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REVIEW ARTICLE

Gap junctional intercellular communication as a target for liver toxicity and carcinogenicity

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Abstract

Direct communication between hepatocytes, mediated by gap junctions, constitutes a major regulatory platform in the control of liver homeostasis, ranging from hepatocellular proliferation to hepatocyte cell death. Inherent to this pivotal task, gap junction functionality is frequently disrupted upon impairment of the homeostatic balance, as occurs during liver toxicity and carcinogenicity. In the present paper, the deleterious effects of a number of chemical and biological toxic compounds on hepatic gap junctions are discussed, including environmental pollutants, biological toxins, organic solvents, pesticides, pharmaceuticals, peroxides, metals and phthalates. Particular attention is paid to the molecular mechanisms that underlie the abrogation of gap junction functionality. Since hepatic gap junctions are specifically targeted by tumor promoters and epigenetic carcinogens, both *in vivo* and *in vitro*, inhibition of gap junction functionality is considered as a suitable indicator for the detection of nongenotoxic hepatocarcinogenicity.

Keywords: *Gap junction; connexin; liver homeostasis; hepatotoxicity; nongenotoxic hepatocarcinogenicity*

Abbreviations: AhR, aryl hydrocarbon receptor; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; CL, cytoplasmic loop; CT, carboxy tail; Cx, connexin; CYP450, cytochrome P450; DDT, dichlorodiphenyltrichloroethane; Dlgh, 1, discs large homolog 1; EGFR, epidermal growth factor receptor; EL, extracellular loop; ERK1/2, extracellular signal-regulated kinase 1/2; FRAP, fluorescence recovery after photobleaching; GJIC, gap junctional intercellular communication; HCB, hexachlorobenzene; HNF1, hepatocyte nuclear factor 1; IP3, inositol trisphosphate; LAMP, local activation of molecular fluorescent probe; LPS, lipopolysaccharide; MAPK(s), mitogen-activated protein kinase(s); MEK, mitogen-activated protein kinase kinase; NP-Cx43, nonphosphorylated Cx43; NT, amino tail; OTA, ochratoxin A; P1/P2/P3-Cx43, phosphorylated Cx43 variants; PAH(s), polycyclic aromatic hydrocarbon(s); PCB(s), polychlorinated biphenyl(s); PCDD(s), polychlorinated dibenzodioxin(s); PCP, pentachlorophenol; PKA/C, protein kinase A/C; PLC, phospholipase C; ROS, reactive oxygen species; Sp1, specificity protein 1; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TM, transmembrane region; TPA, 12-O-tetradecanoylphorbol-13-acetate; 3′/5′-UTR, 3′/5′-untranslated region; ZO-1/2/3, zonula occludens 1/2/3.

Introduction

Because of its unique localization in the organism, the liver is highly exposed to exogenous molecules that enter the body upon oral intake. In order to protect against insults triggered by these xenobiotics, hepatocytes, the most abundant cell population in the liver, utilize a complex enzymatic system to clear these foreign molecules from the organism. This prominent functional feature is called biotransformation (Elaut *et al.*, 2006; Papeleu *et al.*, 2006). Being the main site of biotransformation in the body, however, the liver is also a major target for systemic toxicity. Although a plethora of mechanisms may contribute to the occurrence of liver injury, aberrant cellular signaling seems to be a central event in this process (Mehendale *et al.*, 1994; Jaeschke

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Table 1. Agents that downregulate hepatic gap junctional intercellular communication.

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

et al., 2002; Chipman *et al.*, 2003). In the present paper, the involvement of gap junction-mediated intercellular communication in chemically induced hepatotoxicity and hepatocarcinogenicity is reviewed. In a first part, the current knowledge concerning liver gap junctions is provided, including their biochemical properties, their role in the control of the hepatocyte life cycle and methods to assess their functionality. In a second part, the detrimental outcome of prototypical chemical and biological toxic compounds on hepatic gap junctions is discussed, with the main focus on the mechanistic basis of these effects (Table 1).

Biochemical *curriculum vitae* of liver gap junctions

Structural properties

Gap junctions arise from the head-to-head interaction of 2 hemichannels on adjacent cells, which are hexameric channels composed of connexin (Cx) proteins. More than 20 connexin species have been cloned from rodent and human, and they are expressed in a cell-specific manner. Connexins share a 4-transmembrane (TM) topology with 2 extracellular loops (EL), 1 cytoplasmic loop (CL), 1 cytoplasmic N-terminal area (NT) and 1 C-terminal region (CT). (Figure 1). Connexin nomenclature is based on molecular weight, as predicted by cDNA sequencing (Saez *et al.*, 2003; Vinken *et al.*, 2006a; 2006b; 2008; 2009; Dbouk *et al.*, 2009; Decrock *et al.*, 2009). In the liver, most nonparenchymal cells, including Kupffer cells, stellate cells, endothelial cells and cells of the Glisson's capsule, produce Cx43 (molecular weight 43 kDa) (Berthoud *et al.*, 1992; Greenwel *et al.*, 1993; Saez, 1997), whereas both Cx40 and Cx37 have been detected in liver vascular cells (Shiojiri *et al.*, 2006). Hepatocytes, on the other hand, are loaded with Cx32, representing 90% of the total hepatic connexin content, next to small amounts of Cx26 (Cascio *et al.*, 1995; Neveu *et al.*, 1995). Unlike Cx32, which is uniformely distributed in the liver, Cx26 is preferentially expressed in the periportal acinar area (Spray *et al.*, 1994). Gap junctions occupy about 3% of the hepatocyte membrane surface (Spray *et al.*, 1994) and are organized in plaques that contain 10 to 10 000 channels (Musil *et al.*, 2000).

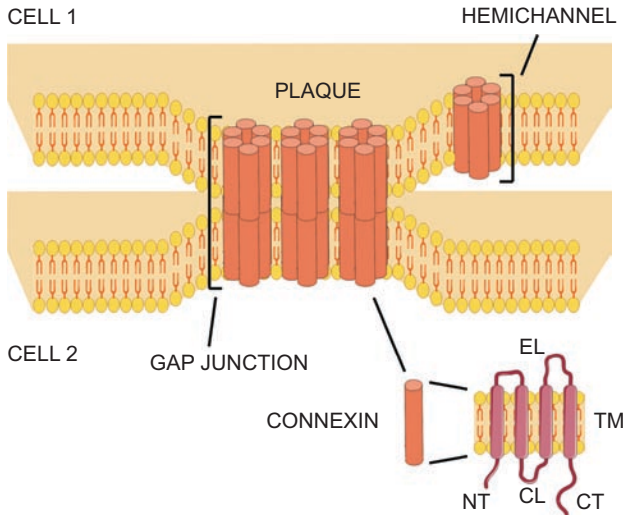

Figure 1. Molecular architecture of gap junctions. Gap junctions are grouped in plaques at the cell plasma membrane surface and are composed of 12 connexin proteins organized as 2 hexameric hemichannels. The connexin structure consists of 4 transmembrane regions (TM), 2 extracellular loops (EL), 1 cytoplasmic loop (CL), 1 cytoplasmic amino tail (NT) and 1 cytoplasmic carboxy tail (CT).

Gap junctions are known to interact with a vast array of partners. Thus, Cx43 binds to adherens junctional proteins (e.g. N-cadherin and β-catenin), tight junctional components (e.g. zonula occludens 1 (ZO-1) and ZO-2), enzymes (e.g. tyrosine kinase *v*-Src, serine/threonine kinase, protein kinase C (PKC), mitogen-activated protein kinase (MAPK) and phosphatases) and a number of other proteins (e.g. caveolin and aquaporin) (Herve

et al., 2004; 2007; Dbouk *et al.*, 2009). Hepatocyte gap junctions, in particular those composed of Cx32, interact with the adherens junction proteins E-cadherin and α-catenin (Fujimoto *et al.*, 1997), the tight junction building stones occludin, claudin-1, ZO-1 and ZO-3 (Kojima *et al.*, 1999; 2001b; 2002), the scaffolding protein discs large homolog 1 (Dlgh1) (Duffy *et al.*, 2007) and the enzymes PKC, protein kinase A (PKA) and Ca^{2+}-calmodulin-dependent protein kinase II (Saez *et al.*, 1990).

Regulatory mechanisms

Gap junctions represent a basic pathway for direct communication between neighboring cells. The flux of molecules through these channels is denoted gap junctional intercellular communication (GJIC) and concerns the passive diffusion of small (<1–1.5 kDa) and hydrophilic molecules, such as glucose, glutamate, glutathione, adenosine trisphosphate (ATP), cyclic adenosine monophosphate (cAMP), inositol trisphosphate (IP3), and ions (e.g. Ca^{2+}, K^+, Na^+) (Alexander and Goldberg, 2003; Decrock *et al.*, 2009). As numerous physiological processes are regulated by substances that are intercellularly exchanged *via* gap junctions, GJIC is considered as a key mechanism in the control of tissue homeostasis (Figure 2) (Vinken *et al.*, 2006a; 2006b; 2008; 2009). The biophysical properties of a given gap junction highly depend on the connexin species that compose the channel. For instance, Cx26-based gap junctions are known to favor cation transfer, whereas gap junctions consisting of Cx32 rather promote anion passage (Bukauskas *et al.*, 1995). In a similar way, ATP is conveyed about 300 times better through gap junctions formed by Cx43 compared with their Cx32-based counterparts (Goldberg *et al.*, 2002).

A myriad of mechanisms regulate GJIC (Figure 3). Long-term control of GJIC mainly concerns regulation at the transcriptional level of connexin expression (Oyamada *et al.*, 2005). The structure of connexin genes is rather simple, consisting of a first exon that contains the 5′-untranslated region (5′-UTR), which is separated from a second exon, bearing the complete coding sequence and the 3′-UTR, by an intron of varying length (Sohl and Willecke, 2004; Oyamada *et al.*, 2005). An exception is the Cx32 gene, which displays differential splicing of the 5′-UTR (Neuhaus *et al.*, 1996; Duga *et al.*, 1999; Sohl *et al.*, 2001). Connexin gene promoters contain binding sites for both ubiquitous transcription factors, such as specificity protein 1 (Sp1) (Oyamada *et al.*, 2005), and tissue-specific transcription factors, such as hepatocyte nuclear factor 1α (HNF1α) in the case of Cx32 (Koffler *et al.*, 2002; Field *et al.*, 2003). Recently, epigenetic mechanisms, including histone acetylation, DNA methylation and microRNA-related control, have also joined in as master regulators of connexin expression (Vinken *et al.*, 2009).

Short-term control of GJIC, so-called gating, is driven by a number of factors, including transmembrane voltage, and H^+ and Ca^{2+} ions (Cottrell and Burt, 2005). Among these actions, connexin phosphorylation, mainly occurring at the CT region, has gained a great deal of attention. With the exception of Cx26, all connexins are phosphoproteins. The regulation of GJIC by connexin phosphorylation is complex, as the outcome of this posttranslational modification is both connexin-inherent and kinase-specific (Laird, 2005; Solan and Lampe, 2005). Cx43 has been most extensively studied in terms of connexin phosphorylation. Cx43 is a substrate for many kinases, including PKA, PKC, members of the MAPK family, casein kinase 1, the cyclin-dependent kinase 1/cyclin B complex and *v*-Src (Solan and Lampe, 2005; 2009). Different from other connexins, shifts in electrophoretic mobility occur upon phosphorylation of Cx43. Typically, 3 bands appear during sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis, representing the fast-migrating nonphosphorylated Cx43 isoform, referred to as NP-Cx43, and 2 slow-migrating phosphorylated Cx43 isoforms, namely P1-Cx43 and

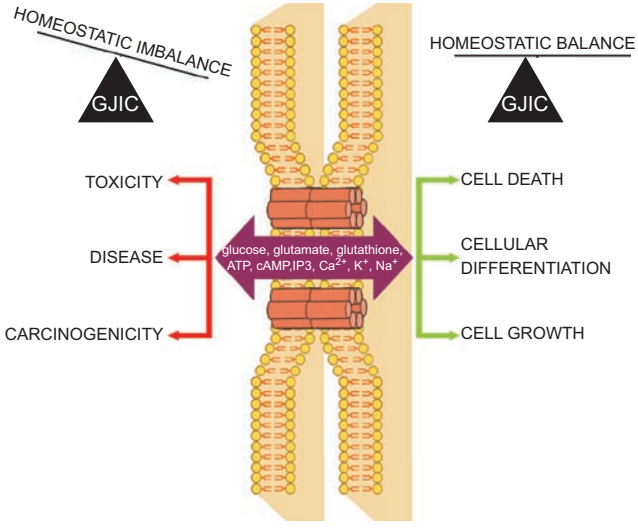

Figure 2. Physiological and pathophysiological role of gap junctions. Gap junctions represent a basic pathway for direct communication between neighboring cells. The flux of molecules through these channels is denoted gap junctional intercellular communication (GJIC) and concerns the passive diffusion of small and hydrophilic molecules, such as glucose, glutamate, glutathione, adenosine triphosphate (ATP), cyclic adenosine monophosphate (cAMP), inositol trisphosphate (IP3), and ions (Ca^{2+}, K^+, Na^+). As numerous physiological processes are regulated by substances that are intercellularly exchanged *via* gap junctions, GJIC is considered as a key mechanism in the control of tissue homeostasis, including cell growth, cellular differentiation and cell death. Not surprisingly, GJIC is frequently impaired upon disruption of the homeostatic balance, as typically occurring during toxicity, disease and carcinogenicity.

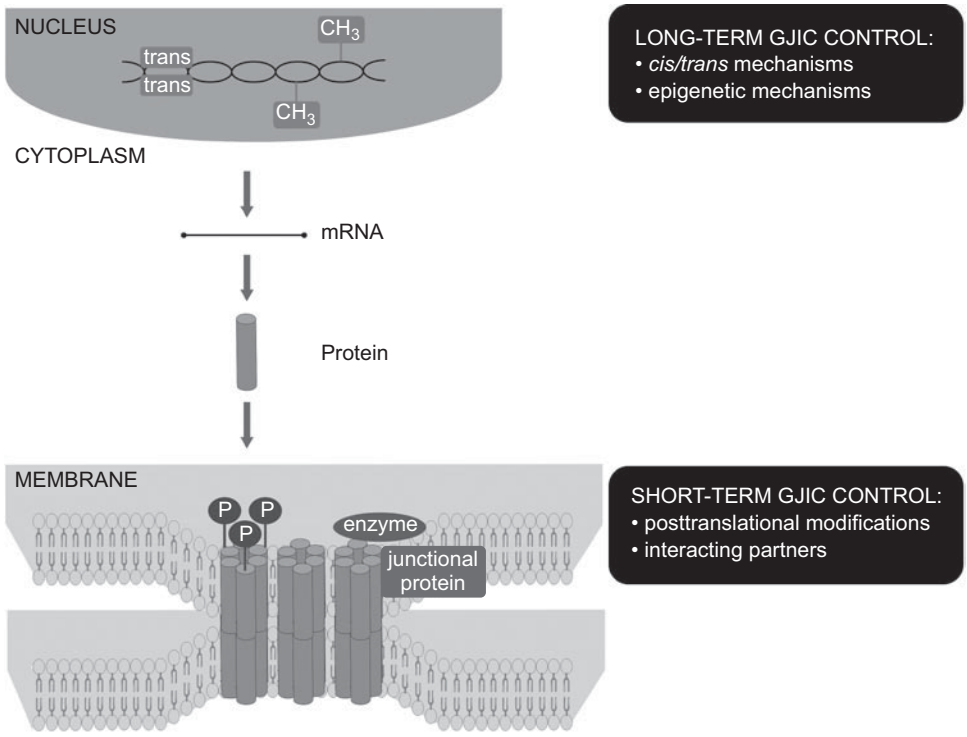

Figure 3. Regulatory mechanisms involved in the control of gap junction functionality. From a kinetic point of view, 2 major regulatory platforms in the control of gap junctional intercellular communication (GJIC) can be distinguished. Long-term control of GJIC mainly concerns regulation of connexin expression at the transcriptional level, relying on both conventional *cis/trans* mechanisms (i.e. the interaction between transcription factors and regulatory elements in the gene promoters regions) and epigenetic mechanisms (e.g. DNA methylation). Following transcription (mRNA) and translation (protein), connexins assemble as gap junctions at the cell membrane surface. Short-term control of GJIC is situated at this level and is mediated by a number of actions, including posttranslational modifications of connexin proteins (e.g. phosphorylation) and regulation through connexin interacting partners (e.g. enzymes and other junctional proteins).

P2-Cx43 (Cooper *et al.*, 2000; Solan and Lampe, 2005; 2009). An additional Cx43 phosphoform, called P3-Cx43 (Kanemitsu *et al.*, 1998; Lampe *et al.*, 1998) or $Cx43_m$ (Xie *et al.*, 1997), has been uniquely detected in mitotic cells.

Cx32 can be phosphorylated by PKC, PKA, the epidermal growth factor receptor (EGFR) and Ca^{2+}-calmodulin-dependent protein kinase II (Lampe and Lau, 2004), whereby phosphorylation by PKA and PKC results in the enhancement of hepatic GJIC (Saez *et al.*, 1986) and the prevention of calpain-mediated proteolysis (Elvira *et al.*, 1993), respectively. Other Cx32-interacting partners can also affect gap junction formation and activity. Thus, assembly of adherens junctions composed of E-cadherin and α-catenin at the hepatocyte cell plasma membrane surface is a prerequisite for the formation of Cx32-based gap junctions (Fujimoto *et al.*, 1997). Inversely, Cx32 expression and activity was reported to be crucial for tight junction formation and function in primary cultures of hepatocytes (Kojima *et al.*, 2001b; 2002). The interaction between Cx32 and the scaffolding protein Dlgh 1 has recently gained particular attention. Dlgh1 acts as a tumor suppressor protein and its presence at the cell plasma membrane surface, bound to Cx32, is associated with a cell cycle block at the G0/G1 phase. Upon release, occurring upon downregulation of Cx32 expression, Dlgh1 translocates to the cell nucleus, which is known to result in increased proliferative activity. Therefore, maintaining Dlgh1 at the cell plasma membrane surface may be a regulatory mechanism by which Cx32 controls hepatocyte proliferation (Duffy *et al.*, 2007).

Role in the control of liver homeostasis

Liver cell growth

A popular experimental model to study the role of GJIC in liver cell growth is the regenerating rodent liver. In normal conditions, the adult liver displays low proliferative activity. Upon partial hepatectomy, however, the remaining intact hepatic lobes start to grow and the original size becomes restored within 7 to 10 days. In general, transiently increased GJIC activity in the G1 phase followed by a dramatic decrease upon initiation of the S phase of the hepatocyte cell cycle has been noticed, both *in vivo* and *in vitro* (Yee and Revel, 1978; Meyer *et al.*, 1981; Traub *et al.*, 1983; Dermietzel *et al.*, 1987; Sugiyama and Ohta, 1990; Miyashita *et al.*, 1991; Kren *et al.*, 1993; Fladmark *et al.*, 1997; Temme *et al.*,

2000a; Kojima *et al.*, 2003). Parallel alterations were observed at the level of Cx32 expression and to a lesser extent in Cx26 expression, whereas Cx43 production remained unchanged (Traub *et al.*, 1983; Kren *et al.*, 1993; Temme *et al.*, 2000a). It has been further shown that the reduced expression of both Cx26 and Cx32 results from decreased mRNA stabilities of the corresponding transcripts (Kren *et al.*, 1993). Similar findings are observed when using an *in vitro* model of hepatocyte proliferation, namely mitogen-stimulated primary hepatocytes (Fladmark *et al.*, 1997; Kojima *et al.*, 1997; 2004). In this system, decreased Cx32 expression is associated with MAPK-mediated phosphorylation (Kojima *et al.*, 2004). Connexin phosphorylation may actually represent a major mechanism responsible for GJIC alterations during liver cell cycling. In a proliferating rat liver cell line, progression from the G0 state to the S phase is related to PKC-dependent phosphorylation of Cx43 and disruption of GJIC (Koo *et al.*, 1997).

The physiological relevance of altered GJIC during cell cycling remains elusive. In the regenerating liver of rats treated with a p38MAPK inhibitor, the disappearance of Cx32 is inhibited without affecting hepatocyte proliferative activity, suggesting that downregulation of GJIC occurs independently of cellular proliferation and, consequently, may be considered as a minor part of the growth response (Kojima *et al.*, 2003). On the other hand, in the regenerating liver of Cx32 knock-out mice, the G0/S transition of the cell cycle, and thus the proliferative activity of the hepatocytes, is not promoted, but the extent of synchronous initiation and termination of DNA synthesis is decreased (Temme *et al.*, 2000a; Dagli *et al.*, 2004). From this perspective, reduction of GJIC does not provide a direct signal for cells to divide, but rather permits cell cycle progression upon mitogenic stimulation. GJIC therefore seems to be coordinated with cell growth and serves a purpose other than triggering proliferation. This purpose may include the functional segregation of the metabolic pools in dividing cells from their quiescent neighbors in order to avoid homeostatic imbalance (Dermietzel *et al.*, 1987; Fladmark *et al.*, 1997; Chipman *et al.*, 2003). Other investigators strongly believe that gap junctions fulfill a determinate function in cell proliferation control, rather than merely an assisting role in growth progression. Gap junctions indeed provide a pathway for the direct exchange of essential growth mediators, such as cAMP (Alexander and Goldberg, 2003). Interestingly, interfering with connexin gene expression often reveals additional mechanisms involved in gap junction-related control of cell proliferation. Thus, transfection of liver-derived cell lines with connexin genes can directly alter gene expression patterns. Forced expression of Cx32 and Cx26 in a rat liver epithelial cell line and human hepatoma cells, for instance, triggers the production of p27 and E-cadherin, respectively, which in turn, negatively affect cell growth (Koffler *et al.*, 2000; Yano *et al.*, 2001).

Liver cell differentiation and functioning

Several groups have shown that connexin expression is modulated during differentiation of early rat hepatic progenitor cells into adult liver parenchymal cells. Oval cells switch from Cx43 to Cx26 expression and, in particular, to Cx32 expression, upon differentiation into hepatocytes, both *in vivo* (Zhang and Thorgeirsson, 1994; Neveu *et al.*, 1995; Paku *et al.*, 2004) and *in vitro* (Zhang and Thorgeirsson, 1994; Rosenberg *et al.*, 1996). Alterations in connexin expression are also seen during liver ontogenesis. Cx26 and Cx32 become detectable in rat liver in the late stage of gestation and their levels culminate about 1 week after birth. At this time, the adult patterns of connexin distribution are established, whereby Cx26 becomes preferentially located in the periportal area (Iwai *et al.*, 2000). This process coincides with the establishment of the glucagon receptor zonation pattern. The latter is mainly detected in the perivenous region, whereas the inverse holds for its ligand (Berthoud *et al.*, 1992). On the other hand, glucagon was found to enhance gene transcription of Cx26 and to a lesser extent that of Cx32 (Kojima *et al.*, 1995). Based on these findings, Cx26 zonation is believed to be controlled at the transcriptional level and glucagon is likely to play a major role in this process (Kojima *et al.*, 1995; Iwai *et al.*, 2000).

Gap junctions fulfill a pivotal function in the maintenance of the differentiated functional phenotype in adult liver. Several liver-specific processes depend on GJIC, including albumin secretion (Yang *et al.*, 2003), ammonia detoxification (Yang *et al.*, 2003), glycogenolysis (Nelles *et al.*, 1996; Stumpel *et al.*, 1998), bile secretion (Nathanson *et al.*, 1999; Temme *et al.*, 2001; Bode *et al.*, 2002) and cytochrome P450 (CYP450)-mediated xenobiotic biotransformation (Neveu *et al.*, 1994a; Shoda *et al.*, 1999; 2000) Hamilton *et al.*, 2001). With respect to the latter, it was found that both the constitutive and drug-induced expression of CYP450 isoenzymes, and more specifically of CYP3A4 and CYP2B6, in primary human hepatocyte cultures require the presence of gap junctions composed of Cx32 (Hamilton *et al.*, 2001).

A number of reports have addressed the mechanistic basis of the involvement of GJIC in glycogenolysis. This process, involving the enzymatic degradation of glycogen to glucose, is activated by both hormonal and nervous stimuli, and mainly occurs in the periportal region. Perivenous hepatocytes also show glycogenolytic activity, albeit to a lesser extent in comparison with their periportal counterparts (Stumpel *et al.*, 1998; Saez *et al.*, 2003). Gap junctions play a key role in the propagation of the glycogenolytic response from the periportal area to the perivenous region. Indeed, gap junctions control the

intercellular passage of IP3, which triggers Ca^{2+} release from the endoplasmic reticulum, in turn causing Ca^{2+} waves along the acinar tract (Saez *et al.*, 2003; Gaspers and Thomas, 2005). It has been shown that Cx32 knockout mice exhibit decreased levels of glucose release into blood upon glycogenolytic stimulation (Nelles *et al.*, 1996; Stumpel *et al.*, 1998). Bile secretion also relies on GJIC-dependent Ca^{2+} signaling. Bile flow consists of both canalicular secretion from hepatocytes and ductular secretion from cholangiocytes. The latter mainly express Cx43 and, as holds for hepatocytes, the propagation of Ca^{2+} waves between these cells controls their secretory activity (Nathanson *et al.*, 1999; Temme *et al.*, 2001).

Liver cell death

Although a number of papers have clearly indicated a role for gap junctions in the occurrence of hepatocyte death, particularly by apoptosis, this research field is still in its infancy. In human hepatoma cells, apoptotic cell death was accelerated following overexpression of Cx26 (Muramatsu *et al.*, 2002). However, during apoptosis induced by choline depletion in human hepatoma cells and in a rat liver epithelial cell line, a decline in GJIC activity was observed. This was associated with cytoplasmic redistribution of Cx43 without alterations in its expression. Restoration of the Cx43 cell plasma membrane localization, and consequently of GJIC, as well as enhanced cell survival was brought about by 8-bromo-cAMP, a well-known inducer of Cx43 phosphorylation (Albright *et al.*, 2001). Induction of apoptosis in rat liver epithelial cells by the hydrophobic platinum IV complex LA-12 was also linked to suppression of GJIC and the disappearance of connexin clusters from the cell plasma membrane surface. LA-12 thereby triggered rapid Cx43 hyperphosphorylation mediated by the mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (MEK/ERK) pathway (Prochazka *et al.*, 2007). Increased connexin phosphorylation during cell death does, however, not always go hand in hand with loss of GJIC. Indeed, the histone deacetylase inhibitor suberoylanilide hydroxamic acid induced apoptosis and simultaneously increased GJIC and Cx43 phosphorylation in rat liver epithelial cells (Ogawa *et al.*, 2005). Wilson and co-workers elegantly demonstrated that GJIC is induced in the early phases of apoptosis in a serum-deprived rat liver epithelial cell line and coincides with increased Cx43 expression and phosphorylation. The latter might be mediated by the cyclin-dependent kinase 1/cyclin B complex, which also controls the G2/M transition of the cell cycle. Upon further progression of cell death, GJIC activity declines, as evidenced by the absence of communication between apoptotic bodies (Wilson *et al.*, 2000). It is thought that the transient induction of GJIC in the early phases of apoptosis could point to a role for gap junctions in the initial spread of a death wave from cell to cell. In this context, Ca^{2+} ions are thought to be the killing messengers. The onset of apoptosis is generally associated with drastic alterations in Ca^{2+} concentration, an ion that is intercellularly exchanged *via* gap junctions. The subsequent reduction in GJIC activity may possibly serve to reduce the flux of toxic metabolites (e.g. nitric oxide and superoxide ions) and thus to protect a healthy cell from its dying neighbor (Krutovskikh *et al.*, 2002; Contreras *et al.*, 2004).

Methods to probe GJIC

Metabolic coupling assays

The metabolic cooperation approach is based upon the monitoring of the transfer of endogenous and biologically relevant compounds. For this procedure, fluorescently marked donor cells are incubated in the presence of radiolabeled precursors, like nucleotides or glucose, and are then co-cultured with unlabeled recipient cells. Subsequently, donor cells are separated from receiver cells through fluorescence-activated cell sorting and the amount of the radioisotope in the receipt cell population is assessed by chromatography and/or quantitative autoradiography (Goldberg *et al.*, 1998; 1999). A more indirect method includes the tracking of Ca^{2+} waves, which correlates with the presence of functional gap junctions. In this technique, cells are loaded with a Ca^{2+}-sensitive fluorescent dye and are stimulated electrically, mechanically or chemically in order to generate IP3, which triggers the actual Ca^{2+} wave. A more sophisticated approach is the local liberation of IP3 from a caged precursor by flash photolysis, which allows the stimulation of single cells (Leybaert and Sanderson, 2001).

Electrical coupling assays

The dual voltage patch clamp technique envisages the recording of gap junctional electrical conductance, whereby originally 2 separate microelectrodes were introduced in each cell of a cell pair, i.e. for current injection and for voltage control (Spray *et al.*, 1979; 1981). This technique was later modified to a double whole cell voltage clamp technique, using only 1 patch pipet per cell, which is a very sensitive method that allows the recording of a single gap junction channel (Hamill *et al.*, 1981; Neyton and Trautmann, 1985). Analysis of gap junctional electrical conductance, however, is a labor-intensive, expensive and rather slow technique that requires appropriate expertise and technical skills (Yamasaki, 1997; Abbaci *et al.*, 2008).

Dye coupling assays

Dye coupling methods are by far the most frequently used ones, mainly because of their ease of use. This kind of assays relies on the introduction of small (< 900 Da)

dyes into living cells that are traced in their intercellular movement. A wide variety of tracers, mostly fluorescent, are used (Meda, 2000; Abbaci *et al.*, 2008), and there are several ways to introduce these reporter dyes into cells, including microinjection (Kanno and Loewenstein, 1964), mechanical loading by scraping (el-Fouly *et al.*, 1987) and electroporation (Raptis *et al.*, 1994; De Vuyst *et al.*, 2008). In addition, a number of noninvasive dye coupling protocols have been established. In the fluorescence recovery after photobleaching (FRAP) analysis, cells are loaded with a lipophilic cell plasma membrane permeable dye, such as calcein acetoxymethyl ester. Upon cellular uptake, this dye is hydrolized by cytoplasmic esterases, yielding a fluorescent and membrane impermeable molecule, *in casu* calcein. Fluorescence in a single cell is then irreversibly photobleached using a high-powered laser beam and subsequent transfer of fluorescent dye from neighboring cells into the target cell is monitored (Wade *et al.*, 1986; Abbaci *et al.*, 2007). Both the preloading assay and the parachute technique also require cell loading with cell plasma membrane permeable dyes. In the former, loaded cells are suspended together with unloaded counterparts and are then allowed to form a confluent monolayer (Goldberg *et al.*, 1995), whereas in the latter, loaded cells in suspension adhere to a monolayer of unloaded cells (Ziambaras *et al.*, 1998). In both cases, the spread of the dye from donor cells to receiver cells is studied by fluorescence microscopy and is a measure for GJIC. Dakin and coworkers introduced the local activation of molecular fluorescent probe (LAMP) method, which is based upon a new generation of caged coumarin-like fluorophores. Like in FRAP, these dyes are processed by intracellular esterases, but they only become fluorescent upon subsequent local illumination with a small dose of ultraviolet light. The latter is unlikely to cause photodamage, in contrast to the high-powered laser beam used in the FRAP approach (Dakin *et al.*, 2005). Recently, an improvement to the LAMP method has been described, the so-called infrared-LAMP assay, which allows to examine cell–cell coupling in 3 dimensions (Yang and Li, 2009).

Effects of toxicants on liver gap junctions

Environmental pollutants

Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) represent a large group of lipophilic environmental pollutants that mainly occur in soil, sediment and oily substances. They are also produced as byproducts during combustion processes and can be found in grilled food. PAHs are of major concern for human health as some of them have been identified as carcinogenic, mutagenic and teratogenic. Their mode of toxic action has been well studied and involves binding to the aryl hydrocarbon receptor (AhR) following cellular uptake. PAH binding results in the translocation of the AhR to the nucleus, where it directly affects gene expression. Many genes related to CYP450-mediated xenobiotic biotransformation are transcriptionally induced upon AhR activation and a number of signaling pathways that control crucial processes like cell cycling become drastically altered (Puga *et al.*, 2009). Benzene, the founding PAH member, but not a PAH itself, did not alter GJIC in a rat liver cell line. Its metabolites, however, especially *trans,trans*-muconaldehyde, strongly counteracted hepatic gap junction functionality, which was associated with decreased Cx43 protein expression and Cx43 phosphorylation mediated by ERK1/2 (Rivedal and Witz, 2005). It has been reported that the ability of PAHs to reduce GJIC is related to their carcinogenic potential (Sharovskaya *et al.*, 2006; Svihalkova-Sindlerova *et al.*, 2007; Machala *et al.*, 2008), whereby GJIC inhibition is not depending on PAH biotransformation or AhR activation (Sharovskaya *et al.*, 2006). Blaha and group screened a wide series of PAHs for their outcome on gap junction functionality in a rat liver cell line and found that only a subset of PAHs inhibit dye coupling, especially those with a lower molecular mass (Blaha *et al.*, 2002). In fact, clear structure–activity relationships have been established with respect to the ability of PAHs to reduce GJIC. Indeed, benzene-type PAHs, naphthalene-type PAHs and fluorene-type PAHs only inhibit hepatic GJIC if a bay-like region is present in their structure (Upham *et al.*, 1998; Weis *et al.*, 1998; Rummel *et al.*, 1999). Marvanova and colleagues further showed that benz[*a*]anthracenes with a methyl group in the bay-like region, which is important for AhR binding, are strong inhibitors of GJIC, but weak inducers of hepatic AhR activity (Marvanova *et al.*, 2008). PAHs with a bay-like region also activate hepatic ERK1/2, which occurs after GJIC inhibition. It is therefore thought that modulation of GJIC might affect ERK activation (Rummel *et al.*, 1999). In line with this finding, 1-methylanthracene, unlike 2-methylanthracene, inhibited dye coupling in a rat liver epithelial cell culture system, which involved phosphatidylcholine-specific phospholipase C (PLC), but not p38MAPK (Upham *et al.*, 2008). Similarly, exposure of a rat liver cell line to diesel exhaust particles, which is a complex mixture of polar PAHs, resulted in inhibition of GJIC, but this was not accompanied by modifications in PKC and MEK activity or an altered Cx43 phosphorylation status (Rivedal *et al.*, 2003). The utilization of advanced oxidation processes, such as ozonation, is a frequently applied strategy to combat PAHs in water. In this respect, the effects of pyrene and benzo[*a*]pyrene and their ozonated products on gap junction functionality have been investigated in rat liver cell lines. It was found that suppression of GJIC by the ozonated products correlated

with oxidation of the aromatic ring framework, whereby extended oxidation, and thus longer ozonation times, yielded low molecular weight products that did not alter gap junction functionality (Luster-Teasley *et al.*, 2005; Ottinger *et al.*, 2005).

Polychlorinated dibenzodioxins

Polychlorinated dibenzodioxins (PCDDs), or simply dioxins, are industrial byproducts that accumulate in fatty tissues in humans upon dietary uptake. PCDDs, like PAHs, are acknowledged ligands of the AhR. The most toxic PCDD congener is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), which has been shown to display teratogenic, endocrine disrupting, immunotoxic, hepatotoxic, mutagenic, and carcinogenic properties in animals (Knerr and Schrenk, 2006a; Pelclova *et al.*, 2006). In experimental models of chemical-induced hepatocarcinogenesis, TCDD is typically used as a tumor promoter following exposure of laboratory animals to tumor initiators such as nitrosamines, whereby deleterious effects on connexin expression are observed (Neveu *et al.*, 1990; 1994b). Single treatment of rats with TCDD also results in decreased hepatic protein levels of Cx32 (Bager *et al.*, 1997; Mally and Chipman, 2002) and Cx26 (Bager *et al.*, 1997). In a liver-based co-culture system, TCDD decreased both Cx32 immunoreactivity and Cx32 gene transcription, which in turn abrogated GJIC (Herrmann *et al.*, 2002). Reduced mRNA levels of Cx32, but not of Cx26, and concomitant inhibition of gap junction functionality was also observed in primary rat hepatocyte cultures and this was mediated, at least in part, through AhR activation (Baker *et al.*, 1995).

Polychlorinated biphenyls

Polychlorinated biphenyls (PCBs) are mixtures of up to 209 different congeners, subdivided into dioxin-like and nondioxin-like PCBs. PCBs have been used for a wide variety of applications, e.g. as coolants, plasticizers, insulating fluids for transformers and fire retardants. Although PCB production has been banned since the 1970s, they still are abundantly present in the environment and accumulate in the food chain. Numerous studies have demonstrated that PCBs exert several toxic effects, including carcinogenicity, immunotoxicity, teratogenicity and reproductive toxicity, in laboratory animals. Among the mechanisms that underlie these toxicological processes, especially for dioxin-like PCBs, AhR activation has been well characterized (Knerr and Schrenk, 2006b; Cillo *et al.*, 2007). Commercial PCB mixtures, marketed as Aroclors or Kanechlors, contain more than 50 congeners and are frequently used for experimental studies because their composition is representative of environmental PCB pollution (Knerr and Schrenk, 2006b). Administration of Aroclor-1260 to male rats resulted in reduced hepatic GJIC and simultaneous decreased Cx32 protein levels. By contrast, the Cx43 protein amount increased, whereas the hepatic Cx26 protein content was not affected by Aroclor-1260 and these changes were not observed at the transcriptional level. Furthermore, both Cx26 and Cx43 were located within the cytosol of hepatocytes from treated animals (Krutovskikh *et al.*, 1995). Treatment of male rats with the dioxin-like PCB126 (3,4,5,3′,4′-pentachlorobiphenyl), but not with the dioxin-like PCB118 (2,4,5,3′,4′-pentachlorobiphenyl) and the nondioxin-like PCB153 (2,4,5,2′,4′,5′-hexachlorobiphenyl) decreased both Cx26 and Cx32 protein levels in the liver (Bager *et al.*, 1994; 1997), but not the corresponding mRNA contents (Bager *et al.*, 1994). *In vitro*, however, PCB153, unlike PCB126, decreased GJIC, which was associated with activation of hepatic ERK1/2, phosphatidylcholine-specific PLC, diacylglycerol lipase and *v*-Src kinase (Machala *et al.*, 2003; Simeckova *et al.*, 2009). PCB153 also altered the Cx43 phosphorylation status, yielding the P3-Cx43 form, and increased both proteasomal and lysosomal Cx43 internalization and degradation (Simeckova *et al.*, 2009). Furthermore, it has been shown that metabolites of PCBs that reduce gap junction functionality, including methylsulfonyl metabolites (Kato *et al.*, 1998), hydroxylated metabolites and to a lesser extent quinoid metabolites (Machala *et al.*, 2004), also act as potent inhibitors of hepatic GJIC.

Biological toxins

Phorbol esters

Phorbol esters are tetracyclic diterpenoids derived from the seed oil of the *Croton tiglium* plant. The most common phorbol ester is 12-O-tetradecanoylphorbol-13-acetate (TPA), also called phorbol-12-myristate-13-acetate, which is used as a prototypical tumor promoter in models of carcinogenesis. TPA mimics the action of diacylglycerol, an activator of PKC, which regulates different signal transduction pathways and other cellular metabolic activities (Goel *et al.*, 2007; Griner and Kazanietz, 2007). It has been demonstrated on many occasions that TPA downregulates GJIC in liver-based *in vitro* models, both primary cells and cell lines. With some exceptions (Asamoto *et al.*, 1991; Matesic *et al.*, 1994), hepatic connexin mRNA levels remained unaffected (Lampe, 1994; Guan *et al.*, 1995; Ren *et al.*, 1998; Nielsen *et al.*, 2000; Kang *et al.*, 2001), whereas both increased (Budunova *et al.*, 1993) and decreased (Asamoto *et al.*, 1991; Kenne *et al.*, 1994; Rivedal *et al.*, 1994) protein contents have been observed in the presence of TPA. The principal mode of TPA action is actually located at the posttranslational level. It has indeed been repeatedly reported that TPA induces Cx43 hyperphosphorylation, which results in the loss of GJIC (Berthoud *et al.*, 1993; Budunova *et al.*, 1993; Kanemitsu and Lau,

1993; Hill *et al.*, 1994; Lampe, 1994; Matesic *et al.*, 1994; Hu and Cotgreave, 1995; Hu *et al.*, 1995a; Guan and Ruch, 1996; Kato and Kenne, 1996; Chaumontet *et al.*, 1997; Upham *et al.*, 1997; Ren *et al.*, 1998; Nielsen *et al.*, 2000; Kang *et al.*, 2001; Rivedal and Opsahl, 2001; Ruch *et al.*, 2001; Klotz *et al.*, 2002; Leithe *et al.*, 2003; Leithe and Rivedal, 2004; Loch-Caruso *et al.*, 2004; Rivedal and Leithe, 2005; Jung *et al.*, 2006; Park *et al.*, 2006; Sirnes *et al.*, 2009). Besides reducing the unphosphorylated Cx43 signal, TPA was also reported to induce the appearance of the P3-Cx43 variant during immunoblot analysis (Matesic *et al.*, 1994; Guan *et al.*, 1995; Ren *et al.*, 1998). Both PKC (Oh *et al.*, 1988; Berthoud *et al.*, 1993; Ren *et al.*, 1998; Rivedal and Opsahl, 2001; Ruch *et al.*, 2001; Leithe *et al.*, 2003; Leithe and Rivedal, 2004; Rivedal and Leithe, 2005; Sirnes *et al.*, 2009) and MAPKs (Rivedal and Opsahl, 2001; Ruch *et al.*, 2001; Jung *et al.*, 2006; Park *et al.*, 2006; Sirnes *et al.*, 2009) have been shown to mediate TPA-induced Cx43 hyperphosphorylation. In fact, TPA induces oxidative stress and activation of PKCα, PKCδ and PKCε (Hu and Cotgreave, 1995; Hu *et al.*, 1995a; Leithe *et al.*, 2003). PKC then directly phosphorylates Cx43 at serine368 in the CT region (Loch-Caruso *et al.*, 2004; Sirnes *et al.*, 2009), followed by its internalization and degradation (Hu and Cotgreave, 1995; Hu *et al.*, 1995a; Ren *et al.*, 1998). Cx43 breakdown also occurs through crosstalk with the MAPK pathway, since both PKC and ERK1/2 are involved in TPA-triggered phosphorylation of Cx43 at serine255 and serine262, both located within the CT region, leading to ubiquitination, internalization and ultimately to Cx43 degradation (Leithe and Rivedal, 2004; Rivedal and Leithe, 2005; Sirnes *et al.*, 2009).

Lipopolysaccharide

Lipopolysaccharide (LPS), found in the outer membrane of Gram negative bacteria, acts as an endotoxin and elicits strong immune and inflammatory responses in animals (Gingalewski *et al.*, 1996; De Maio *et al.*, 2000). GJIC drastically decreases upon administration of LPS to male rodents, which is accompanied by reduced Cx32 immunostaining (Gingalewski *et al.*, 1996; De Maio *et al.*, 2000; Correa *et al.*, 2004). The latter results from decreased Cx32 mRNA stability due to shortening of the poly(A)tail (Theodorakis and De Maio, 1999). By contrast, LPS increased hepatocellular Cx26 mRNA and protein contents (De Maio *et al.*, 2000; Temme *et al.*, 2000b; Romualdi *et al.*, 2002). LPS also enhanced Cx43 expression in rat liver Kupffer cells and stellate cells, both *in vitro* and *in vivo* (Gonzalez *et al.*, 2002; Fischer *et al.*, 2005; Eugenin *et al.*, 2007).

Ochratoxin A

Ochratoxin A (OTA) is a mycotoxin produced by some species of *Aspergillus* and *Penicillium* during storage of food. OTA exerts several toxic effects, mainly involving the kidney and the liver (Horvath *et al.*, 2002; Gagliano *et al.*, 2006). In a rat liver epithelial cell line, but not in a human kidney cell line, OTA inhibited GJIC. This was associated with activation of MAPK, p38MAPK and ERK, and concomitant modifications in Cx43 phosphorylation (Horvath *et al.*, 2002). Furthermore, administration of OTA to male rats negatively affected the production of Cx26, Cx32 and Cx43 transcripts in the liver (Gagliano *et al.*, 2006).

Patulin

Patulin is a reactive mycotoxin commonly contaminating agricultural products, including fruit products (Kabak *et al.*, 2006). In an attempt to elucidate its mechanism of cytotoxicity, Barhoumi and Burghardt found that patulin decreased fluorescence recovery during FRAP analysis in cultures of rat liver epithelial cells. In fact, suppression of GJIC and depletion of intracellular glutathione are the first events triggered by patulin in these cells. This was followed by the generation of reactive oxygen species (ROS), mitochondrial membrane depolarization, increase of intracellular Ca^{2+} concentration, cytoplasmic acidification and plasma membrane depolarization (Barhoumi and Burghardt, 1996).

Gossypol

Gossypol is a toxic pigment present in cottonseed meal that acts as a nonsteroid antifertility agent and a nonspecific enzyme inhibitor (Herve *et al.*, 1996). Similar to patulin, inhibition of GJIC precedes an increase of intracellular Ca^{2+} concentration during gossypol-induced cytotoxicity in cultured rat liver epithelial cells (Barhoumi and Burghardt, 1996). Furthermore, suppression of gap junction activity in these cells was associated with alterations in the Cx43 phosphorylation status and was attenuated by a cAMP analogue (Hutchinson *et al.*, 1998).

Organic solvents

Ethanol

Ethanol is a prototypical organic solvent, but is better known as the type of alcohol that is found in alcoholic beverages. Many mechanisms are involved in ethanol-induced liver injury, including oxidative stress (Lu and Cederbaum, 2008), nitrosative stress (Cooper and Magwere, 2008) and induction of liver cell death (McVicker *et al.*, 2007), all of which may eventually burgeon into the onset of liver cancer. Exposure of primary rat hepatocytes (Abou Hashieh *et al.*, 1996) and a rat liver-based cell line (Bokkala *et al.*, 2001) to ethanol was found to decrease GJIC. This was not a result from changes in gap junction plaques (Abou Hashieh *et al.*, 1996) or modifications in connexin gene transcription

(Bokkala *et al.*, 2001), but from decreased connexin protein biosynthesis (Abou Hashieh *et al.*, 1996). In the case of the primary hepatocyte culture system, ethanol-induced gap junction dysfunction was closely related to ethanol metabolism, since inhibition of alcohol dehydrogenase, which catalyzes the oxidation of ethanol to acetaldehyde, abolished this effect (Abou Hashieh *et al.*, 1996).

Carbon tetrachloride

Carbon tetrachloride was formerly often used as a solvent, and nowadays is applied as an experimental tool to provoke liver fibrosis and cirrhosis in laboratory animals (Wasser and Tan, 1999). Carbon tetrachloride induces liver cancer in rodents, primarily by causing oxidative and lipid peroxidative damage, which in turn indirectly triggers genotoxicity (Eastmond, 2008). In primary cultures of rat hepatocytes, carbon tetrachloride decreased junctional conductance and its biotransformation was a prerequisite for this outcome (Saez *et al.*, 1987). Administration of carbon tetrachloride to male rats reduced hepatic Cx32 immunoreactivity (Cowles *et al.*, 2007) and protein levels (Miyashita *et al.*, 1991), but increased the Cx32 mRNA content (Nakata *et al.*, 1996). However, this did not result in the abrogation of gap junction functionality in the liver (Cowles *et al.*, 2007).

Trichloroethylene

Trichloroethylene is an industrial solvent used as a chemical additive and for metal degreasing. It is known to cause hepatotoxicity (Brautbar and Williams, 2002) and to induce liver tumorigenesis through both genotoxic and nongenotoxic pathways (Shiao, 2009). Trichloroethylene was reported to reduce dye coupling in primary cultured hepatocytes from mouse, but not from rat. Its inhibitory effect on GJIC relied on metabolic capacity, as no alterations in gap junction functionality were noticed upon simultaneous exposure of primary mouse hepatocytes to SKF-525A, an inhibitor of CYP450 isoenzymes (Klaunig *et al.*, 1989).

Pesticides

Organophosphorous pesticides

Organophosphorous pesticides, such as parathion, methylparathion, diazinon and malathion have been widely used as insecticides and to a lesser extent as herbicides. They act through inhibition of acetylcholinesterase, which underlies most of their adverse affects (Maroni *et al.*, 2000). However, organophosphorous pesticides also elicit toxicity *via* other mechanisms, including cytotoxicity (Wagner *et al.*, 2005), disruption of sex hormone homeostasis (Okamura *et al.*, 2005) and genotoxicity (Rahman *et al.*, 2002). It has been demonstrated that parathion, methylparathion, diazinon and malathion inhibit dye coupling in cultures of rat liver epithelial cells. Notably, their ozonated byproducts, formed upon ozonation of drinking water, do not affect gap junction functionality (Masten *et al.*, 2001; Wu *et al.*, 2007).

Cyclodiene organochlorine pesticides

Cyclodiene organochlorine compounds, such as endosulfan, chlordane, heptachlor and dieldrin represent a subgroup of versatile pesticides of which most have been banned since the 1970s. Nevertheless, their residues are still continuously detected in food and in the environment (Manclus *et al.*, 2004). Most of the cyclodiene organochlorine pesticides are nongenotoxic (hepato)carcinogens (Ruch *et al.*, 1990) and some were also found to display estrogenic activity (Soto *et al.*, 1995). Endosulfan, chlordane, heptachlor and dieldrin all reduced GJIC in cultures of rat liver epithelial cells, which was linked to an altered Cx43 phosphorylation status (Kenne *et al.*, 1994; Matesic *et al.*, 1994; Warngard *et al.*, 1996; Rivedal and Opsahl, 2001). In the case of heptachlor and dieldrin, this was also associated with reduced protein levels Cx26 and Cx43, whereby only the latter was reflected at the transcriptional level (Matesic *et al.*, 1994). The inhibitory action of endosulfan, heptachlor and chlordane on GJIC in primary mouse hepatocyte cultures did not depend on xenobiotic phase I biotransformation capacity, as concomitant exposure to a panel of inhibitors of CYP450 isoenzymes did not affect their outcome (Ruch *et al.*, 1990). Furthermore, the actions of dieldrin seem to be species-specific, since this versatile insecticide abrogated gap junction functionality in primary cultures of mouse hepatocytes, but not of rat hepatocytes (Klaunig and Ruch, 1987).

Dichlorodiphenyltrichloroethane

The prototypical pesticide 1,1,1-trichloro-2,2 bis (4-chlorophenyl)ethane, commonly known as dichlorodiphenyltrichloroethane (DDT), is a formerly used nonsystemic insecticide which strongly persists in the environment and accumulates in animal fats (Maroni *et al.*, 2000). DDT is neurotoxic, causes endocrine disruption and acts as a tumor promoter, whereby the liver is a principal target (Beard, 2006). Early freeze-fracture studies showed that the size of gap junction plaques on hepatocytes from rats exposed to DDT is reduced (Sugie *et al.*, 1987). Subsequent animal experiments demonstrated that DDT provokes gap junction closure in male rat liver, which is associated with decreased Cx32 immunoreactivity and/or protein levels, aberrant Cx32 localization, but not with changes in the Cx32 phosphorylation status and mRNA content (Ito *et al.*, 1993; Tateno *et al.*, 1994; Harada *et al.*, 2003; Cowles *et al.*, 2007). DDT did not affect the overall Cx26 protein and mRNA levels (Tateno *et al.*, 1994; Cowles *et al.*, 2007), but enhanced

Cx26 protein production by perivenous hepatocytes (Krutovskikh *et al.*, 1995) and negatively affected Cx26 immunoreactivity in the periportal region (Tateno *et al.*, 1994). Furthermore, DDT also promoted the appearance of Cx43 in the cytoplasm of hepatocytes when administered to male rats (Krutovskikh *et al.*, 1995). *In vitro* studies, carried out in primary hepatocyte cultures from rat and mouse, showed that the inhibition of GJIC caused by DDT did not depend on CYP450-mediated biotransformation capacity (Klaunig *et al.*, 1990) and was likely to be triggered by the formation of radical intermediates following lipid peroxidation (Leibold and Schwarz, 1993). In rat liver epithelial cell line models, DDT also reduced GJIC (Budunova *et al.*, 1993; Ruch *et al.*, 1994; Ren *et al.*, 1998; Nielsen *et al.*, 2000), which was accompanied by decreased Cx43 protein levels (Budunova *et al.*, 1993), an altered Cx43 phosphorylation status (Budunova *et al.*, 1993; Ruch *et al.*, 1994; Ren *et al.*, 1998) and increased endocytosis of gap junctions and lysosomal degradation of the P2-Cx43 variant (Guan and Ruch, 1996), whereas changes in Cx43 gene transcription were not observed (Ruch *et al.*, 1994).

Lindane

Lindane or γ-hexachlorocyclohexane is a broad spectrum insecticide that has been used since the early 1950s to protect seeds, soil, timber, stored materials, animals and men against ectoparasites (Marvanova *et al.*, 2008). Lindane is neurotoxic, embryotoxic and hepatocarcinogenic in rodent bioassays, whereby the latter does not result from genetic damage (Guan *et al.*, 1995). It has also been found to act as an endocrine disruptor, both *in vitro* and *in vivo* (Tiemann, 2008). Lindane was reported to inhibit gap junction activity in cultured rat liver epithelial cells, which was accompanied by increased endocytosis of gap junctions and lysosomal degradation of P2-Cx43 (Guan and Ruch, 1996) and by elevated immunostaining for serine368-phosphorylated Cx43 (Loch-Caruso *et al.*, 2004). Guan and colleagues investigated the time-dependent outcome of lindane on gap junction functionality in primary rat hepatocyte cultures and found that short-term exposure (minute range) resulted in inhibition of dye coupling without changes in Cx43 expression. Mid-term exposure (hour range) of hepatocytes to lindane led to the reduced presence of Cx43-based gap junction plaques at the cell plasma membrane surface and changes in Cx43 phosphorylation, which in turn downregulated gap junction activity. Inhibition of GJIC following long-term treatment (day range) with lindane resulted from the loss of Cx43 expression (Guan *et al.*, 1995).

Hexachlorobenzene

Hexachlorobenzene (HCB) has been used as a fungicide and is also a byproduct of industrial processes. While the use of HCB has been banned in most industrialized countries, it is still present in the environment. Exposure to HCB has been linked to the development of porphyria and hepatic cancer, especially in female rodents (Plante *et al.*, 2002; 2006; 2007.). In line with this, administration of HCB to female but not to male rats resulted in decreased hepatic GJIC, associated with downregulated Cx26 and Cx32 productions, both at the transcriptional and at the translational level (Plante *et al.*, 2002). *In vitro* studies in rat hepatoma cells further showed that HCB-induced downregulation of Cx32 mRNA levels is linked to the activation of the integrin-linked kinase pathway. The latter triggers nuclear translocation of Akt, which is believed to affect transcription factors that control Cx32 gene transcription, such as Sp1 and HNF1α (Plante *et al.*, 2006). This finding was corroborated *in vivo*, whereby administration of HCB to rats resulted in decreased binding of transcriptional complexes Fr26 and Fr110, known to be controlled by Akt, to the Cx32 gene promoter in female liver, but not in its male counterpart (Plante *et al.*, 2007).

Pentachlorophenol

Pentachlorophenol (PCP) is a halogenated phenolic compound that, besides being a general herbicide, is used to control termites. Its sodium salt is applied as a disinfectant, whereas its laurate ester is used to protect wood from fungal rot and wood-boring insects (Maroni *et al.*, 2000). PCP negatively affected GJIC in normal and transformed rat liver epithelial cells, which resulted from downregulated Cx43 mRNA and/or protein levels (Sai *et al.*, 1998; 2001). Furthermore, administration of PCP to male mice caused decreased hepatic gap junction functionality and reduced Cx32 immunoreactivity (Sai *et al.*, 2000).

Pharmaceuticals

Hypolipidemic drugs

Clofibrate (Fidaleo, 2008), nafenopin (Roberts *et al.*, 2002) and Wy-14,643 (Gonzalez and Shah, 2008) are lipid-lowering agents that represent an important class of so-called peroxisome proliferators. These compounds bind to the peroxisome proliferator-activated receptor α, which modulates gene expression programs in favor of proliferative activity. Not surprisingly, long-term treatment of rodents with peroxisome proliferators has been associated with hepatocarcinogenesis (Roberts *et al.*, 2002; Fidaleo, 2008; Gonzalez and Shah, 2008). Both *in vitro* (Elcock *et al.*, 1998; Kamendulis *et al.*, 2002) and *in vivo* (Krutovskikh *et al.*, 1995; Cowles *et al.*, 2007), it has been found that clofibrate, nafenopin and Wy-14,643 reduce GJIC between hepatocytes. Inhibition of GJIC by these agents occurs in a species-specific way, since it was observed in primary cultured hepatocytes

from rat, mouse and hamster, but not from monkey and human (Kamendulis *et al.*, 2002). Similarly, treatment of primary hepatocytes from rat, but not from guinea pig, with nafenopin resulted in the disappearance of GJIC. The latter did not result from altered Cx26 and Cx32 protein levels or modifications in the cellular localization of Cx32, but was linked to PKC-mediated phosphorylation of Cx32 (Elcock *et al.*, 1998). By contrast, clofibrate (Krutovskikh *et al.*, 1995; Tsuda *et al.*, 1995) and Wy-14,643 (Cowles *et al.*, 2007) reduced hepatic Cx26 and Cx32 protein amounts. In addition, clofibrate enhanced the appearance of Cx43 in the cytoplasm of hepatocytes (Krutovskikh *et al.*, 1995).

Phenobarbital

Phenobarbital or phenobarbitone is a widely used antiepileptic drug that also has sedative and hypnotic properties. It is frequently applied as a model tumor promoter in rodent liver (Moennikes *et al.*, 2000; Luebeck *et al.*, 2005), whereby the expression of a broad set of genes is altered, of which genes related to CYP450-dependent xenobiotic biotransformation have gained most attention (Stahl *et al.*, 2005). The presence of functional gap junctions consisting of Cx32, but not of Cx26, is a prerequisite for the promotional activity of phenobarbital, since Cx32 knock-out mice (Moennikes *et al.*, 2000; Luebeck *et al.*, 2005), unlike Cx26 knock-out animals (Marx-Stoelting *et al.*, 2008), are resistant to promotion of hepatocarcinogenesis by the barbiturate. Furthermore, a subset of genes is differentially affected by phenobarbitone in the liver of Cx32-deficient mice compared to their wild-type counterparts (Stahl *et al.*, 2005), thus further pointing to a critical role for GJIC in phenobarbital-mediated tumor promotion. It has been shown by several groups that gap junction activity becomes reduced upon administration of phenobarbitone to rodents (Neveu *et al.*, 1990; 1994a; Krutovskikh *et al.*, 1995; Ito *et al.*, 1998; Jeong *et al.*, 2000; Warner *et al.*, 2003). This was associated with abnormal frequency and size of gap junctions on the hepatocyte plasma membrane surface (Sugie *et al.*, 1987), decreased Cx32 immunoreactivity (Neveu *et al.*, 1990; 1994a; Ito *et al.*, 1998; Okamiya *et al.*, 1998) and aberrant Cx32 localization (Krutovskikh *et al.*, 1995), whereas Cx26 expression was not affected (Neveu *et al.*, 1990; 1994a; Ito *et al.*, 1998). Both unchanged (Neveu *et al.*, 1994a; Warner *et al.*, 2003) and decreased (Beer *et al.*, 1988; Mesnil *et al.*, 1988) hepatic Cx32 mRNA levels were reported in phenobarbital-treated rodents. Interestingly, phenobarbital specifically reduced Cx32 protein production in perivenous hepatocytes of male rodent liver (Ito *et al.*, 1998; Neveu *et al.*, 1990; 1994a), which is the acinar area where the phenobarbital-induced expression of CYP2B1/2 is mostly manifested. These colocalized modifications in Cx32 production and CYP2B1/2 expression are believed to be physiologically important for the effective biotransformation of xenobiotics, *in casu* phenobarbital, by limiting the cytoplasmic diffusion of toxic reactive intermediates (Neveu *et al.*, 1994a). As shown in rodent models both *in vivo* (Warner *et al.*, 2003) and *in vitro* (Klaunig and Ruch, 1987; Ren *et al.*, 1998), the reduction of GJIC by phenobarbitone occurs in a strain-specific way. Furthermore, the inhibitory effect of phenobarbital on GJIC between primary cultured mouse hepatocytes depends on xenobiotic biotransformation capacity, as it was abolished by a CYP450 inhibitor (Klaunig *et al.*, 1990).

Methapyrilene

Methapyrilene is an antihistaminic drug with strong sedative properties that has been mainly prescribed to treat insomnia. It has been banned in most countries because of its potential to cause serious liver damage (Auman *et al.*, 2007). In recent years, methapyrilene has been tested in several toxicogenomics studies (Hamadeh *et al.*, 2002; Waring *et al.*, 2004; Beekman *et al.*, 2006; Auman *et al.*, 2007; Uehara *et al.*, 2008) and even in integrated systems toxicological trials (Craig *et al.*, 2006) as a typical nongenotoxic hepatocarcinogen, whereby it became clear this drug induces numerous alterations in critical metabolic and signaling pathways. With respect to intercellular communication mediated by gap junctions, it has been reported that the number and size of Cx32-containing gap junctions plaques in liver is negatively affected upon treatment of male rats with methapyrilene (Mally and Chipman, 2002).

Miscellaneous

Peroxides

ROS, including peroxides, free radicals and oxygen ions, are natural byproducts of oxygen metabolism that play important roles in cellular signaling. Under stress conditions, however, levels of ROS may drastically increase, resulting in damage of cellular structures, a situation referred to as oxidative stress (Imlay, 2008; Jones, 2008). Oxidative stress has been involved in many toxicological and pathological processes and is counteracted through glutathione metabolism (Jones, 2008). Hydrogen peroxide, a prominent ROS, was reported to inhibit GJIC in a number of liver-based cell lines (Upham *et al.*, 1997; Huang *et al.*, 1999; Kang *et al.*, 2000; Cho *et al.*, 2002; Hwang *et al.*, 2005; 2008; Jung *et al.*, 2006), which involved glutathione and which was not a consequence of free radical damage (Upham *et al.*, 1997). This was associated with Cx43 hyperphosphorylation (Huang *et al.*, 1999; Kang *et al.*, 2000; Cho *et al.*, 2002; Hwang *et al.*, 2005; 2008; Jung *et al.*, 2006), in turn resulting from the activation of EGFR (Huang *et al.*, 1999; 2001), Akt (Hwang *et al.*, 2008), p38MAPK, ERK1/2 and c-jun N-terminal

kinase (Cho *et al.*, 2002; Lee, KW *et al.*, 2004; Hwang *et al.*, 2005; Jung *et al.*, 2006). Other peroxides, such as dicumyl peroxide (Upham *et al.*, 2007) and benzoyl peroxide (Hu and Cotgreave, 1995; Upham *et al.*, 2007) as well as compounds that induce the generation of hydrogen peroxide, such as gallic acid (Lee *et al.*, 2005a; Kim *et al.*, 2009a), (-)-epigallocatechin gallate (Kang *et al.*, 2008), 25-hydroxycholesterol (Guo *et al.*, 1993), paraquat (Ruch and Klaunig, 1988), TPA (Hu and Cotgreave, 1995) and DDT (Harada *et al.*, 2003), also negatively affect hepatic GJIC. *Vice versa*, a number of substances have been found to counteract hydrogen peroxide-mediated inhibition of GJIC in liver-based *in vitro* models, mostly by normalizing the Cx43 phosphorylation status, including pterostilbene (Kim *et al.*, 2009b), indole-3-carbinol (Hwang *et al.*, 2008), resveratrol (Upham *et al.*, 2007; Kim *et al.*, 2009a), cacao been husk extract (Lee *et al.*, 2005b), trichostatin A (Jung *et al.*, 2006), Chinese cabbage extracts, sulforaphane (Hwang *et al.*, 2005), *Abies nephrolepis* leaf phenolics (Lee, SJ *et al.*, 2004), mushroom *Phellinus linteus* extract (Cho *et al.*, 2002), epicatechin, ginsenoside Rb2 (Kang *et al.*, 2000), boldine, glaucine and probucol (Hu *et al.*, 1995b).

Metals

A number of metals, e.g. mercury and aluminum, have been shown to interfere with gap junction functionality in specific cell types, *in casu* renal proximal epithelial cells (Yoshida *et al.*, 1998) and astroglial cells (Theiss and Meller, 2002), respectively. With regard to hepatic GJIC, specific attention has been paid to cadmium, which is a major environmental pollutant. Exposure of the general population to cadmium mainly occurs through cigarette smoke and to a much lesser extent *via* food and water (Jeong *et al.*, 2000; Siu *et al.*, 2009). Acute and chronic exposure to cadmium lead to renal tubular damage (Fukumoto *et al.*, 2001). The liver is also a major target for cadmium toxicity, whereby chronic liver toxicity is manifested as granulomatous inflammation, cell proliferation, nodular hyperplasia and apoptosis (Jeong *et al.*, 2000; Waalkes, 2000). Upon administration of cadmium chloride to male mice, a time- and concentration-dependent reduction of GJIC in the liver was observed, which was associated with decreased Cx26 and Cx32 immunoreactivities (Jeong *et al.*, 2000). Cadmium chloride also negatively affected dye coupling, reduced the number of gap junctions and induced cell proliferation in a liver-based cell line (Jeon *et al.*, 2001).

Phthalates

Phthalates are a group of esters of phthalic acid that are used worldwide, mainly as plasticizers to soften polyvinylchloride in a variety of commercial products. Since the phthalates are not chemically bound to polyvinylchloride, they can freely migrate into food or evaporate into air. Human exposure to phthalates occurs through ingestion, inhalation and dermal exposure during the whole lifetime (Heudorf *et al.*, 2007). Phthalates, such as di-2-ethyl hexyl phthalate, are known reproductive and developmental toxicants in animals and suspected endocrine disruptors in humans, by abolishing androgenic action (David, 2006; Heudorf *et al.*, 2007; Hu *et al.*, 2009). They also act as peroxisome proliferators and increase cellular proliferation, as well as the incidence of hepatocellular adenomas in mice and rats (Corton and Lapinskas, 2005; David, 2006; Rusyn *et al.*, 2006). Kamendulis and colleagues tested a set of 8 phthalates and found that GJIC was significantly reduced in cultures of primary hepatocytes from mouse and rat, but not from hamster, cynomolgus and human (Kamendulis *et al.*, 2002). Similar observations were reported in *in vivo* studies (Isenberg *et al.*, 2000, 2001; Smith *et al.*, 2000). Thus, the inhibitory effects of phthalates on GJIC are strictly species-specific and may actually not be relevant for human beings (McKee, 2000).

Conclusions and perspectives

Gap junctions are essential effectors of hepatocellular collaboration, as they foresee a direct pathway for intercellular communication. The establishment of a well-orchestrated GJIC network between hepatocytes has been demonstrated numerous times as a prerequisite for the appropriate performance of hepatic functionality (Nelles *et al.*, 1996; Stumpel *et al.*, 1998; Nathanson *et al.*, 1999; Hamilton *et al.*, 2001; Temme *et al.*, 2001; Bode *et al.*, 2002; Yang *et al.*, 2003). In addition, gap junctions act as major gatekeepers in the control of liver cell death (Wilson *et al.*, 2000) and proliferation (Yee and Revel, 1978; Meyer *et al.*, 1981; Traub *et al.*, 1983; Dermietzel *et al.*, 1987; Sugiyama and Ohta, 1990; Miyashita *et al.*, 1991; Kren *et al.*, 1993; Fladmark *et al.*, 1997; Temme *et al.*, 2000a; Kojima *et al.*, 2001a; 2003). It is therefore not astonishing that gap junctions are frequently involved during disturbance of hepatic homeostasis, such as in the case of hepatotoxicity and hepatocarcinogenicity (Figure 2). Indeed, Cx32 dominant-negative mutant transgenic rats were reported to be resistant to hepatic damage induced by chemicals like carbon tetrachloride (Asamoto *et al.*, 2004). Likewise, Cx32 knock-out mice displayed lack of promotion of hepatocarcinogenesis by phenobarbital (Moennikes *et al.*, 2000; Luebeck *et al.*, 2005; Stahl *et al.*, 2005) and Wy-14,643 (Moennikes *et al.*, 2003). Although controversy exists (Ott *et al.*, 2006), however, most evidence points to a rather defensive function for hepatic gap junctions, particularly those composed of Cx32 (Temme *et al.*, 1997; Dagli *et al.*, 2004; King and Lampe, 2004; King *et al.*, 2005; Hokaiwado *et al.*, 2005; 2007;

Gotow *et al.*, 2008). For instance, a high incidence of both spontaneous and chemically induced liver tumors was observed in mice deficient for Cx32 (Temme *et al.*, 1997; Dagli *et al.*, 2004). The concept of a cytoprotective role for gap junctions is further supported by the abundant number of reports that describe disruption of GJIC by hepatotoxicants and hepatocarcinogens, both *in vitro* and *in vivo*.

In the current paper, the effects of the most relevant and best-studied chemical and biological toxic compounds on hepatic gap junction functionality have been discussed, including environmental pollutants, biological toxins, organic solvents, pesticides, pharmaceuticals and a heterogeneous group of peroxides, metals and phthalates (Table 1). Clearly, this list is not exhaustive, since additional though less investigated chemicals like chlorohydroxyfuranones (Hakulinen *et al.*, 2006) and chlorinated paraffins (Kato and Kenne, 1996) have also been reported to negatively affect hepatic GJIC. In the vast majority of the cases discussed, inhibition of GJIC is an early event, occurring before the actual onset of toxicity. It is, however, not always entirely clear how decreased GJIC subsequently leads to cytotoxicity. With respect to the molecular mechanisms that underlie the abrogation of GJIC, most of the effects triggered by the hepatotoxicants and hepatocarcinogens are targeted towards translational and posttranslational control, but do not involve the most upper regulatory levels of connexin expression. Furthermore, their deleterious outcome on gap junction production and functioning is frequently manifested in a species-specific and tissue-specific manner. Such specificity in performing detrimental cellular actions as well as the lack of causing direct DNA damage are typical features of nongenotoxic carcinogenicity. Many of the chemical and biological compounds that suppress hepatic gap junction functioning are indeed tumor promoters or epigenetic carcinogens. As a matter of fact, inhibition of GJIC may represent an interesting biomarker for the detection of nongenotoxic carcinogens in general (Ruch and Klaunig, 1986; Budunova and Williams, 1994; Mesnil *et al.*, 1995; Combes, 2000; Mally and Chipman, 2002; Cowles *et al.*, 2007). This may be challenging from an experimental toxicologist's perspective, since no validated *in vitro* assays are currently available for the testing of nongenotoxic carcinogenicity. When developing such *in vitro* screens, care should be taken while selecting the cellular system. Thus, to allow reliable detection of nongenotoxic hepatocarcinogens, the liver-based *in vitro* model must exhibit the *in vivo*-like hepatic connexin expression pattern. For instance, nontumorigenic rat liver epithelial WB-F344 cells, frequently used to study nongenotoxic hepatocarcinogenicity with respect to GJIC inhibition, intensively express Cx43 but not Cx32, which is in sharp contrast to the hepatic *in vivo* situation (Neveu *et al.*, 1994c; Rae *et al.*, 1998). Another crucial parameter includes the metabolic competence of the selected *in vitro* system. As outlined in this paper, several compounds rely on biotransformation in order to perform their harmful effects on GJIC. Human hepatoma-derived HepG2 cells, another regularly applied cell line for *in vitro* toxicological purposes, lacks expression of many CYP450 isoenzymes, which are abundantly found in the liver *in vivo* (Elaut *et al.*, 2006; Vinken *et al.*, 2006a). Primary hepatocytes may here be a better option, since they possess sufficient biotransformation capacity, at least during short-term cultivation regimes (Elaut *et al.*, 2006). Moreover, upon provision of appropriate culture conditions, Cx32 is strongly expressed and GJIC can be measured at an acceptable level for extended periods in this *in vitro* setting (Vinken *et al.*, 2006a). Hence, a primary hepatocyte culture system may currently be the best *in vitro* model to establish a hepatic GJIC inhibition assay. Another advantage is its compatibility with most currently applied GJIC methods, though dye coupling assays could be preferred, especially when intending routine use (Yamasaki, 1997).

In a first instance, a well-developed and validated hepatic GJIC inhibition assay could serve regulatory nongenotoxic carcinogenicity testing. It has been recommended to implement the GJIC inhibition method in a standard battery together with assays that detect alternative nongenotoxic endpoints (Blaha *et al.*, 2002), a strategy that is widely followed for genotoxicity testing (Ellinger-Ziegelbauer *et al.*, 2009). The GJIC inhibition approach has also been shown effective for the toxicological evaluation of complex mixtures, like coal tar (Reeves *et al.*, 2001) and cigarette smoke (van der Zandt *et al.*, 1990; McKarns *et al.*, 2000; Upham *et al.*, 2008), whether or not in combination with other toxicity tests (Reeves *et al.*, 2001). The outcome of the GJIC inhibition test not only is of relevance for the detection and prediction of nongenotoxic (hepato)carcinogenicity as such, but can also form the basis for the toxicological ranking of compounds. This idea has been elegantly exemplified for PAHs, whereby arbitrary hepatic GJIC inhibition equivalency factors were calculated based on the ratio of the experimentally assessed half maximal inhibitory concentration value of the reference PAH benzo[*a*]pyrene to that of individual PAHs (Blaha *et al.*, 2002).

In conclusion, inhibition of GJIC can be considered as a reliable toxicological marker, in particular in the context of nongenotoxic (hepato)carcinogenicity testing. Further efforts should be focused on the optimization and standardization of test conditions before a solid GJIC inhibition assay for routine use can be delivered. It can be expected that the resulting validated *in vitro*

GJIC inhibition test will be a valuable tool, with clear-cut *in vivo* relevance, for the evaluation of the hazardous potential of chemical compounds during the process of risk assessment.

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