Structurally-dependent hepatotoxic effects of phthalates on liver homeostasis

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SUMMARY & CONCLUSION

•The effects of phthalates were investigated in a rat liver progenitor / liver epithelial cell line WB-F344 characterized by functional GJIC.

•The modulation of GJIC by phthalates depends on their structure and molecular weight. The highest level of GJIC inhibition was observed after exposures to phthalic acid diesters with side chains made by 4-6 carbons.

•GJIC was affected even after short exposure times (<10 minutes), which implies, that apart from mechanisms of genomic signaling through nuclear receptors, the effects of phthalates can be mediated also through rapid mechanisms of non-genomic signaling.

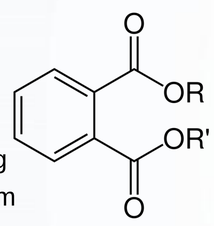
•Except first group of low molecular weight phthalates, all other groups did activate phosphorylation of MAPK, which is considered to be one of the mechanisms of dysregulation GJIC

THE AIM OF THIS STUDY WAS TO ASSESS THE HEPATOTOXIC EFFECT OF STRUCTURALLY DIFFERENT PHTHALATES ON NON-DIFERENTIATED OVAL CELLS IN LIVER. AS A MARKER OF HEPATOTOXICITY AND DISRUPTION OF LIVER HOMEOSTASIS WAS EVALUATED CELL-TO-CELL COMMUNICATION AND ACTIVATION OF MAPK/ERK PATHWAY.

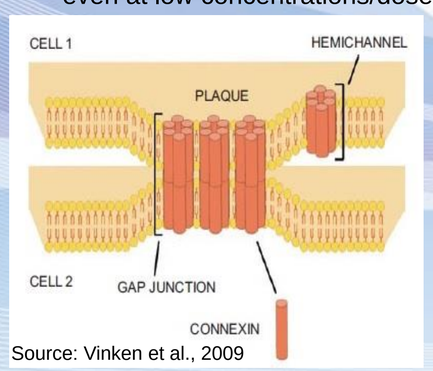
RAT LIVER EPITHELIAL CELL LINE WB-F344

- Liver epithelial cells from Fischer F344 rats
- Normal, non-cancerous diploid cells
- Hepatic progenitor cells / stem-like cells
- Multipotent, capable of *in vitro* and *in vivo* differentiation into hepatocytes, cardiomyocytes, and biliary duct cells
- Expression of connexin 43 (*Cx43*) and functional gap junctional intercellular communication (GJIC),
- Not expressing PPARα

PHTHALATES



- Esters of phthalate acid
- Used as major plasticizers
- Humans are exposed to phthalates leaking from plastics from the environment and from cosmetics or food on a daily basis.
- Phthalates are showing low acute toxicity
- Studies indicated other negative effects after chronic exposure even at low concentrations/doses



Source: Vinken et al., 2009

GJIC

• Gap junctional intercellular communication (GJIC) represents a key mechanism involved in the maintenance of tissue homeostasis including liver and it is a central regulator of cell signalling and gene expression.

•The downregulation of GJIC and the activation of mitogen-activated protein kinases (MAPKs), specifically extracellular receptor kinases 1 and 2 (ERK 1 and ERK 2), have been strongly linked to the tumor-promoting phase of cancer.

METHODS

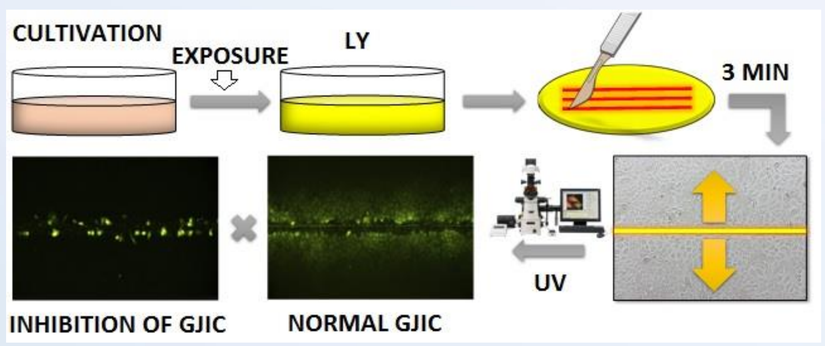
•WB-F344 cells were seeded and cultured for 48 hours to reach complete confluence before the addition of each chemical.

•The confluent cultures were exposed to the individual phthalate of interest, phorbol ester 12- *O* -tetradecanoylphorbol 13-acetate (TPA) as well as to solvent for 30 min and 24 h

•TPA was used as positive control, on the base of TPA is a known GJIC inhibitor via protein kinase C (PKC) as well as mitogen-activated protein kinase-extracellular receptor kinase 1/2 (MAPK ERK1/2)-dependent

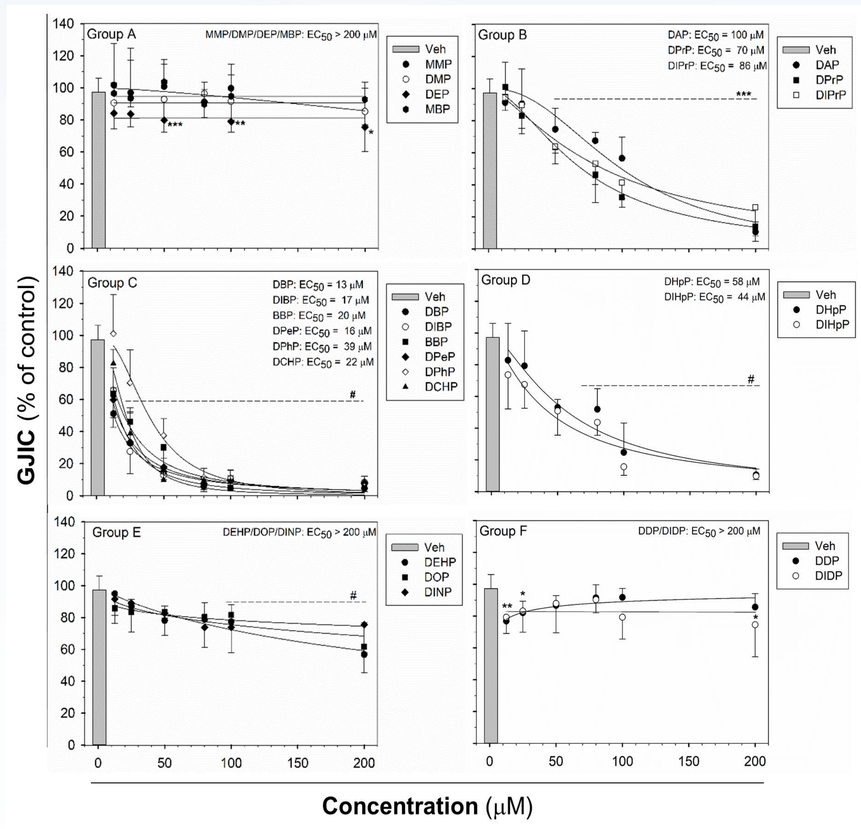
•The scrape load-dye transfer (SL-DT) technique was used: Lucifer Yellow (LY) dilithium salt was added to the cells and the dye was introduced to the monolayer by three parallel cuts per dish with a surgical scalpel blade. LY was allowed to diffuse through gap junctions for 3 min, followed by a thorough rinse of cells with CaMgPBS and a fixation step with a 4 % (vol/vol) formaldehyde solution in PBS

• A representative microscopic image of the LY transfer was taken and the extent of GJIC was evaluated as an area of the cells stained with LY



A. CONCENTRATION- AND TIME-DEPENDENT GJIC DYSREGULATION INDUCED BY PHTHALATES

Concentration dependent GJIC inhibition



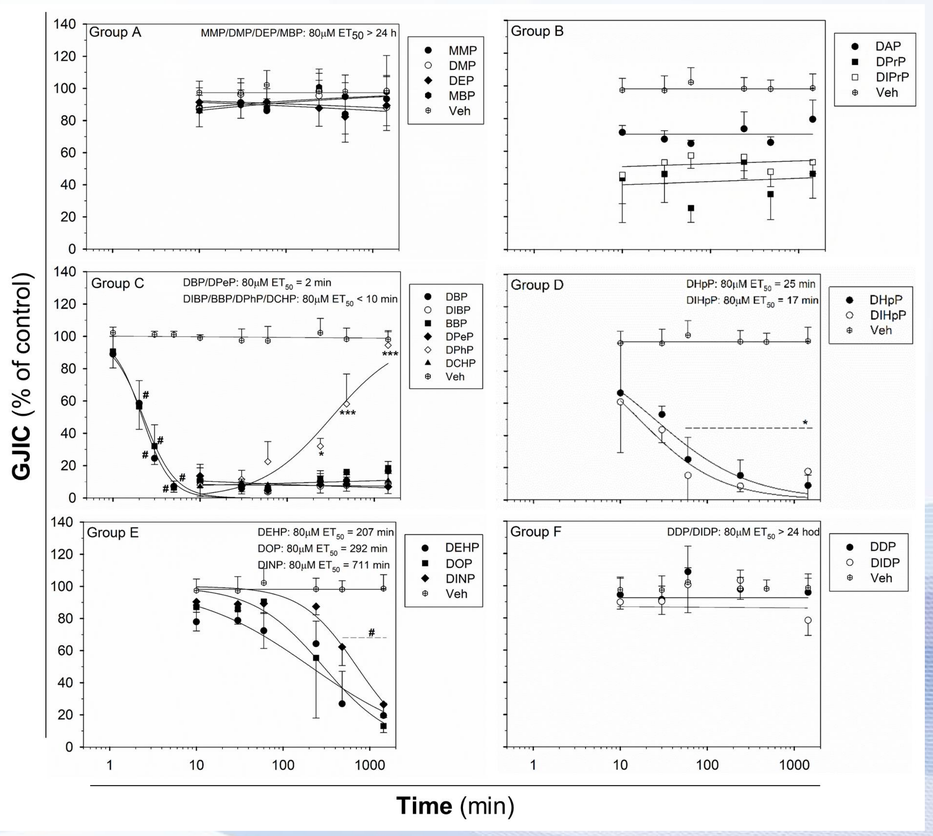
The phthalates were shown as potent GJIC-inhibitors in liver oval cells and the effects were structure-dependent. Based on the data from GJIC evaluation, phthalates could be distinguished in six groups according to the effects observed to their molecular weight and effects on intracellular communication:

1. **Group A** (low-molecular-weight phthalates with short or medium carbon side chain length - MMP, DMP, DEP and MBP) → any significant effect on GJIC even after longer exposure time.
2. **Group B** (molecular weight of 246–251 g.mol-1 and short 3C side chains - DAP, DPrP and DIPrP)
   - ➢inhibited GJIC in higher concentrations
   - ➢after 30min EC_{50} values of 70-100 mM
   - ➢effect did not change within time.
3. **Group C** (molecular weight of 278–330 g.mol-1 and medium carbon side chain - DBP, DIBP, BBP, DPeP, DPhP and DCHP)
   - ➢dysregulated GJIC with the highest potency with the $_{30min}EC_{50}$ values of 10-40 µM.
   - ➢GJIC dysregulation induced by these phthalates occurred rapidly within first 10 min
   - ➢ did not significantly change during the extended exposure times (up to 24 h) except for DPhP.

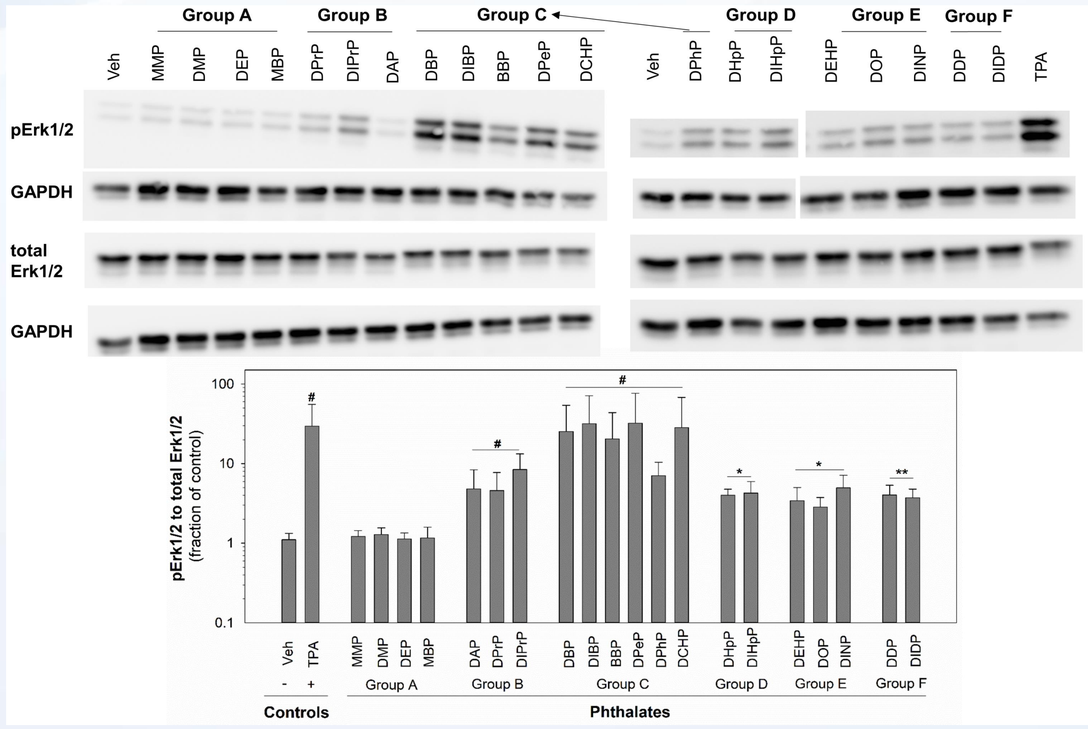
* The inhibitory effect of DPhP on GJIC was transient. DPhP also caused strong inhibition of GJIC within several minutes, but the communication was restored after longer exposition, and after 1440 min of exposure GJIC level was not significantly different from the solvent control.

4. **Group D** (DHP and DIHP)
   - ➢ medium effect on liver GJIC with the $_{30min}EC_{50}$ values of 10-40 µM.
   - ➢ time required for 50% inhibition of GJIC was estimated to be 20 min for the concentration of 80 µM ($_{80\mu M}ET_{50}$).
5. **Group E** (molecular weight of 363–419 g.mol-1 and long carbon side chain - DEHP, DOP, DINP)
   - ➢ stronger inhibition effect after longer time of exposure with the $_{80\mu M}ET_{50}$ values of 3-11 h.
6. **Group F** (the highest molecular weight, DOP and DIDP)
   - ➢ any significant effect neither in higher concentrations, neither after the longest tested time of 1440 min.

Time dependent GJIC inhibition



B. MAP KINASE (MAPK) ACTIVATION IN LIVER OVAL CELLS IN RESPONSE TO PHTHALATES



As a positive control of GJIC inhibition there was used TPA which is known to initiate phosphorylation of MAPK kinases. After 30 min exposure to 10 nM TPA the phosphorylation of both ERK1 and 2 rapidly significantly increased (30-fold increase) in WB-F344 cells.

As expected, phthalates from the **Group A** did not induce phosphorylation. After 30 min exposure of WB-F344 cells to phthalates from the other groups, the phosphorylation of ERK1/2 always increased.

Phthalates from the **Group B** are weak activators of phosphorylation which was only 5-8× higher than in the control.

The strongest activators were shown to be phthalates from the **Group C**, which activated phosphorylation of ERK1/2 from 7 to 30 times. Phthalates from rest of the groups can be considered as weak activators as they activate phosphorylation 4× (**Groups D and F**) and 3-5× (**Group E**), respectively.