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Targeting Gap Junctional Intercellular Communication by Hepatocarcinogenic Compounds

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Abstract:

Gap junctions in liver, as in other organs, play a critical role in tissue homeostasis. Inherently, these cellular constituents are major targets for systemic toxicity and diseases, including cancer. This review provides an overview of chemicals that compromise liver gap junctions, in particular biological toxins, organic solvents, pesticides, pharmaceuticals, peroxides, metals and phthalates. The focus in this review is placed upon the mechanistic scenarios that underlie these adverse effects. Further, the potential use of gap junctional activity as an *in vitro* biomarker to identify non-genotoxic hepatocarcinogenic chemicals is discussed.

Key words: connexin, gap junction intercellular communication, hepatocarcinogenicity, non-genotoxic carcinogenic compounds, risk assessment, hemichannel, *in vivo*, *in vitro*.

List of abbreviations:

Ac	Acetylation
AhR	Aryl hydrocarbon receptor
Akt	Protein kinase B
cAMP	Cyclic adenosine monophosphate
CCl_4	Carbon tetrachloride
Cd	Cadmium
Cx	Connexin
CYP450	Cytochrome P450
DDT	Dichlorodiphenyltrichloroethane
DEHP	Di(2-ethylhexyl) phthalate
ERK	Extracellular signal-regulated kinase
GJIC	Gap junction intercellular communication
HCB	Hexachlorobenzene
HCC	Hepatocellular carcinoma
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase kinase
NO	Nitric oxide
OC	Organochlorine
OP	Organophosphorus
PAHs	Polycyclic aromatic hydrocarbons
PCBs	Polychlorinated biphenyls
PCDDs	Polychlorinated dibenzodioxins
PCP	Pentachlorophenol

PC-PLC	Phosphatidylcholine-specific phospholipase C
PKC	Protein kinase C
PPIs	Protein-protein interactions
PTMs	Posttranslational modifications
PVC	Polyvinyl chloride
ROS	Reactive oxygen species
Src	Rous sarcoma oncogene
TCE	Trichloroethylene
TF	Transcription factor

Introduction

The liver is a versatile organ responsible for many essential processes, such as lipid metabolism, carbohydrate metabolism and xenobiotic biotransformation. The latter includes the chemical modification of drugs and detoxification of harmful chemicals (Karin and Dhar 2016). Because of this, the liver is often one of the first organs to be targeted by noxious chemicals, including carcinogenic compounds. From a mechanistic point of view, cancer can result from genotoxic and non-genotoxic actions. Genotoxic carcinogenic chemicals induce damage to the genetic information through mutagenicity, clastogenicity and aneugenicity, which can occur in virtually all cell types (Guo et al. 2020). In contrast, non-genotoxic carcinogenic compounds act in a tissue-specific and species-specific way, relying on a wide diversity of mechanisms, such as modulation of cell death, induction of inflammation and inhibition of gap junctional intercellular communication (GJIC) (Hernandez et al. 2009; 2013; Vinken et al. 2008a). Since chemicals are major inducers of cancer, various types of chemicals are subjected to investigation for carcinogenic potential *prior* to reaching the market. Typically, a 2-year rodent bioassay is used for this purpose. This bioassay is not only criticized from the ethical point of view, but is also expensive and rather poorly predictive of human carcinogenicity. Genotoxicity testing is usually performed *prior* to potential carcinogenicity of a chemical can be ruled out (Doktorova et al. 2012; 2014a ; 2014b). The most frequently utilized tests are the Ames test and the mammalian gene mutation test, which both detect DNA damage. In addition, genotoxicity screening is done in cytogenetic assays, such as the chromosome aberration assay and the micronucleus assay (Guo et al. 2020). Although these *in vitro* methods have proven their value, the 2-year rodent assay still remains the golden standard when it comes to carcinogenicity testing (Nohmi 2018). For testing non-genotoxic carcinogenic potential, no *in vitro* assays are currently available, which may be attributed to the plethora of specific mechanisms involved in this type of adverse effects. In the 1990s, considerable attention was paid to inhibition of GJIC

as a potential biomarker for non-genotoxic carcinogenic compounds (Yamasaki et al. 1996; Yamasaki 1995). Throughout this research, focus was placed upon the liver (Chipman et al. 2003), which is not surprising given its key function as detoxification hub in the body. The shutdown of GJIC leads to an escape from homeostatic control, ultimately resulting in carcinogenesis (Yamasaki et al. 1999). The present review examines the role of liver gap junctions as targets for non-genotoxic carcinogenic chemicals. As such, this review serves an update of a paper with similar scope previously published by Vinken et al. (2009).

General Features of Liver Gap Junctions

Structural Properties

Gap junctions are a group of cell-to-cell contacts composed of 2 hemichannels of adjacent cells, which in turn are built up by 6 connexin (Cx) proteins (Figure 1) (Cooreman et al. 2019). Thus far, more than 21 human connexin family members were identified, all that are expressed in a cell type-specific manner (Cooreman et al. 2019; Tachikawa et al. 2020). In liver, hepatocytes abundantly produce Cx32 and to a lesser extent Cx26, whereas non-parenchymal hepatic cell populations, including Kupffer cells, endothelial cells and stellate cells, mainly express Cx43 (Berthoud et al. 1992; Greenwel et al. 1993; Saez 1997; Fischer et al. 2005). Cx32 is uniformly produced by hepatocytes, while Cx26 is preferentially expressed in the periportal acinar area (Rosenberg et al. 1992). All connexin proteins are named after their respective molecular weight and share the same topology consisting of 4 transmembrane regions, 2 extracellular loops, 1 cytoplasmic loop and an intracellular *C*-terminus and *N*-terminus. Approximately 3% of the membrane surface of hepatocytes is covered with gap junctions, which are organized in plaques. A gap junction typically measures 180Å in length and 15Å in diameter, enabling passive diffusion of small and hydrophilic molecules, such as glutamate, glucose, inositol trisphosphate, glutathione, adenosine trisphosphate and cyclic adenosine monophosphate (cAMP), as well as ions, including calcium, potassium and sodium (Vinken et

al. 2008b). Thus, gap junctions control all facets of the cellular life cycle ranging from cell growth to cell death (Vinken et al. 2006).

Regulatory Mechanisms

GJIC is mainly controlled at 2 levels, namely at the expression level and at the functionality level (Figure 2) (Nielsen et al. 2012). The latter, also called gap junction gating, encompasses many different factors, such as pH, calcium levels, transmembrane voltage, redox potential, interactions with other proteins and posttranslational modifications, including phosphorylation, glycosylation, *N*-acetylation, ubiquitination, methylation, *S*-tyrosination and SUMOylation (Leithe et al. 2018; Garcia et al. 2018; Vinken 2016; Sorgen et al. 2018). Phosphorylation is probably the best-studied posttranslational connexin modification, being well documented for Cx43. Phosphorylation predominantly occurs at serine, threonine and tyrosine residues of the *C*-terminal tail, and might be generated by a multitude of kinases, such as protein kinase C (PKC), protein kinase A, tyrosine kinase 2, mitogen-activated protein kinase (MAPK), Rous sarcoma oncogene (*v*-Src) and casein kinase 1 (Leithe et al. 2018). Another gating mechanism relates to changes in intracellular pH or calcium levels (Wei et al. 2019; Garciarena et al. 2018). Thus, Cx43 gap junctions open in alkaline conditions, whereas they close upon acidification (Garciarena et al. 2018). Gating in response to calcium is mediated by calmodulin, since gap junctions themselves do not possess high affinity for this ion (Peracchia 2020). Further, functional control also includes voltage gating depending upon the voltage gradient alongside the pore (Bargiello et al. 2018).

In addition to regulation at the cell plasma membrane, GJIC is also modulated at the expression level (Solan and Lampe 2018). The first type of expression control is epigenetic mechanisms, such as histone acetylation, DNA methylation and microRNA interactions. Histone acetylation is carried out by histone acetyltransferases. These enzymes stimulate transcriptional activation through chromatin decondensation, while gene suppression is

achieved by actions of histone deacetylases that condensate chromatin. Various inhibitors of histone deacetylases were linked to enhanced connexin production and gap junction functionality (Vinken 2016). In this respect, trichostatin A re-establishes GJIC in rat liver epithelial cells (Jung et al. 2006) and downregulates Cx43 production in human liver cancer, while leaving Cx26 and Cx32 unaffected (Yamashita et al. 2004). This has been associated with suppression of tumor aggressiveness due to reduction of tumor invasiveness and proliferation mediated by Cx43 (Menezes et al. 2019). Another epigenetic mechanism is facilitated by DNA methyltransferase enzymes. These enzymes typically hypermethylate gene promotors leading to inhibition of gene transcription. In liver cancer, the decrease of Cx26 expression was associated with enhanced levels of DNA methyltransferase mRNA (Shimizu et al. 2007) and methylated CpG dinucleotides located within the Cx26 gene promotor (Piechocki et al. 1999). Similarly, Cx32 and Cx43 gene promotor sites are methylated in liver epithelial cells lacking Cx32 expression and a rat hepatoma cell line missing Cx43, respectively (Piechocki et al. 1999). MicroRNA-related mechanisms constitute another level of epigenetic control. MicroRNAs are small complementary sequences that bind mRNA target molecules, thereby inhibiting translation or cleavage of mRNA (Vinken 2016; Wang et al. 2019a; 2020). Cx43 may be regulated by microRNA-206 amongst many others, thereby influencing metastasis (Lin et al. 2016) and differentiation (Anderson et al. 2006). Connexin gene transcription is also modulated by conventional *cis/trans* mechanisms. Both general and tissue-specific transcription factors are involved in this process. In liver, Cx32 gene expression is mediated, at least in part, by the ubiquitous transcription factor specificity protein 1 as well as by the liver-enriched transcription factor hepatocyte nuclear factor 1α (Plante et al. 2006; Koffler et al. 2002).

Role in Liver Disease

Given their critical role in maintaining homeostasis, it is not surprising that gap junctions are frequently involved in pathological conditions (Yamasaki et al. 1999). A wide spectrum of

alterations were found in connexin expression and gap junction functionality in liver diseases, including cholestasis (Fallon et al. 1995), non-alcoholic steatohepatitis (Luther et al. 2018), fibrosis (Fischer et al. 2005), cirrhosis (Yang et al. 2019) and liver cancer (Xiang et al. 2019). The nature of the GJIC and/or connexin modifications depends upon the type of cell and disease involved (Hernandez-Guerra et al. 2019; Maes et al. 2015a). In acute liver failure (Maes et al. 2016) and hepatitis (Balasubramaniyan et al. 2013), Cx32 and Cx26 production is typically downregulated, while Cx43 expression is upregulated. Generally, Cx43 appears elevated at the expense of Cx32 production in liver disease, owing to both increased production by non-parenchymal cells as well as through *de novo* expression by hepatocytes (Cooreman et al. 2019).

The same overall trend may be seen in hepatocellular carcinoma (HCC) (Yu et al. 2017). In normal liver, Cx43 expression is not detected in parenchymal liver cells (Zhang et al. 2007), whereas in liver cancer, Cx43 is present in these cells, and enhances invasion, migration and lung metastasis in rats (Ogawa et al. 2012). Cx43 expression levels, which are inversely correlated with GJIC, and aberrant localization correspond with malignancy levels of HCC (Zhang et al. 2007; Kawasaki et al. 2007). This has been clearly indicated in metastatic tumors expressing higher levels of Cx43 compared to less severe metastatic tumors or normal liver tissue (Ogawa et al. 2012). These effects are mediated through the binding of the tumor suppressor SEMA3F to the cytoplasmic loop of Cx43 (Kawasaki et al. 2007) as well as by methylation of its gene promotor (Piechocki et al. 1999). Cx43 expression in HCC might also be modulated by *cis/trans* mechanisms. In this respect, both *c*-fos and *c*-jun are upregulated during hepatocarcinogenesis (Elaut et al. 2006). These proto-oncogenes form the transcription factor activator protein 1, which is known to activate Cx43 expression (Oyamada et al. 2005). Overall, Cx43 is believed to play a tumor promoting role (Hernandez-Guerra et al. 2019).

Studies with Cx32 knock-out rats indicate increased susceptibility to tumor development compared to wild-type counterparts (Hokaiwado et al. 2007; Kato et al. 2016), while Cx32

overexpression was reported to inhibit metastasis and proliferation of HCC cells *via* p53 and protein kinase B (Akt) pathways (Zhao et al. 2015). In addition, Cx32-based gap junctions are crucial to avoid chemotherapy resistance, as these mediate the so-called bystander effect, including passage of anti-tumor agents to neighboring tumor cells (Yu et al. 2017; Xiang et al. 2019; Hong et al. 2012). The same bystander effect was also noted for Cx26 (Yang et al. 2016), of which the expression is decreased in HCC through methylation of its gene promotor (Vinken et al. 2012). These insights into the bystander effect of Cx26 and Cx32 are crucial for the exploration of new therapeutic strategies, since a major issue in HCC therapy is the resistance to chemotherapeutic agents (Sanchez et al. 2018).

Despite the large body of studies regarding altered expression and distribution of connexin proteins in HCC, the implications, the role and molecular mechanisms underlying these observations are still elusive. Contradictory to the above-mentioned investigations, overexpression of Cx32 in the intracellular space of non-metastatic HuH7 HCC cells enables invasion, metastasis and enhancement of self-renewal (Kawasaki et al. 2011; Li et al. 2007). When upregulated and internalized, Cx32 is linked to an advanced cancer stage and poor prognosis for HCC patients (Xiang et al. 2019). Similarly, cytoplasmic Cx32 exerts an anti-apoptotic function in HCC, protecting tumor cells from chemotherapeutic agents *via* epidermal growth factor receptor activation. The role of Cx32 in metastasis and oncogenesis is likely to be regulated by intracellular signal transduction pathways and transcriptional changes (Yu et al. 2017). Despite the contradictions, all studies indicate the importance of functional GJIC to maintain normal homeostasis. This does not only apply to the role of gap junctions in liver, but might also be expanded to other organs, such as heart or lungs. Similarly, abrogation of GJIC in these organs is associated with disease development (Li et al. 2018; Spannbrucker et al. 2019).

Effects of Chemicals on Liver Gap Junctions

Polycyclic aromatic hydrocarbons, Polychlorinated dibenzodioxins, Polychlorinated biphenyls

Polycyclic aromatic hydrocarbons (PAHs)

Polycyclic aromatic hydrocarbons (PAHs) are toxic environmental pollutants that act through the aryl hydrocarbon receptor (AhR) (Kabatkova et al. 2015), a receptor controlling migration, proliferation, cell adhesion and cell-cell communication (Andrysik et al. 2013). PAHs with lower molecular weight, consisting of maximum 4 benzene rings, are less genotoxic compared to PAHs that consist of at least 5 benzene rings. The former, however, inhibit GJIC and disrupt contact inhibition, and are therefore involved in tumor promotion (Kabatkova et al. 2015). In rat epithelial cells, PAHs reduce Cx43 levels and gap junction plaques, possibly through upregulated proteasomal degradation (Andrysik et al. 2013). GJIC is inhibited through phosphatidylcholine-specific phospholipase C (PC-PLC) and MAPK/extracellular signal-regulated kinase (ERK) pathways, but not through mitogen-activated protein kinase kinase (MEK) mechanisms (Upham et al. 2008; Sovadinova et al. 2015).

Polychlorinated dibenzodioxins (PCDDs)

Polychlorinated dibenzodioxins (PCDDs), also known as dioxins, are organic byproducts of combustion and industrial processes, which are released in the environment through air and contaminated soil and water. Eventually, PCDDs accumulate in human fatty tissues through food contamination. Dioxins might induce cancer (Lin et al. 2012), damage the immune system (Mrema et al. 2013), disrupt the endocrine system (Maqbool et al. 2016), and produce developmental (Maqbool et al. 2016) and reproductive problems (Lin et al. 2012), mediated by the AhR (Patrizi and Siciliani de Cumis 2018). The most toxic and most frequently used dioxin in research is 2,3,7,8-tetrachlorodibenzo-*p*-dioxine (Patrizi and Siciliani de Cumis 2018). This compound disrupts contact inhibition in rat liver epithelial cells, reduces gap junction plaques

and inhibits GJIC (Andrysik et al. 2013). The latter occurs in a time-dependent and concentration-dependent manner (Baker et al. 1995), yet multiple studies indicated that it takes 48 hr *prior* to changes in GJIC becoming manifested (Warngard et al. 1996; Bager et al. 1997). Further, protein levels of Cx43 are reduced, possibly because of increased proteasomal activity (Andrysik et al. 2013) and decreased Cx43 mRNA quantities (Bager et al. 1997). Cx32 mRNA levels and plasma membrane localization are also adversely affected (Herrmann et al. 2002).

Polychlorinated biphenyls (PCBs)

Polychlorinated biphenyls (PCBs) are a structurally diverse group of industrial chemicals and known environmental pollutants (Machala et al. 2003; Bager et al. 1994). During their production process, mixtures are formed instead of individual congeners. The best-known mixtures are Aroclor and Kanechlor. Until their ban in 1979, PCBs were used for all types of applications, including adhesives, plasticizers and paints. Even though the ban was enforced more than 40 years ago, PCBs are still present in the environment and pose a threat to human health (Grimm et al. 2015). Non-planar PCBs inhibit GJIC in rat cells, while co-planar PCBs do not (Machala et al. 2003). One of the most studied non-planar PCBs is the non-dioxin-like PCB153 (Machala et al. 2003). This compound induced long-term GJIC inhibition through PC-PLC and *v*-Src kinases (Machala et al. 2003; Sovadinova et al. 2015).

Simeckova et al. (2009) showed that ERK1/2 increases Cx43 phosphorylation, which might downregulate Cx43 protein levels and gap junction plaques because of enhanced internalization and subsequent proteasomal and lysosomal degradation. Cx43 mRNA levels remain unaffected. In contrast, a reduction of Cx43 phosphorylation was also reported upon loss of GJIC, while this was alleviated when GJIC was restored (Bager et al. 1997). Similarly, Bager et al. (1994) demonstrated that PCB126 decreases protein, but not mRNA levels of Cx32 and Cx26 in rat. In addition, Pierucci et al. (2017) noted that PCB153 differentially changes sphingolipid

sphingosine 1-phosphate/ceramide levels, which are crucial determinants of cell fate and regulators of Cx43, in a time-dependent manner.

Biological Toxins

Phorbol esters

Phorbol esters are plant-derived tetracyclic diterpenoids, of which 4β-12-*O*-tetradecanoylphorbol-13-acetate has been well documented (Goel et al. 2007). The compound is derived from the croton plant and used commonly in research for tumor promoting and GJIC inhibiting activities (Goel et al. 2007; Roemer et al. 2013). Phorbol esters act as activators of PKC, which is responsible for signal transduction and developmental processes (Goel et al. 2007). Through direct action of PKC, 4β-12-*O*-tetradecanoylphorbol-13-acetate is able to dysregulate GJIC (Sovadinova et al. 2015; Rivedal and Opsahl 2001). This is, however, not the only pathway, as hyperphosphorylation of Cx43 on serine368 (Loch-Caruso et al. 2004) through MEK1/2, a mitogen-activated protein kinase, contributes to this inhibitory effect by producing internalization of Cx43 (Sai et al. 1998; Sovadinova et al. 2015; Rivedal and Opsahl 2001). ERK1/2 phosphorylation also inhibits GJIC (Jung et al. 2006). Further, Cx43 is internalized and degraded after ubiquitination mediated by both the PKC and the MAPK pathway (Rivedal and Leithe 2005; Leithe and Rivedal 2004).

Lipopolysaccharide (LPS)

Lipopolysaccharide (LPS) is an endotoxin located in the outer membrane of Gram-negative bacteria that initiates acute inflammation in animals. A number of studies reported marked reduction of Cx32 production in response to administration of LPS *in vitro* (Yang et al. 2019) and *in vivo* in both mice (Temme et al. 2000) and rats (Correa et al. 2004; Gingalewski et al. 1996; Gonzalez et al. 2002). This reduction may be attributed to enhanced protein degradation and inhibition of Cx32 mRNA expression (Yang et al. 2019). This is associated with lowered mRNA stability induced by alterations in posttranscriptional modifications of Cx32 gene

expression, such as shortening of the poly(A)tail (Gingalewski et al. 1996; Theodorakis and De Maio 1999). Concurrently, Gonzalez et al. (2002) noted that GJIC was inhibited. Based upon data from rat hepatocytes, it was postulated that not LPS alone, but activated Kupffer cells and their secreted pro-inflammatory mediators are responsible for reduced number of GJIC. In contrast, GJIC is induced in rat hepatic stellate cell cultures and Kupffer cell cultures upon exposure to pro-inflammatory compounds because of enhanced protein and mRNA Cx43 expression (Eugenin et al. 2007; Fischer et al. 2005). Results for Cx26 are not consistent, as both upregulation (Temme et al. 2000; De Maio et al. 2000) and downregulation (De Maio et al. 2000; Gonzalez et al. 2002) of its protein and mRNA content were detected.

Ochratoxin A

Ochratoxin A is a mycotoxin frequently produced by species of the genera *Aspergillus* and *Penicillium* during storage of agricultural products (Gagliano et al. 2006). Studies in endemic regions demonstrated adverse effects on kidney and liver after chronic exposure to mycotoxins (Huong et al. 2016; Oyedele et al. 2017). The loss of GJIC induced by ochratoxin A might contribute to its renal toxicity and carcinogenicity (Mally et al. 2006). Over the years, multiple investigators confirmed that ochratoxin A exerts oxidative stress (Rasic et al. 2018; 2019; Shin et al. 2019; Gagliano et al. 2006). Decreased GJIC as a consequence of reduced ability to cope with oxidative stress was proposed as a contributing factor in liver carcinogenicity in rats (Gagliano et al. 2006). Recently, Shin et al. (2019) confirmed that ochratoxin A triggered oxidative stress in human hepatoma HepG2 cells, which might contribute to hepatocarcinogenic effects *in vivo*.

Patulin

Patulin is a mycotoxin that acts as a toxin in vertebrates (Kabak et al. 2006). The main route of exposure for humans is through moldy fruit, vegetables and other contaminated foods. Exposure of an immortalized rat hepatocyte cell line to patulin induced cellular injury *via* a

cascade of effects (Barhoumi and Burghardt 1996). Patulin produced suppression of GJIC followed by the generation of reactive oxygen species (ROS), which in turn depolarizes the inner mitochondrial membrane (Barhoumi and Burghardt 1996). This was followed by a rise in intracellular calcium levels and cytoplasmic acidification leading to depolarization of the cell plasma membrane (Barhoumi and Burghardt 1996).

Gossypol

Gossypol is a toxic compound derived from cotton seed oil that was used as an anti-fertility agent for men (Ye et al. 1990). Cell-cell communication is inhibited upon gossypol-induced toxicity in rat liver cells (Ye et al. 1990). The mechanism of action in rat liver epithelial cells is similar to that of patulin, namely inhibition of GJIC and generation of ROS that leads to acidification of the cytoplasm, which ultimately activates depolarization of the cell plasma membrane (Barhoumi and Burghardt 1996; Ye et al. 1990). Subsequent studies demonstrated concentration-dependent alterations of Cx43 phosphorylation and attenuation of GJIC suppression by a cAMP analogue (Hutchinson et al. 1998; Ye et al. 1990).

Organic Solvents

Ethanol

Ethanol is an alcohol best known for its presence in alcoholic beverages. Excessive alcohol intake over extended periods of time is linked to chronic liver disease, which ultimately may lead to onset of HCC (Liu 2014). Various mechanisms, including oxidative stress (Lu and Cederbaum 2008), nitrosative stress (Cooper and Magwere 2008), acetaldehyde toxicity (Setshedi et al. 2010), induction of liver cell death (McVicker et al. 2007) and lipid peroxidation (Setshedi et al. 2010), are involved in alcohol-induced liver injury. Several investigators found that ethanol exposure downregulates GJIC (Abou Hashieh et al. 1996; Bokkala et al. 2001). More specifically, ethanol decreased connexin protein biosynthesis in primary rat hepatocytes (Bokkala et al. 2001) and acted as a GJIC inhibitor through its intracellular metabolism in

cultured hepatocytes (Abou Hashieh et al. 1996). This is in contrast to the mechanism of long-chain alcohols, which inhibit GJIC directly on the extracellular part of the cell plasma membrane (Abou Hashieh et al. 1996). Using Cx32 knock-out rats, Kato et al. (2016) demonstrated the protective role of Cx32 in HCC, indicating that GJIC dysfunction in chronic liver disease enhanced ethanol-induced tumor development.

Carbon tetrachloride

Carbon tetrachloride (CCl_4) is a pro-fibrogenic agent employed in animal models to induce liver fibrosis and cirrhosis (Cogliati et al. 2016; Liang et al. 2017). In mice, Cx32 protects the liver from CCl_4-induced fibrosis compared to Cx32 knock-out animals (Cogliati et al. 2016). In rats, CCl_4-induced cirrhotic livers display significantly reduced Cx43 and Cx40 protein expression despite simultaneous elevation in mRNA production (Hernandez-Guerra et al. 2014). These findings are in agreement with previous *in vivo* studies in rats, where lower protein levels, but a rise in mRNA was seen, albeit for Cx32 (Nakata et al. 1996; Miyashita et al. 1991; Cowles et al. 2007). However, Yang et al. (2019) recently noted a fall in Cx32 and Cx26 mRNA levels upon CCl_4 administration. Despite this contradiction with earlier studies, the previously reported functional inhibition of GJIC was observed (Yang et al. 2019; Nakata et al. 1996). This inhibitory effect of CCl_4 is associated with cytotoxicity (Cowles et al. 2007), primarily mediated by oxidative and lipid peroxidative damage indirectly triggering genotoxicity (Eastmond 2008). In contrast to the findings in rats, Cx43 expression was enhanced in CCl_4-treated mice, although aberrantly located in hepatic stellate cells. Cx32 and Cx26 levels were unaffected (Cogliati et al. 2011).

Trichloroethylene (TCE)

Trichloroethylene (TCE) is a widely used industrial organic solvent known to induce HCC (Jiang et al. 2017). The precise underlying mechanism is not fully elucidated, but both genotoxic and non-genotoxic pathways are involved (Jiang et al. 2017; Rusyn et al. 2014). Jiang

et al. (2014) suggested that epigenetic alterations, more specifically altered DNA methylation, play a critical role in TCE-triggered HCC. Changes in microRNA expression are also involved (Jiang et al. 2017). Additional non-genotoxic pathways include alterations in proliferation and apoptosis, activation of peroxisome proliferator-activated receptor α, secondary oxidative stress (Rusyn et al. 2014) and inhibition of GJIC (Klaunig et al. 1989). The latter requires phase I biotransformation capacity, as no GJIC inhibition occurs when an inhibitor of cytochrome P450 (CYP450) is added simultaneously (Klaunig et al. 1989).

Pesticides

Organophosphorus (OP) pesticides

Organophosphorus (OP) pesticides are an important class of pesticides mainly used as insecticides and, to a lesser extent, as herbicides. Nearly all insecticides display neurotoxic effects in humans through the inhibition of acetylcholinesterase (Maroni et al. 2000). In addition, OP pesticides initiate toxicity *via* genotoxicity (Rahman et al. 2002), cytotoxicity (Wagner et al. 2005), immunotoxicity (Yeh et al. 2005) and disruption of sex hormones and reproduction (Okamura et al. 2005). The neurotoxic effects are often induced by the metabolites following biotransformation of the parent compound by CYP450 (Li et al. 2019). Most reports focused on neurotoxicity, while far less is known regarding the non-cholinergic effects, especially actions on liver connexins. Wu et al. (2007) demonstrated that parathion, methyl parathion, diazinon and malathion inhibited GJIC in rat liver cell cultures. These OP compounds elicit this effect themselves, since their oxons and ozonation byproducts produced no marked effect. A study aiming to identify altered quantities of membrane proteins in mouse livers revealed alterations in proteins that prevent oxidative stress, but not in connexin levels (Seifert 2014).

Cyclodiene organochlorine (OC) pesticides

Aldrin, dieldrin, endrin, heptachlor, chlordane and endosulfan all are pesticides with a chlorinated dicyclopentadiene ring (Kataoka et al. 2016). Because of their non-genotoxic (hepato)carcinogenic effects (Manclus et al. 2004), their use is prohibited since the 1970s. However, given their long half-life, they are persistent organic pollutants able to remain present in agriculture fields for extended periods of time (Kataoka et al. 2016). In rat liver epithelial cells, GJIC is inhibited by endosulfan, chlordane, heptachlor or dieldrin (Kenne et al. 1994; Matesic et al. 1994; Rivedal and Opsahl 2001; Warngard et al. 1996). This phenomenon does not depend upon xenobiotic phase 1 biotransformation capacity, as indicated by experiments using CYP450 inhibitors in mouse hepatocytes, but is associated with alterations in Cx43 phosphorylation for endosulfan, heptachlor and chlordane (Ruch et al. 1990). In addition, dieldrin and heptachlor reduced Cx26 and Cx43 protein levels (Matesic et al. 1994).

Dichlorodiphenyltrichloroethane (DDT)

Dichlorodiphenyltrichloroethane (DDT) was a widely used OC pesticide until its withdrawal in the 1970s given its liver tumor promoting action and prolonged environmental persistence (VoPham et al. 2017; IARC 2018). Together with its metabolite dichlorodiphenyldichloroethylene, DDT produces HCC in rodents through the inhibition of GJIC (VoPham et al. 2017). In male rats, DDT exposure leads to diminished GJIC even though Cx32 and Cx26 levels are not affected, and are not differentially located or modified at posttranslational levels (Cowles et al. 2007). However, these findings are not entirely confirmed by other *in vivo* studies. In this respect, Krutovskikh et al. (1995) demonstrated that decreased amounts of gap junctions, aberrant localization of Cx32 and minor changes in protein expression of Cx32 might be induced by DDT. In addition, Cx26 production was upregulated, but only in centrilobular groups of hepatocytes. Further, Cx43 expression was enhanced, yet is only found in the cytoplasm (Krutovskikh et al. 1995). Overall, evidence indicates that GJIC is

inhibited, but the underlying molecular mechanism was only elucidated decades after the withdrawal of the pesticide. *In vitro* studies using a pluripotent rat liver epithelial oval cell line showed that DDT inhibited GJIC through PC-PLC and not *via* MAPK pathways, which are well known for GJIC-dysregulating mechanisms (Sovadinova et al. 2015).

Lindane

Lindane (γ-hexachlorocyclohexane) is a halogenated aromatic hydrocarbon pesticide with insecticidal properties, which is mainly used to protect wood, seed, fruit and vegetables crops. In addition, lindane is present in products as lotions or shampoos to treat head lice. During the 1970s, multiple studies reported the hepatocarcinogenic potential of lindane in murine models (IARC 2018). Lindane executes its carcinogenic effect through inhibition of GJIC (Klaunig et al. 1990). However, the mechanism by which GJIC is regulated by lindane is not entirely clear. Sovadinova et al. (2015) suggested involvement of MEK1/2 using a rat liver epithelial cell line, indicating that GJIC is modulated through phosphorylation of connexin proteins, more specifically Cx43. However, MEK1/2 alone was not sufficient (Sovadinova et al. 2015). A second factor is activation of the MAPK/ERK pathway leading to serine368 phosphorylation. These kinases promote Cx43 endocytosis resulting in impaired GJIC (Mograbi et al. 2003). Further, the effect of lindane is time-dependent (Guan et al. 1995). Short-term exposure (min) results in inhibition of GJIC without changes in Cx43 expression. Mid-term exposure (hr) reduces GJIC associated with alterations in Cx43 phosphorylation and subsequent endocytosis. Long-term exposure (days) leads to loss of Cx43 expression (Guan et al. 1995).

Hexachlorobenzene (HCB)

Hexachlorobenzene (HCB) is a non-genotoxic hepatocarcinogenic chemical thought to induce cancer *via* inhibition of GJIC (Mally and Chipman 2002). The compound was mainly used as a fungicide to protect seeds and wheat until it was banned in 1965 (Pohanish 2015). HCB effects on connexins, mainly Cx32, were examined both *in vitro* and *in vivo* (Plante et al.

2002, 2006; 2007). Noteworthy is the sexual dimorphism. Because of the occurrence of ovarian hormones, HCB produces liver tumors in female rats, but not in males. Upon HCB administration, GJIC is inhibited, and mRNA levels of Cx26 and Cx32 are significantly reduced (Plante et al. 2002). *In vitro* studies linked this decrease to activation of the integrin-linked kinase pathway, which induced and translocated Akt that subsequently triggered transcription factors changing Cx32 expression, such as specificity protein 1 and hepatocyte nuclear factor 1α (Plante et al. 2006). This observation was confirmed *in vivo*, where HCB administration produced decreased binding of transcriptional complexes Fr26 and Fr110, known to be controlled by Akt, to the Cx32 gene promotor in female, but not in male rat liver (Plante et al. 2007).

Pentachlorophenol (PCP)

Pentachlorophenol (PCP) is a halogenated phenolic herbicide primarily known for termite control. The sodium salt is applied as a general disinfectant, whereas the ester is used to protect wood from fungal rot and insects (Maroni et al. 2000). PCP downregulates GJIC in rat liver epithelial cells accompanied by diminished Cx43 mRNA and/or protein levels (Sai et al.1998; 2001). Both studies failed to detect a change in the phosphorylation status of Cx43. Although PCP activates kinases of the MAPK/ERK pathway, this compound does not dysregulate GJIC through MEK1/2, a MAPK involved in GJIC control for several other pesticides, like lindane (Sovadinova et al. 2015). *In vivo* studies reported normal localization of Cx32, but a reduced density of Cx32 gap junction plaques leading to inhibited GJIC (Sai et al. 2000).

Pharmaceuticals

Hypolipidemic drugs

WY-14.643 and fibrates, such as clofibrate or ciprofibrate, are peroxisome proliferators that are used as lipid-lowering agents (Kersten and Stienstra 2017). In rodents, this class of compounds induces hepatocarcinogenesis by reducing GJIC both *in vitro* (Elcock et al. 1998)

and *in vivo* (Mally and Chipman 2002; Krutovskikh et al. 1995). Elcock et al. (2000) showed the involvement of PKC in the mechanism of action of nafenopin in rats. GJIC was lowered in rat hepatocytes in a time-dependent and concentration-dependent manner, but neither Cx32 and Cx26 protein levels nor their location were changed. The inhibitory effect is a result of altered phosphorylation of Cx32 (Elcock et al. 2000). However, not all hypolipidemic drugs share the same outcome and/or mechanism of action. Thus, WY-14.643 decreased the expression of Cx32 and gap junction plaque area in contrast to nafenopin in rats (Mally and Chipman 2002). The same holds true for clofibrate. In this case, the expression of Cx32 is also lowered, but the main reason for reduced GJIC lies in the aberrant localization of Cx32 rather than changes at the transcriptional or translational level (Krutovskikh et al. 1995). In addition, WY-14.643 decreased Cx26 expression in rats (Cowles et al. 2007). The effects in rodents cannot be reproduced in humans (Corton et al. 2014).

Phenobarbital

Phenobarbital is an anti-epileptic drug frequently used as a liver tumor promotor in rodents (Stahl et al. 2005). Upon exposure, this non-genotoxic CYP450 inducer decreases GJIC by reducing Cx32 expression (Kushida et al. 2005; Neveu et al. 1990). Although carcinogenesis is enhanced in Cx32 knock-out mouse models (Moennikes et al. 2000), downregulation of Cx32 production in rats is not sufficient to trigger carcinogenesis (Neveu et al. 1990). Surprisingly, the carcinogenic effect of phenobarbital only manifests when functional Cx32 is present, as Cx32-null mice do not develop liver cancer after drug treatment (Moennikes et al. 2000). Unlike Cx32 knock-out mice, Cx26 knock-out animals are prone to hepatocarcinogenic effects of phenobarbital, indicating a minor role for Cx26 in tumor development (Marx-Stoelting et al. 2008). Moreover, gene expression in response to phenobarbital only takes place in highly differentiated hepatocytes (Sidhu et al. 2004), mainly affecting drug-metabolizing enzymes, such as CYP2B6 and CYP3A4 (Page et al. 2007). The carcinogenic potential of phenobarbital

is strain-specific, since it is a potent hepatocarcinogen in B6C3F1, but not in C57BL/6 mice. Accordingly, GJIC is inhibited in B6C3F1 mice, but not in C57BL/6 mice upon phenobarbital treatment (Warner et al. 2003).

Methapyrilene

Methapyrilene is an anti-histaminic drug that was withdrawn in 1979 because of its non-genotoxic hepatocarcinogenic potential (Braeuning et al. 2019). Mally and Chipman (2002) noted in male rats a reduced number and size of Cx32 gap junction plaques, specifically in liver. No further studies were performed to test the effects of methapyrilene on connexins. The use of methapyrilene in rat hepatocyte cultures demonstrated concentration-dependent effects on mitochondrial proteins, specifically energy supply pathways, ammonia and amino acid metabolism (Braeuning et al. 2019). *In vivo* data showed effects on mitochondrial pathways, such as mitochondrial oxidative phosphorylation, albeit at the transcriptional level (Ito et al. 2019). In addition, methapyrilene enhanced cell proliferation (Ito et al. 2019). Overall, the effects of methapyrilene are believed to be mediated through alterations in transcription of multiple genes (Tryndyak et al. 2019) and modulation of the DNA methylation footprint (Tryndyak et al. 2018).

Miscellaneous

Peroxides

ROS, such as hydrogen peroxide, are byproducts of oxygen metabolism. Besides their role in cellular signaling, ROS may also produce damage under stress conditions (Imlay 2008). Hydrogen peroxide, benzoyl peroxide and dicumyl peroxide were all reported to inhibit GJIC in rat cell lines (Upham et al. 2007), indicating their tumor promoting potential, which involves alterations in the phosphorylation status of Cx43. Thus, dicumyl peroxide elicits its effects through PC-PLC and the MAPK/ERK pathway (Sovadinova et al. 2015; Upham et al. 2007). Benzoyl peroxide also activates MAPK/ERK (Upham et al. 2007), but not MEK1/2 or PC-PLC

(Sovadinova et al. 2015). In the presence of specific inhibitors of these kinases, benzoyl peroxide is still able to inhibit GJIC. Benzoyl peroxide may also initiate its effects through other kinases, such as PKC, protein kinase A, Akt, p38, redox-dependent regulatory mechanisms or phospholipases (Sovadinova et al. 2015).

Metals

Toxic metals, such as cadmium (Cd), pose an increasing worldwide health problem related to expanding industrialization. When it comes to the effect of metals on hepatic gap junction activity, Cd is the best-studied case. This metal is mainly taken up in the body through ingestion and smoking. Cd targets the liver and kidneys, where it accumulates because of its long half-life. In mice, Cd exerts time-dependent and concentration-dependent inhibition of GJIC together with decreased expression of Cx26 and Cx32 (Jeong et al. 2000). In rat cell lines, the same inhibition of GJIC is seen together with decreased expression of Cx43-based gap junctions (Jeon et al. 2001) and elevated occurrence of phosphorylated Cx43 (Hu et al. 2016). Cd increases intracellular calcium concentrations (Hu et al. 2016), induces autophagy (Zou et al. 2015a) and triggers apoptosis through changes in expression of Bax, Bcl-2, ERK and p38 signaling pathways (Hu et al. 2016). When the latter MAPK proteins are inhibited, Cd-induced hepatotoxicity can be attenuated (Zou et al. 2015b).

Phthalates

Phthalates are diesters of phthalic acid mainly used as plasticizers in polyvinyl chloride (PVC) or other industrial products. The most frequently applied phthalates in commercial products are di(2-ethylhexyl) phthalate (DEHP), benzyl butyl phthalate and diisodecyl phthalate. These compounds are taken up by humans through dietary sources, air inhalation and dermal absorption, resulting in endocrine disruption and adverse reproductive effects (Wang et al. 2019b). Kamendulis et al. (2002) tested 8 monoesters, which are the metabolites of the diester, and revealed species-specific effects. More specifically, GJIC was inhibited in mouse

and rat hepatocytes in a concentration-dependent manner, but not in human, hamster and monkey hepatocytes (Kamendulis et al. 2002). The same species specificity was observed with the diester forms (Isenberg et al. 2000). *In vivo* tests confirmed these *in vitro* findings, as treatment of *cynomolgus* monkeys with diisononyl phthalate and di(2-ethylhexyl) phthalate did not markedly affect GJIC (Pugh et al. 2000), while these compounds induced GJIC inhibition in F344 rats and B6C3F1 mice (Isenberg et al. 2001; Smith et al. 2000).

Conclusions and Perspectives

Gap junctions play a crucial role in the hepatocyte's life cycle by maintaining tissue homeostasis (Yamasaki et al. 1999). GJIC may be inhibited upon exposure of liver cells to a number of compounds of diverse nature, leading to an escape from homeostatic control and ultimately triggering carcinogenesis (Chipman et al. 2003; Maes, et al. 2015b). It has been hypothesized that reduction of GJIC might serve as an indicator for non-genotoxic carcinogenicity (Maes et al. 2015b). In this respect, the non-genotoxic carcinogenic compounds described in this review adversely affected GJIC through a variety of regulatory mechanisms, such as altered posttranslational modifications, changes in subcellular localization, increased proteasomal degradation and modifications in the epigenetic machinery (Table 1 and Figure 3). A multitude of experimental systems and models is used throughout this research field. While some animal models of liver cancer might provide a reliable reflection of the corresponding human pathology and thus have high translational value, these encompass a number of issues. In this respect, besides the obvious ethical constraints, animal models are not always well suited for mechanistic investigation, because of their complex dynamic nature. *In vitro* models seem better fit for this purpose, as these provide a more controllable experimental setting that enables dissection of specific signaling pathways, including connexin-based communication.

To date, no *in vitro* tests to detect non-genotoxic carcinogenic properties of chemicals exist (Nohmi 2018; Wilde et al. 2018). In this respect, the loss of GJIC might be considered as a new

in vitro biomarker to detect non-genotoxic carcinogenicity (Maes et al. 2015b). Although changes in gap junction functionality are not to be used as a single endpoint for non-genotoxic carcinogenicity screening, these alterations might be included in a battery of tests that encompasses other targets for non-genotoxic carcinogenic substances. Such additional assays may focus on monitoring cell death, cell morphology, proliferation or mitochondrial activity, all processes that are known to be affected by non-genotoxic carcinogenic compounds (Wilde et al. 2018). This approach would be similar to the screening panel applied to identify genotoxic carcinogenic compounds, which comprises multiple tests that pick up different endpoints of genotoxicity (Nohmi 2018).

Over the past 2 decades, connexin hemichannels have gained increasing attention (Decrock et al. 2009; 2011; Vinken et al. 2006). Historically, connexin hemichannels were solely seen as the structural precursors of gap junctions, yet it now is clear that these constituents also provide a pathway for communication on their own, albeit between the intracellular and extracellular environment. Overall, connexin hemichannels act as pathological pores, which is in sharp contrast to their full channel gap junction counterparts, responsible for tissue homeostasis (Cooreman et al. 2019). Indeed, connexin hemichannels have been characterized as key players in liver disease, including acute liver failure (Maes et al. 2017), fibrosis (Crespo Yanguas et al. 2018) and non-alcoholic steatohepatitis (Willebrords et al. 2017a). In the latter case, inhibition of Cx32 and Cx43 hemichannels reduces inflammation and oxidative stress (Willebrords et al. 2017a). The same holds true for liver fibrosis, where inhibition of Cx43 hemichannels lowers disease progression (Crespo Yanguas et al. 2018). Given their implication in chronic liver disease, which often results in HCC, connexin hemichannels might also be involved in hepatocarcinogenesis. Their specific function in cancer is still unclear (Sinyuk et al. 2018), but several investigators suggested a tumor promoting role (Schalper et al. 2014; Aasen et al. 2019). Further, their role in inflammation and cell death, which are prominent mechanisms of non-

genotoxic carcinogenicity (Hernandez et al. 2009; Vinken, Doktorova et al. 2008), suggests that they might underlie this type of carcinogenesis (Willebrords et al. 2017a; Crespo Yanguas et al. 2018). For this reason, future studies need to focus on the role of connexin hemichannels in HCC and their involvement in non-genotoxic carcinogenicity. Na et al. (1995) showed that GJIC abrogation is not unique to non-genotoxic carcinogenic chemicals. In addition, Yamasaki et al. (1996) reported that not all non-genotoxic carcinogenic compounds inhibit GJIC. However, investigating the function and role of connexin hemichannels, *in casu* in liver cancer, remains a technical challenge. Since gap junctions and connexin hemichannels are built up by connexin proteins and mediate trafficking of identical messengers, current functionality methods barely distinguish between both channel types (Schalper et al. 2014). A number of techniques exist to monitor the functionality of connexin hemichannels, yet these are based upon measurement of extracellular release of widely occurring messengers, such as adenosine triphosphate, or the cytosolic uptake of tracer dyes, which are rather unspecific and may be mediated by many other families of channels (Spray et al. 2006). Progress has been made over the past few years with the generation of antibodies (Riquelme et al. 2013) and connexin mimetic peptides (Willebrords et al. 2017b) that seem to specifically target connexin hemichannels. Research in this direction in the upcoming years needs to be strongly encouraged, as it opens new perspectives for development of *in vitro* methods to test non-genotoxic carcinogenic potential of chemicals.

Conflicts of interest: The authors report no conflicts of interest.

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Table 1: *Overview of non-genotoxic carcinogenic compounds that compromise GJIC in liver.*

Polycyclic aromatic hydrocarbons
Polychlorinated dibenzodioxins
Polychlorinated biphenyls
Biological toxins
Phorbol esters
Lipopolysaccharide
Ochratoxin A
Patulin
Gossypol
Organic solvents
Ethanol
Carbon tetrachloride
Trichloroethylene
Pesticides
Organophosphorus pesticides
Cyclodiene organochlorine pesticides
Dichlorodiphenyltrichloroethane
Lindane
Hexachlorobenzene
Pentachlorophenol
Pharmaceuticals
Hypolipidemic drugs
Phenobarbital
Methapyrilene
Miscellaneous
Peroxides
Metals
Phthalates

Figure 1: *Structure of gap junctions.* Connexin proteins consist of 4 transmembrane domains, 2 extracellular loops, 1 intracellular loop, an intracellular *N*-terminal tail and an intracellular *C*-terminal tail. As such, 6 connexin proteins form a hemichannel, and 2 hemichannels of adjacent cells can dock together to form a gap junction. Multiple gap junctions assemble into plaques at the cell plasma membrane surface.

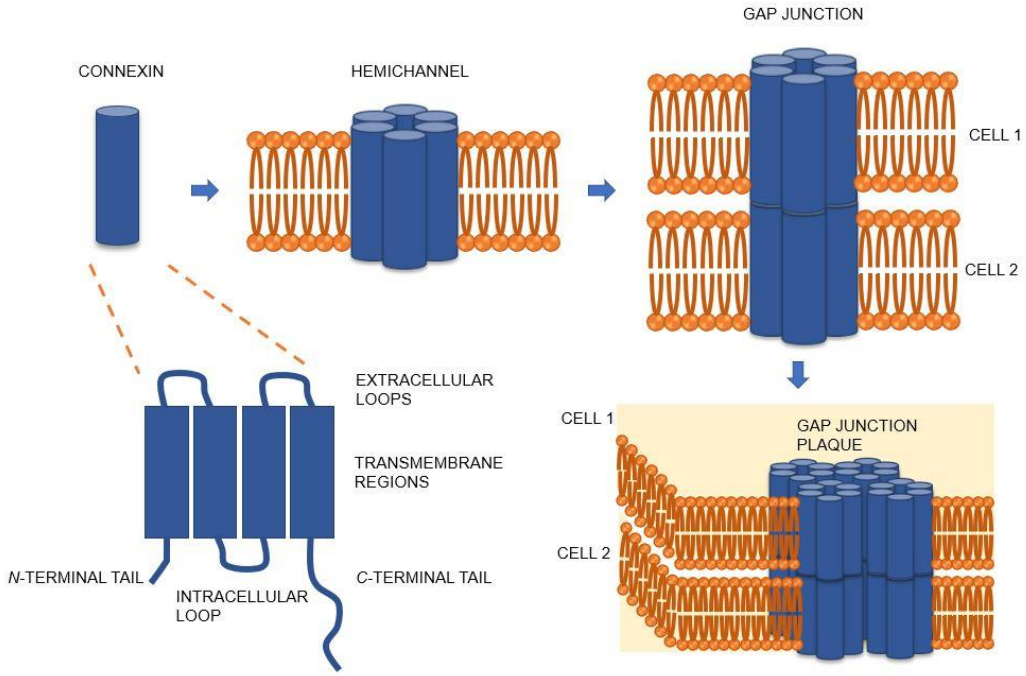

Figure 2: *Overview of GJIC regulating mechanisms.* GJIC can be modulated at the expression level and at the functionality level. The latter includes many different factors, such as transmembrane voltage, pH, calcium levels, redox potential (mainly carried out by nitric oxide: NO), protein-protein interactions (PPI) and posttranslational modifications (PTM). Connexin expression is managed by transcription factors (TF) and epigenetic mechanisms, such as histone acetylation (Ac), DNA methylation and microRNAs.

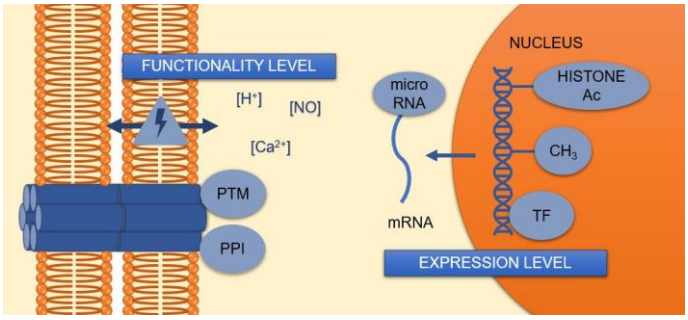

Figure 3: *Overview of mechanisms leading to GJIC inhibition in liver by non-genotoxic carcinogenic compounds.* GJIC can be inhibited by alterations in posttranslational modifications *via* different kinases, which may trigger proteasomal degradation. Similarly, altered transcriptional activity *via* transcription factors (TF), aberrant DNA methylation patterns, reduced mRNA stability, decreased transcriptional and translational activity lead to reduced expression of liver connexins. Other GJIC inhibitory mechanisms underlying non-genotoxic effects involve proinflammatory mediators, oxidative stress and divergent subcellular localization.