Chemico-Biological Interactions xxx (2009) xxx–xxx

Contents lists available at ScienceDirect

Chemico-Biological Interactions

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

Gap junction intercellular communication and benzene toxicity

Edgar Rivedal[a,*], Gisela Witz[b], Edward Leithe[a]

[a] Department of Cancer Prevention, Institute for Cancer Research, Norwegian Radium Hospital, Oslo University Hospital and Centre for Cancer Biomedicine, University of Oslo, Oslo, Norway
[b] Department of Environmental and Occupational Medicine, Robert Wood Johnson Medical School/UMDNJ, USA

ARTICLE INFO

Article history:
Available online xxx

Keywords:
Benzene
Muconaldehyde
Cancer
Hematopoiesis
Gap junctions
Connexin43

ABSTRACT

Aberrant regulation of gap junction intercellular communication (GJIC) has been linked to several human diseases, including cancer and abnormal hematopoietic development. Benzene exposure has been shown to cause hematotoxicity and leukemia, but the underlying mechanisms involved remain unclear. We have observed that several metabolites of benzene have the ability to block gap junction intercellular communication. The ring-opened *trans,trans*-muconaldehyde (MUC) was found to be the most potent inhibitor of gap junction channels. MUC was found to induce cross-linking of the gap junction protein connexin43, which seemed to be responsible for the induced inhibition of GJIC. Glutaraldehyde, which has a similar molecular structure as MUC, was found to possess similar effects on gap junctions as MUC, while the mono-aldehyde formaldehyde shows lower potency, both as a connexin cross-linker, and as an inhibitor of GJIC. Both glutaraldehyde and formaldehyde have previously been associated with induction of leukemia and disturbance of hematopoiesis. Taken together, the data support a possible link between the effect of MUC on gap junctions, and the toxic effects of benzene.

1. Introduction

Gap junctions are specialized plasma membrane domains enriched in intercellular channels that provide for transport of ions, metabolites and cell signalling molecules between adjacent cells [1]. The gap junction channels consist of transmembrane proteins called connexins and have been shown to play an important role in maintaining normal cell growth and tissue homeostasis [2–4]. Aberrant regulation of GJIC has been associated with several human pathological conditions [5,6], and compounds with the ability to block gap junction intercellular communication (GJIC) have been linked to cancer induction, as well as interference with normal hematopoietic development [7–12].

The best-studied connexin isoform, connexin43 (Cx43), is ubiquitously expressed in various tissues and is involved in bone marrow hematopoiesis [13]. Reduced Cx43 expression during embryogenesis has been shown to compromise normal progression of T and B lymphopoiesis [14]. Gap junction dependent interaction with stromal cells in the bone marrow and thymus seems necessary for normal hematopoietic development [15], and inhibitors of GJIC have been found to interfere with such normal regulation [16–18].

The toxicity and cancer-causing abilities of benzene were recognized many years ago [19,20]. The underlying mechanisms are, however, poorly understood, although metabolism has been shown to be required for the induced toxicological effects. Chromosomal aberrations [21–24], sister chromatid exchange [25], micronuclei formation [26,27], chromosomal loss and DNA strand breaks [28,29] have been observed, but the role of direct DNA reactivity, DNA adducts and mutations in benzene toxicity is still questioned [30].

The ring-opened six-carbon benzene metabolite *trans,trans*-muconaldehyde (MUC) has been shown to cause bone marrow depression [31] and reduced erythropoiesis in mice [32], and is one of the most potent bone marrow depressive benzene metabolites. We have observed that MUC is a potent inhibitor of gap junction channels and studied its underlying mechanisms of action [33,34]. MUC-induced inhibition of GJIC is associated with loss of Cx43 visualized by Western blotting, mediated through its ability as a protein cross-linker. The ability of benzene metabolites to interfere with gap junction functionality could be involved in benzene-induced hematotoxicity and leukemia.

2. Materials and methods

2.1. Cells

The rat liver epithelial cell line IAR20 obtained from International Agency for Research on Cancer, Lyon, France were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The cells are well coupled by connexin43-containing gap junctions.

* Corresponding author. Tel.: +47 22 78 17 18.
E-mail address: edgarr@rr-research.no (E. Rivedal).

0009-2797/$ – see front matter © 2009 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.cbi.2009.11.013

2.2. Chemicals

MUC was obtained from Calbiochem (San Diego, CA, USA), benzene and formaldehyde from Merck (Darmstadt, Germany), chlordane from Sulpelco (Bellefonte, PA, USA), and glutaraldehyde and TPA from Sigma. The chemicals were dissolved in DMSO.

2.3. Measurement of gap junction intercellular communication

GJIC was determined by scrape loading of Lucifer yellow (LY) and quantitative determination of dye spreading by image analysis [35]. 10^6 IAR20 cells were plated onto 60 mm Petri-dishes (Costar, Cambridge, MA, USA). Two days later the dishes were washed with PBS and 0.05% (w/v) LY dissolved in PBS was added prior to cutting the monolayer with a surgical scalpel and incubating 3.5 min at room temperature. The dye was removed, cells rinsed and fixed in 4% formalin. Digital mages were acquired and analysed for Lucifer yellow diffusion.

2.4. Western blotting

Cells were seeded and treated as described in the cell communication experiments. Following exposure as indicated the cells were scraped into 500 μl SDS electrophoresis sample buffer (10 mM Tris pH 6.8, 15% (w/v) glycerol, 3% (w/v) SDS, 0.01% (w/v) bromophenol blue and 5% (v/v) 2-mercaptoethanol), sonicated, heated at 95 °C and loaded on SDS-polyacryamide gels. Following electrophoresis protein was electro blotted onto nitrocellulose membranes and incubated with the relevant antibodies.

3. Results

We have shown that several metabolites of benzene block GJIC [33]. Benzene itself had no effect on GJIC even at very high concentrations. The most potent benzene metabolite, MUC, blocks GJIC with increasing effect with time up to 5 h. This is different from what is observed for many other GJIC inhibiting compounds such as the phorbol ester TPA and chlordane, which induce complete block in cell communication via gap junctions after a few minutes [36].

Exposure to communication inhibiting concentrations of MUC for more than 1 h results in the disappearance of Cx43 on Western blots [33]. As shown in Western blots in Fig. 1A, Cx43 disappears after exposure to 10 μM or higher concentrations of MUC after 5 h exposure. Slot blot analysis revealed however no loss (data not shown).

We have previously shown that TPA as well as epidermal growth factor (EGF) induce internalization and degradation of Cx43, in a process involving Cx43 ubiquitination [37,38]. Our data suggest, however, that the loss of Cx43 in MUC-exposed cells is mediated through a different mechanism. One explanation is that MUC exposure causes cross-linking of Cx43. MUC has previously been shown to function as a cross-linking agent [39], and this could explain why Cx43 from MUC-exposed cells did not appear in the normal position in homogeneous SDS poly-acrylamide gels with stacking gel. When samples were run on gradient poly-acrylamide gels without stacking gel, Cx43 bands with molecular weights of approximately 80 and 120 kD appeared (Fig. 1D), suggesting cross-linking of Cx43.

MUC and glutaraldehyde are dialdehydes with similar molecular structure, MUC being one carbon atom longer (Fig. 1B). Due to more double bonds MUC is also conformationally restrained compared to glutaraldehyde, which could have an impact on the efficiency of connexin cross-linking. We therefore tested the effect of glutaraldehyde on gap junctions and observed that it induced the same disappearance of Cx43 in Western blots as shown for MUC

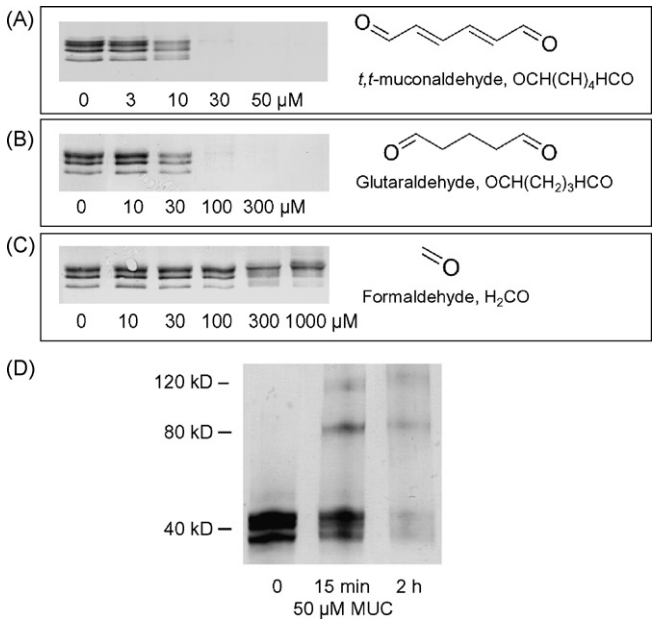

Fig. 1. Concentration-dependent effect of MUC (A), glutaraldehyde (B) and formaldehyde (C) on Cx43 in Western blot after 5 h exposure of IAR20 cells. (D) Western blotting of Cx43 in gradient SDS poly-acrylamide gel. Exposure to 50 μM MUC resulted in bands with 2× and 3× increased molecular weight, suggesting cross-linking of Cx43.

(Fig. 1B) with approximately three times lower potency; i.e. three times higher concentration was required to induce the same effect as for MUC.

Formaldehyde is a short one-carbon mono-aldehyde, known to cross-link via CH_2 bridges. Short cross-linking distance may prohibit cross-linking between individual Cx43 molecules and explain why Cx43 does not disappear from the Western blots in formaldehyde-exposed cells (Fig. 1C).

The effect of the three aldehydes on GJIC is shown in Fig. 2. Glutaraldehyde completely blocks GJIC at concentrations where Cx43 disappears on Western blots and 50% at about 50 μM, while MUC was about three times more potent. This difference in potency is in concordance with the difference in ability to cross-link Cx43 as judged from the Western blots in Fig. 1. Formaldehyde was also far less effective than MUC and glutaraldehyde in inhibiting GJIC, and only 20–30% inhibition was observed at the most effective concentrations (Fig. 2).

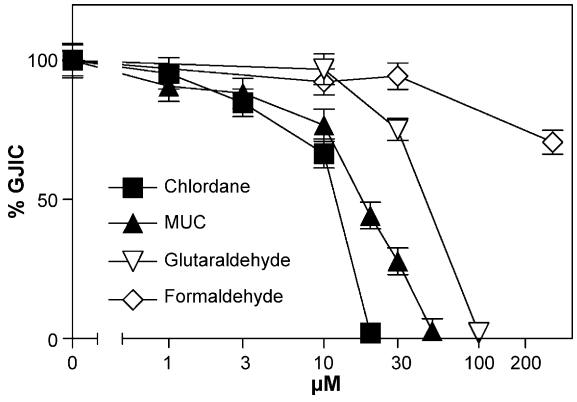

Fig. 2. Effect of exposure to different concentrations of MUC (5 h), glutaraldehyde (2 h), formaldehyde (2 h) and chlordane (1 h) on GJIC in IAR20 cells. Mean ± SD, $n = 15$.

Please cite this article in press as: E. Rivedal, et al., Gap junction intercellular communication and benzene toxicity, Chem. Biol. Interact. (2009), doi:10.1016/j.cbi.2009.11.013

4. Discussion

Benzene causes myelodysplastic syndrome, aplastic anaemia, and acute myelogenous leukaemia [17,18]. The mechanism underlying these effects is however poorly understood although previous studies have focused on chromosomal effects [20,40], DNA-protein cross-links and DNA strand breaks [39]. We have shown that some benzene metabolites are potent inhibitors of gap junction intercellular communication [33]. The strongest GJIC inhibitor of the benzene metabolites is the dialdehyde *trans,trans*-muconaldehyde. The mono-aldehyde benzene metabolites, 6-hydroxy-*trans,trans*-2,4-hexadienal and 6-oxo-*trans,trans*-2,4-hexadienoic acid, are weaker inhibitors of GJIC, while *trans,trans*-muconic acid has no inhibitory effect [41]. A similar order of potency has been shown for hematotoxic effects in mice [42].

Loss of GJIC has under different circumstances been related to downregulated transcription or post-translational modification of connexins. A number of kinases, including MAP kinase and Protein kinase C have been shown to inhibit GJIC through direct phosphorylation of Cx43.

Our data suggest that the apparent loss of Cx43 observed in Western blots is caused by cross-linking of Cx43. Immunohistochemistry has shown that remaining Cx43 after MUC exposure is localized in the plasma membrane [33], differently from what is observed after exposure to TPA, where the Cx43 plaques are endocytosed [43]. We have previously shown that TPA-induced internalization and degradation of Cx43 involves a phosphorylation- and ubiquitination-dependent mechanism [37]. MUC seems not to have this capability due to cross-linking in the plasma membrane. The slow removal of plasma membrane-associated Cx43 plaques in MUC-exposed cells may suggest that such plaques are more prone to being cross-linked than intracellular Cx43. Glutaraldehyde, as well as MUC are potent activators of ERK1/2, but it seems that Cx43 phosphorylation is of lesser importance than Cx43 cross-linking for their induced inhibition of GJIC [34]. Formaldehyde, being a mono-aldehyde, is inefficient as a cross-linker of Cx43 and far less efficient as inhibitor of GJIC. The major inhibitory effect of formaldehyde on GJIC seems to be related to activation of ERK1/2 [34].

Glutaraldehyde has been extensively tested for toxicological effects and conflicting data is reported regarding its genetic activity [44]. Bone marrow hyperplasia and enhanced levels of leukemia were observed in a chronic drinking water study in rats [45]. This is of interest since this is similar to the effects observed after benzene exposure, and highlights the possible role of MUC in benzene toxicity.

Formaldehyde is classified as Group 1 carcinogen by IARC [46,47] based on the finding of enhanced levels of different types of cancer in exposed humans, including nasopharyngeal cancer, leukemia and sinonasal cancer. Inhalation studies in rats have shown induction of squamous carcinomas of the nasal cavities. When exposed to formaldehyde in drinking water, forestomach and gastrointestinal tumors were observed, in addition to lymphomas and leukemias. Co-exposure with different model carcinogens resulted in enhanced levels of different tumors, indicating co-carcinogenic or tumor promoting effects of formaldehyde [46,47].

It is difficult to compare doses of reactive compounds used in *in vitro* cell assays with the relevant *in vivo* situation. Aldehydes and other reactive substances will be able to reach other cellular targets when being formed through metabolism, such as acetaldehyde through the metabolism of ethanol, or muconaldehyde from benzene.

The presented data may suggest that effects of MUC on gap junction function, caused by cross-linking of connexin, could be involved in the hematotoxic effects of benzene.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

We would like to thank Astri Nordahl for excellent technical assistance. The work is supported by the Norwegian Cancer Society.

References

[1] C.J. Wei, X. Xu, C.W. Lo, Connexins and cell signaling in development and disease, Annu. Rev. Cell Dev. Biol. 20 (2004) 811–838.
[2] H. Yamasaki, C.C. Naus, Role of connexin genes in growth control, Carcinogenesis 17 (1996) 1199–1213.
[3] E. McLachlan, Q. Shao, H.L. Wang, S. Langlois, D.W. Laird, Connexins act as tumor suppressors in three-dimensional mammary cell organoids by regulating differentiation and angiogenesis, Cancer Res. 66 (2006) 9886–9894.
[4] G.S. Goldberg, J.F. Bechberger, Y. Tajima, M. Merritt, Y. Omori, M.A. Gawinowicz, R. Narayanan, Y. Tan, Y. Sanai, H. Yamasaki, C.C. Naus, H. Tsuda, B.J. Nicholson, Connexin43 suppresses MFG-E8 while inducing contact growth inhibition of glioma cells, Cancer Res. 60 (2000) 6018–6026.
[5] M. Mesnil, S. Crespin, J.L. Avanzo, M.L. Zaidan-Dagli, Defective gap junctional intercellular communication in the carcinogenic process, Biochim. Biophys. Acta 1719 (2005) 125–145.
[6] E.H. Chang, G. Van Camp, R.J. Smith, The role of connexins in human disease, Ear Hear. 24 (2003) 314–323.
[7] H.S. Rosenkranz, N. Pollack, A.R. Cunningham, Exploring the relationship between the inhibition of gap junctional intercellular communication and other biological phenomena, Carcinogenesis 21 (2000) 1007–1011.
[8] L. Blaha, P. Kapplova, J. Vondracek, B. Upham, M. Machala, Inhibition of gap-junctional intercellular communication by environmentally occurring polycyclic aromatic hydrocarbons, Toxicol. Sci. 65 (2002) 43–51.
[9] M. Machala, L. Blaha, J. Vondracek, J.E. Trosko, J. Scott, B.L. Upham, Inhibition of gap junctional intercellular communication by noncoplanar polychlorinated biphenyls: inhibitory potencies and screening for potential mode(s) of action, Toxicol. Sci. 76 (2003) 102–111.
[10] S.H. Swierenga, H. Yamasaki, C. Piccoli, L. Robertson, L. Bourgon, N. Marceau, D.J. Fitzgerald, Effects on intercellular communication in human keratinocytes and liver-derived cells of polychlorinated biphenyl congeners with differing in vivo promotion activities, Carcinogenesis 11 (1990) 921–926.
[11] E. Rivedal, S.O. Mikalsen, T. Sanner, Morphological transformation and effect on gap junction intercellular communication in Syrian hamster embryo cells as screening tests for carcinogens devoid of mutagenic activity, Toxicol. In Vitro 14 (2000) 185–192.
[12] E. Rivedal, O. Myhre, T. Sanner, I. Eide, Supplemental role of the Ames mutation assay and gap junction intercellular communication in studies of possible carcinogenic compounds from diesel exhaust particles, Arch. Toxicol. 77 (2003) 533–542.
[13] B. Foss, T. Hervig, O. Bruserud, Connexins are active participants of hematopoietic stem cell regulation, Stem Cells Dev. 18 (2009) 807–812.
[14] E. Montecino-Rodriguez, H. Leathers, K. Dorshkind, Expression of connexin 43 (Cx43) is critical for normal hematopoiesis, Blood 96 (2000) 917–924.
[15] E. Montecino-Rodriguez, K. Dorshkind, Regulation of hematopoiesis by gap junction-mediated intercellular communication, J. Leukoc. Biol. 70 (2001) 341–347.
[16] R.E. Ploemacher, A.E. Mayen, A.E. de Koning, T. Krenacs, M. Rosendaal, Hematopoiesis:, Gap junction intercellular communication is likely to be involved in regulation of stroma-dependent proliferation of hemopoietic stem cells, Hematology 5 (2000) 133–147.
[17] E.C. Vigliani, A. Forni, Benzene and leukemia, Environ. Res. 11 (1976) 122–127.
[18] B.D. Goldstein, Benzene toxicity: a critical evaluation: hematotoxicity in humans, J. Toxicol. Environ. Health Suppl. 2 (1977) 69–105.
[19] B.T. Golding, W.P. Watson, Possible mechanisms of carcinogenesis after exposure to benzene, IARC Sci. Publ. (1999) 75–88.
[20] R. Snyder, Benzene and leukemia, Crit. Rev. Toxicol. 32 (2002) 155–210.
[21] M.T. Smith, L. Zhang, Biomarkers of leukemia risk: benzene as a model, Environ. Health Perspect. 106 (Suppl. 4) (1998) 937–946.
[22] L. Zhang, N. Rothman, Y. Wang, R.B. Hayes, G. Li, M. Dosemeci, S. Yin, P. Kolachana, N. Titenko-Holland, M.T. Smith, Increased aneusomy and long arm deletion of chromosomes 5 and 7 in the lymphocytes of Chinese workers exposed to benzene, Carcinogenesis 19 (1998) 1955–1961.
[23] L. Zhang, Y. Wang, N. Shang, M.T. Smith, Benzene metabolites induce the loss and long arm deletion of chromosomes 5 and 7 in human lymphocytes, Leuk. Res. 22 (1998) 105–113.
[24] I.M. Tough, W.M. Brown, Chromosome aberrations and exposure to ambient benzene, Lancet 10 (1965) 684–685.
[25] R.R. Tice, D.L. Costa, R.T. Drew, Cytogenetic effects of inhaled benzene in murine bone marrow: induction of sister chromatid exchanges, chromosomal aberrations, and cellular proliferation inhibition in DBA/2 mice, Proc. Natl. Acad. Sci. U.S.A. 77 (1980) 2148–2152.
[26] M. Hite, M. Pecharo, I. Smith, S. Thornton, The effect of benzene in the micronucleus test, Mutat. Res. 77 (1980) 149–155.

Please cite this article in press as: E. Rivedal, et al., Gap junction intercellular communication and benzene toxicity, Chem. Biol. Interact. (2009), doi:10.1016/j.cbi.2009.11.013

[27] G.L. Erexson, J.L. Wilmer, W.H. Steinhagen, A.D. Kligerman, Induction of cytogenetic damage in rodents after short-term inhalation of benzene, Environ. Mutagen. 8 (1986) 29–40.
[28] U. Plappert, E. Barthel, K. Raddatz, H.J. Seidel, Early effects of benzene exposure in mice. Hematological versus genotoxic effects, Arch. Toxicol. 68 (1994) 284–290.
[29] H. Chen, D.A. Eastmond, Synergistic increase in chromosomal breakage within the euchromatin induced by an interaction of the benzene metabolites phenol and hydroquinone in mice, Carcinogenesis 16 (1995) 1963–1969.
[30] J. Whysner, M.V. Reddy, P.M. Ross, M. Mohan, E.A. Lax, Genotoxicity of benzene and its metabolites, Mutat. Res. 566 (2004) 99–130.
[31] G. Witz, G.S. Rao, B.D. Goldstein, Short-term toxicity of trans,trans-muconaldehyde, Toxicol. Appl. Pharmacol. 80 (1985) 511–516.
[32] R. Snyder, E. Dimitriadis, R. Guy, P. Hu, K. Cooper, H. Bauer, G. Witz, B.D. Goldstein, Studies on the mechanism of benzene toxicity, Environ. Health Perspect. 82 (1989) 31–35.
[33] E. Rivedal, G. Witz, Metabolites of benzene are potent inhibitors of gap-junction intercellular communication, Arch. Toxicol. 79 (2005) 303–311.
[34] E. Rivedal, E. Leithe, The benzene metabolite trans,trans-muconaldehyde blocks gap junction intercellular communication by cross-linking connexin43, Toxicol. Appl. Pharmacol. 232 (2008) 463–468.
[35] H. Opsahl, E. Rivedal, Quantitative determination of gap junction intercellular communication by scrape loading and image analysis, Cell Adhes. Commun. 7 (2000) 367–375.
[36] E. Rivedal, E. Leithe, Connexin43 synthesis, phosphorylation, and degradation in regulation of transient inhibition of gap junction intercellular communication by the phorbol ester TPA in rat liver epithelial cells, Exp. Cell Res. 302 (2005) 143–152.
[37] E. Leithe, E. Rivedal, Ubiquitination and down-regulation of gap junction protein connexin-43 in response to 12-O-tetradecanoylphorbol 13-acetate treatment, J. Biol. Chem. 279 (2004) 50089–50096.
[38] E. Leithe, E. Rivedal, Epidermal growth factor regulates ubiquitination, internalization and proteasome-dependent degradation of connexin43, J. Cell Sci. 117 (2004) 1211–1220.
[39] R.P Amin, G. Witz, DNA-protein crosslink and DNA strand break formation in HL-60 cells treated with trans,trans-muconaldehyde, hydroquinone and their mixtures, Int. J. Toxicol. 20 (2001) 69–80.
[40] M.T. Smith, Mechanistic studies of benzene toxicity—implications for risk assessment, Adv. Exp. Med. Biol. 387 (1996) 259–266.
[41] E. Rivedal, G. Witz, Benzene metabolites block gap junction intercellular communication. Role in hematotoxicity and leukemia? Chem. Biol. Interact. 153–154 (2005) 257–260.
[42] Z. Zhang, S.A. Kline, T.A. Kirley, B.D. Goldstein, G. Witz, Pathways of trans,trans-muconaldehyde metabolism in mouse liver cytosol: reversibility of monoreductive metabolism and formation of end products, Arch. Toxicol. 67 (1993) 461–467.
[43] E. Rivedal, H. Yamasaki, T. Sanner, Inhibition of gap junctional intercellular communication in Syrian hamster embryo cells by TPA, retinoic acid and DDT, Carcinogenesis 15 (1994) 689–694.
[44] E. Zeiger, B. Gollapudi, P. Spencer, Genetic toxicity and carcinogenicity studies of glutaraldehyde–a review, Mutat. Res. 589 (2005) 136–151.
[45] J.P. Van Miller, S.J. Hermansky, P.E. Losco, B. Ballantyne, Chronic toxicity and oncogenicity study with glutaraldehyde dosed in the drinking water of Fischer 344 rats, Toxicology 175 (2002) 177–189.
[46] IARC, Formaldehyde, 2-butoxyethanol and 1-tert-butoxypropan-2-ol, IARC Monogr. Eval. Carcinog. Risk Hum. 88 (2006) 1–478.
[47] V.J. Cogliano, Y. Grosse, R.A. Baan, K. Straif, M.B. Secretan, G.F. El, Meeting report: summary of IARC monographs on formaldehyde, 2-butoxyethanol, and 1-tert-butoxy-2-propanol, Environ. Health Perspect. 113 (2005) 1205–1208.

Please cite this article in press as: E. Rivedal, et al., Gap junction intercellular communication and benzene toxicity, Chem. Biol. Interact. (2009), doi:10.1016/j.cbi.2009.11.013