triggered by exposure to DINP and DINCH.

Lipids play a significant role in multiple biological processes, such as membrane composition, hormonal signaling and sources of energy.[64,65] Several studies have identified that a number of lipid molecules could be used as biomarkers for different metabolic disorders and diseases.[66–68] It has also been indicated that dysregulation of lipid metabolism is a key feature of metabolic diseases and cancer.[69] In the present study, we performed a comparative toxicity study to determine the negative effects associated with DINP and DINCH using 3T3-L1 cell line. We found that both plasticizers can regulate lipid metabolism; however, DINP seems to be more active than DINCH. First, we observed a dose-dependent increase in lipid content in response to both DINP and DINCH. The higher concentration (100 μM) of both compounds showed a similar pattern of lipid accumulation with the positive control (Rosi). The data also demonstrated that DINP exposure resulted in a higher lipid accumulation than DINCH. In line with this, in a previous study, exposure to DINP (ranging from 0.01 to 25 μM) for 10 days exerted a dose-dependent lipid accumulation in 3T3-L1 cells.[70] In another study, it was also shown that 10 days of exposure to DINP showed an increase in adipogenesis and lipid content in 3T3-L1 cells.[28] In our previous study, we determined a modest lipid accumulation in the yolk region of zebrafish in response to DINCH.[47] However, in another study, DINCH did not show any significant effect, while its primary metabolite induced lipid accumulation in preadipocytes of rats.[38] Several other studies have also shown an altered fatty acid composition[71] and a significant induction in phospholipids diacylglycerides[72] in mice, and an increase in lipids and triglycerides,[73] and saturated and unsaturated lipids[74] in the liver of gilthead seabream, suggesting a lipid accumulation in response to DINP.

To further confirm these findings, we performed qPCR analysis for genes involved in lipid metabolism pathways. We observed a significant upregulation of *Fabp5* in response to 100 μM dose of both compounds, whereas *Fabp4* was upregulated only by a higher dose of DINP. Fatty acid-binding proteins are a family of lipid chaperones involved in systemic metabolic regulation through various lipid signaling pathways.[75] *Fasn* plays a role in storing triacylglycerides in adipose tissue by converting acetyl-CoA and malonyl-CoA into palmitate and then esterifying palmitate into triacylglycerides.[76] We determined a significantly repressed expression of *Fasn* in response to all doses of DINP. The protein expression level was also correlated with gene expression data, as we observed a significant downregulation of FASN upon exposure to DINP. Interestingly, DINCH did not alter the expression of *Fasn* at any exposure condition, while the protein expression level of FASN was repressed by 100 μM dose. We suggest that although DINCH did not affect *Fasn* mRNA level, it demonstrated its impact on protein level due to complex post-transcriptional mechanisms.[77,78] *Srebp1*, a transcription factor that regulates the expression of genes involved in the uptake and synthesis of cholesterol and fatty acids,[79] was also not affected by any doses of either compounds. This is in line with another study, where DINP showed no effect on the expression of *Srebp1* in 3T3-L1 cells.[28] We also observed a downregulation of *Maf1* in response to higher dose of DINCH. *Maf1* is a negative regulator of RNA polymerase III, and it has been demonstrated that it is involved in regulating lipid metabolism and homeostasis.[69,80,81]

Altered lipid metabolism is linked to several metabolic disorders including obesity and type II diabetes.[82] Adipogenesis is a complex cascade and is regulated by a number of transcription factors including master regulators *Pparγ* and *C/EBPα*[83] and a negative regulator *Gata2*.[84] Overexpression of *Gata2* can suppress adipocyte differentiation via direct binding to *Pparγ* transcription factor and downregulating its activity. Besides, the interaction between *Gata2* and *C/EBPα* is of critical for *Gata2* to negatively regulate adipocyte differentiation.[84] In the present study, we observed a significant downregulation of *Gata2*, while *Pparγ* and *C/EBPα* were highly induced by higher dose of both DINP and DINCH. In the meantime, another transcription factor *Pparα*, involved in regulation of β-oxidation,[85] was also significantly upregulated by higher dose of both compounds. In consistent with our data, in a previous study, it has been demonstrated that *Pparγ* and *C/EBPα* were significantly upregulated in response to DINP indicating an induced adipogenesis to adipocytes in 3T3-L1 cells.[28] Interestingly, in a study on Sprague Dawley rats, it has been demonstrated that *C/EBPα* was induced, while *Pparα* and *Pparγ* were significantly downregulated.[46] In another study, DINP was found to activate PPARγ in transient transfection assay.[70] In our previous study on zebrafish, we also observed an altered expression of PPAR genes in response to DINCH.[47] The study by Campioli et al.[38] showed that DINCH could induce the expression of *Pparg2* in rats. In an in vitro study, it has also been demonstrated that DINCH metabolites altered PPARα- and PPARγ-dependent reporter gene activities in a dose-dependent manner.[42] Although there is no direct evidence that DINP and DINCH cause specific diseases, several studies describe effects such as alteration of lipid mechanisms or endocrine systems. These effects, although not direct, may indirectly promote various diseases, as we hypothesized in this study. Altogether, we suggested that DINP and DINCH have the potential to alter lipid accumulation by regulating adipogenesis in 3T3-L1 cells. The present data also indicate that the induced lipid accumulation in cells is more distinct upon DINP exposure than DINCH.

5 | CONCLUSION

Proper regulation of lipid metabolism is critical in maintaining lipid homeostasis and the physiological functions. Several PEs and their metabolites have been shown to be associated with several diseases, including obesity and diabetes. DINP and DINCH are alternative plasticizers that are not properly assessed for possible adverse effects. A better understanding of the molecular mechanisms of their negative impacts could aid in their regulation, use, and prevention of associated metabolic diseases. In this study, we showed that DINP and DINCH could alter lipid accumulation in 3T3-L1 cells. We also identified altered expression of several genes that resulted in disruption of lipid homeostasis and adipogenesis. The impact of DINP is more severe compared to DINCH. In conclusion, the present study suggests that alternative plasticizers DINP and DINCH could be associated with

lipid accumulation and this subsequently could trigger off adipogenesis. Besides, this metabolic change may stimulate various cancers and neurodegenerative diseases through common patterns of altered gene expression. To better reveal this, further analysis needs to be done in in vivo model organisms. The level of alternative plasticizers in the environment is increasing gradually; hence, their use should be monitored and regulated carefully.

AUTHOR CONTRIBUTIONS

Conceptualization: Ajay Pradhan, Ceyhun Bereketoglu. Methodology: Ajay Pradhan, Ceyhun Bereketoglu, Isabel Häggblom, Beste Turanlı. Data acquisition: Ajay Pradhan, Ceyhun Bereketoglu, Isabel Häggblom, Beste Turanlı. Data analysis and interpretation: Ceyhun Bereketoglu, Ajay Pradhan, Beste Turanlı. Manuscript writing: Ajay Pradhan, Ceyhun Bereketoglu, Beste Turanlı. Manuscript Review and Editing: Ceyhun Bereketoglu, Ajay Pradhan, Beste Turanlı. Funding Acquisition: Ajay Pradhan, Ceyhun Bereketoglu.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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