Toxic Effects of Methylated Benzo[a]pyrenes in Rat Liver Stem-Like Cells

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Effects of methylated chrysenes on AhR-dependent and -independent toxic events in rat liver epithelial cells

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ABSTRACT

Methylated chrysenes (MeChry) are important cigarette smoke constituents and 5-MeChry has been listed as possibly carcinogenic to humans. Although a major attention has been in past paid especially to mutagenic, tumor-initiating effects of MeChry, little is known about toxic effects of MeChry related to tumor promotion. As the position of methyl group has been repeatedly observed to determine genotoxic effects of MeChry, we examined both genotoxic and nongenotoxic effects of MeChry, using rat liver cell lines as experimental models. All six MeChry were relatively efficient aryl hydrocarbon receptor (AhR) agonists, with 3- and 6-MeChry being the most potent inducers of the AhR-mediated reporter gene activity. All six compounds disrupted contact inhibition in rat liver epithelial WB-F344 cells, a process previously reported to be AhR-dependent, suggesting that MeChry may interfere with cell cycle control in an AhR-dependent manner. In contrast, only 5- and 6-MeChry were found to acutely inhibit gap junctional intercellular communication (GJIC), another parameter correlating with tumor promoting effects of xenobiotics. Both 5- and 6-MeChry were efficient inducers of mRNA expression of enzymes involved in metabolic activation of polycyclic aromatic hydrocarbons, including cytochromes P450 1A1/1B1 and aldo-keto reductase 1C9. However, only 5-MeChry, and not 6-MeChry, induced significant formation of DNA adducts in rat liver epithelial cells, which corresponded with its ability to induce high accumulation of cells in S-phase. On the other hand, 5-MeChry induced neither apoptosis related to DNA damage nor phosphorylation of p53 tumor suppressor. Taken together, our results suggest that methyl group position may affect both genotoxic and nongenotoxic effects of MeChry, such as formation of DNA adducts and inhibition of GJIC. All MeChry showed a potency to disrupt cell proliferation control, while 5-MeChry was a single compound inducing DNA damage, disruption of cell cycle control and inhibition of GJIC in rat liver cells.

1. Introduction

Alkylated polycyclic aromatic hydrocarbons (PAHs) are important constituents of complex environmental mixtures of organic contaminants (Boström et al., 2002). A particular attention has been paid to some methylated PAHs, which are carcinogenic to experimental animals and suspected human carcinogens (IARC, 1983). Among methylated chrysenes (MeChry), 5-methylchrysene (5-MeChry) has been widely studied as mutagenic, tumor-initiating compound, which is substantially more carcinogenic than chrysene itself or other MeChry isomers (Hecht et al., 1974; Hoffmann et al., 1974). Relatively high levels of MeChry are present in mainstream tobacco smoke, where their total levels may represent up to 50% of chrysene concentration (Hecht et al., 1974). Nevertheless, it has been shown that these compounds are present at significant levels also in some environmental compartments, such as freshwater sediments (Brack and Schirmer, 2003).

The tumor-initiating properties of MeChry have been studied in both mouse skin and in newborn mice, revealing that especially 5-MeChry is a potent initiating compound (Hecht et al., 1974, 1987; Hoffmann et al., 1974). 5-MeChry is also a potent lung carcinogen in strain A/J mice (You et al., 1994), and it has been found to be mutagenic in bacterial assays (Coombs et al., 1976; Cheung et al., 1993) and to form DNA adducts in mammalian cells (Melikian et al., 1991). The presence of a methyl group adjacent to the bay region in (+/−)-*anti*-5-methylchrysene-1,2-diol-3,4-epoxide (5-MCDE), the ultimate mutagenic metabolite of 5-MeChry, is thought to induce steric hindrance responsible

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for its high mutagenicity and carcinogenicity, while corresponding (+/−)-*anti*-6-methylchrysene-1,2-diol-3,4-epoxide (6-MCDE) is only weakly mutagenic in many test systems, probably due to increased repair of adducts, or increased detoxification of 6-MCDE (Amin et al., 1985; Hecht et al., 1987; Melikian et al., 1991). Thus, the position of methyl group in bay region seems to be a crucial determinant of mutagenic and genotoxic effects of 5-MeChry. However, 5-MeChry, and to a lesser extent also other MeChry, are complete carcinogens (Hecht et al., 1974; You et al., 1994) and it has been suggested that 5-MeChry or its metabolites may activate cellular signaling pathways contributing to tumor promotion (Li et al., 2004). Therefore, position of methyl group might also affect nongenotoxic effects of MeChry, which are related to tumor promotion.

The methyl group reportedly affects the ability of monomethylated PAHs to bind and activate the aryl hydrocarbon receptor (AhR), or to inhibit intercellular communication mediated by gap junctions (GJIC). The inhibition of GJIC could be closely related to tumor promotion, therefore being a parameter reflecting possible promoting activity of studied chemicals (Rosenkranz et al., 2000). The closure of gap junctions by tumor promoting chemicals may lead to a release of the cells, including genotoxically damaged cells, from the control of neighbouring cells and consequently to disruption of homeostasis and cell-to-cell communication (Trosko and Upham, 2005). Acute inhibition of GJIC does not seem to depend on AhR activation. We have recently observed that methyl substitution may differentially affect AhR activation or GJIC inhibition by a closely related group of compounds – methylated benz[*a*]anthracenes (Marvanová et al., 2008). Interestingly, 5-MeChry, and not chrysene, has been found to inhibit GJIC in rat liver epithelial cells (Bláha et al., 2002). Other methylated derivatives of PAHs found at high levels in cigarette smoke, such as methylated anthracenes, have been also shown to inhibit GJIC in a manner strictly dependent on position of methyl group (Upham et al., 1996). Therefore, we hypothesized that methyl substitution may also significantly modulate the tumor promoting effects of chrysene derivatives. However, there is currently no comprehensive information on the ability of MeChry to inhibit GJIC.

The AhR activation has been suggested, in addition to control of expression of xenobiotic metabolizing enzymes being involved in metabolic activation of mutagenic PAHs, to play a role in possible tumor promoting effects of PAHs (Sjögren et al., 1996; Boström et al., 2002). AhR plays a significant role in carcinogenic effects of complex environmental mixtures of contaminants containing high levels of PAHs, such as extracts of airborne particulate matter (Matsumoto et al., 2007). Activation of AhR by various toxicants has been shown to induce perturbations of cell signaling pathways regulating cell proliferation, differentiation and apoptosis (Bock and Köhle, 2005; Marlowe and Puga, 2005). For example, it has been shown that PAHs, as well as more persistent AhR ligands, may disrupt contact inhibition in liver epithelial cells in the AhR-dependent manner (Andrysík et al., 2007; Weiss et al., 2008). Disruption of contact inhibition is a mechanism, which has been suggested to participate in tumor promotion (Oesch et al., 1988; Dietrich et al., 2002). Therefore, different relative potencies to activate AhR might determine the ability of MeChry to induce cytochrome P450 (CYP) enzymes (Cheung et al., 1993), involved in their metabolic activation, and to modulate cell cycle control.

To study metabolic activation, genotoxic and apoptotic events and AhR-mediated effects of PAHs, various liver cells, including primary hepatocytes, hepatoma cell lines or liver epithelial cells can be used. As the rat liver epithelial WB-F344 cells, a model of liver progenitor cells (Tsao et al., 1984; Shafritz and Dabeva, 2002), have been shown to express AhR-regulated enzymes necessary for metabolic activation of PAHs, wild-type p53 tumor suppressor or to have functional GJIC (Bláha et al., 2002; Andrysík et al., 2007; Topinka et al., 2008), we selected this model to study effects of MeChry on deregulation of cell proliferation in contact-inhibited cells and inhibition of GJIC. We also used rat liver hepatoma H4IIE cells stably transfected with DRE-controlled luciferase reporter gene (Sanderson et al., 1996) to study the effects of MeChry on AhR activation. We then selected 5-MeChry and 6-MeChry as model compounds (first being potent carcinogen and the second compound being mostly inactive in various carcinogenicity models), in order to study induction of enzymes involved in metabolic activation of MeChry, formation of DNA adducts, phosphorylation of p53 tumor suppressor, deregulation of cell cycle or induction of apoptosis in rat liver epithelial WB-F344 cells.

2. Materials and methods

2.1. Chemicals

1-Methylchrysene (1-MeChry), 2-methylchrysene (2-MeChry), 3-methylchrysene (3-MeChry), 4-methylchrysene (4-MeChry), 5-methylchrysene (5-MeChry) and 6-methylchrysene (6-MeChry) were obtained from Promochem (Wesel, Germany). Dibenzo[*a*,*l*]pyrene (DBalP) was purchased from AccuStandard (New Haven, CT). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) was from Cambridge Isotope Laboratories (Andover, MA). Stock solutions were prepared in dimethyl sulfoxide (DMSO) and kept in a dark. Concentration used for dose–response studies of effects of MeChry were selected based on our previous experiments with PAHs in WB-F344 and H4IIE cells (Machala et al., 2001; Chramostová et al., 2004). Polyclonal antibody to Ser-15 phosphorylated p53 tumor suppressor was obtained from Cell Signaling Technology (Beverly, MA). Mouse monoclonal antibody to β-actin was obtained from Sigma–Aldrich. Polyvinylidene difluoride (PVDF) membrane Hybond-P, and chemiluminescence detection reagents (ECL + Plus) were purchased from Amersham (Aylesbury, UK). 4′6-Diamidino-2-phenylindole dihydrochloride (DAPI) was purchased from Fluka (Buchs, Switzerland). Spleen phosphodiesterase was purchased from ICN Biomedicals; ribonuclease A and T1, proteinase K, micrococcal nuclease, nuclease P1, and protein assay kit (No. 5656) were from Sigma–Aldrich; polyethylene-imine cellulose TLC plates (0.1 mm) from Macherey-Nagel (Düren, Germany); T4 polynucleotide kinase from USB; and γ-^{32}P-ATP (3000 Ci/mmol, 10 μCi/μl) from Amersham. All other chemicals were provided by Sigma–Aldrich.

2.2. Cells

WB-F344 rat liver epithelial cells (Tsao et al., 1984) were kindly provided by Dr. J.E. Trosko (Michigan State University, East Lansing, MI). The cells were cultured in D-MEM/F-12 Medium (Invitrogen, Carlsbad, CA), supplemented with 5% heat-inactivated fetal bovine serum as described previously (Umannová et al., 2007). Only the cells at passage levels 15–24 were used for the study. The rat hepatoma H4IIEGud.Luc1 cells (BioDetection Systems, Amsterdam, The Netherlands) were cultured in the alpha modification of Minimal Essential Medium, supplemented with 10% heat-inactivated fetal bovine serum. Both cell lines were incubated in a humidified atmosphere of 5% CO_2 at 37 °C. The cells were routinely maintained in 75 cm^2 flasks and subcultured twice a week. All tissue culture reagents were obtained from Sigma–Aldrich.

2.3. Detection of the AhR-mediated activity

The rat hepatoma H4IIEGud.Luc1.1 cell line, stably transfected with a luciferase reporter gene under the control of dioxin responsive elements (Sanderson et al., 1996), were used for estimation of the AhR-mediated activity of methylated chrysenes in the DR-CALUX® assay as described previously (Machala et al., 2001). Cells were seeded in 96-well cell culture plates and grown 24 h to 90–100% confluence. They were exposed to test or reference compounds dissolved in DMSO (maximum concentration 0.04%, v/v) for 24 h. The medium was removed, cells were washed with PBS and luciferase was extracted with the low salt lysis buffer (10 mM Tris, 2 mM DTT, 2 mM 1,2-diamin cyclic hexane-*N*,*N*,*N*′,*N*′-tetraacetic acid, pH 7.8). The plates were frozen at −80 °C and luciferase expression was then measured on a microplate luminometer using the Luciferase Monitoring Kit (Labsystems, Helsinki, Finland).

2.4. Detection of gap junctional intercellular communication (GJIC)

The modified scrape loading/dye transfer assay was performed as described previously (Bláha et al., 2002). WB-F344 cells were grown to confluence in 24-well plates and exposed to test compounds, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (20 nM, a reference inhibitor of GJIC), or DMSO for 60 min. After exposure, cells were washed twice with 0.5× PBS; fluorescent dye was added (Lucifer Yellow 0.05%, w/v

in PBS) and cells were scraped using a surgical blade. The cells were washed twice by 0.5× PBS after 4 min and then fixed with 4% formaldehyde (v/v). The dye migration was evaluated using an epifluorescence microscope and its distance from a scrape line was measured at six randomly chosen spots per scrape using Lucia image analysis software (Laboratory Imaging, Prague, Czech Republic). Three independent experiments were carried out in duplicate and at least three scrapes per well were evaluated.

2.5. Determination of cell numbers and cell cycle analysis

The analysis of cell proliferation of contact-inhibited WB-F344 cells was carried out as previously described (Švihálková-Šindlerová et al., 2007). The confluent cells were exposed to test compounds for 72 h, and medium was changed daily. Following the exposure, medium was removed, cells were harvested by trypsinization and counted with a Coulter Counter (Model ZM, Coulter Electronics, Luton, UK). Cells were then washed with phosphate-buffered saline (PBS) and fixed in 70% ethanol at 4 °C overnight. Fixed cells were washed with PBS and stained with propidium iodide (PI) as described previously (Švihálková-Šindlerová et al., 2007). Cells were then analyzed on FACSCalibur, using 488-nm (15 mW) air-cooled argon-ion laser for PI excitation, and CELLQuest software for data acquisition (Becton Dickinson, San Jose, CA). A minimum of 15,000 events was collected per sample. Data were analyzed using ModFit LT Version 2.0 software (Verity Software House, Topsham, ME).

2.6. Real-time RT-PCR

Following the 24 h exposure of cells to MeChry or control compounds, total RNA was isolated from cells using the NucleoSpin RNA II kit (Macherey-Nagel). The levels of CYP1A1, CYP1B1 and AKR1C9 mRNAs were determined by quantitative real-time RT-PCR, using primer and probe sequences published previously (Vondráček et al., 2006). The primers were designed to flank the exon junctions of the transcripts for amplification of cDNA only. The amplifications were run on the LightCycler (Roche Diagnostics GmbH, Mannheim, Germany) using the conditions described previously (Vondráček et al., 2006). All PCR reactions were performed in triplicates and changes in gene expression were calculated using the comparative threshold cycle method (Livak and Schmittgen, 2001).

2.7. Detection of DNA-PAH adducts

Cells were grown on 35-mm-diameter cell culture dishes and incubated for 24 h with test compounds or DMSO as a solvent control (0.1%). After exposure, cells were washed with cold PBS, scraped into the Eppendorf tubes, centrifuged and the cell pellets were stored at −80 °C. The cell pellets were homogenized in a solution of 20 mM Tris–HCl, 10 mM EDTA, and 0.5% SDS, pH 8.0. DNA was isolated using RNAses A and T1 and proteinase K treatment followed by phenol/chloroform/isoamylalcohol as previously described (Švihálková-Šindlerová et al., 2007). DNA concentration was estimated spectrophotometrically by measuring the UV absorbance at 260 nm. DNA samples were kept at −80 °C until analysis. ^{32}P-postlabeling analysis was performed as previously described (Švihálková-Šindlerová et al., 2007). DNA samples (6 μg) were digested by a mixture of micrococcal endonuclease and spleen phosphodiesterase for 4 h at 37 °C. Nuclease P1 was used for adduct enrichment. The labeled DNA adducts were resolved by two-directional thin layer chromatography. Autoradiography was carried out at −80 °C for 6 h. The radioactivity of distinct adduct spots was measured by liquid scintillation counting. To determine the exact amount of DNA in each sample, aliquots of DNA enzymatic digest (1 μg of DNA hydrolysate) were analyzed for nucleotide content by reverse-phase HPLC with UV detection. DNA adduct levels were expressed as adducts per 10^8 nucleotides. BPDE-DNA adduct standard derived from the liver of rats treated intraperitoneally with 100 mg benzo[*a*]pyrene was run in triplicate in each postlabelling experiment to control for interassay variability and to normalize the calculated DNA adduct levels.

2.8. Western blotting

Cells were grown on 35-mm-diameter cell culture dishes and incubated with tested compounds for 24 h. Cells were washed with PBS and lysed using 1% sodium dodecyl sulphate (SDS) lysis buffer (10% glycerol, 100 mM Tris, pH 7.4), and protein concentration was estimated using Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA). Equal amounts of protein were separated on 10% SDS-polyacrylamide gel and transferred onto a polyvinyldifluoride membrane. After incubation with primary and secondary antibodies, detection was performed using ECLPlus Western blotting detection system (GE Healthcare, Little Chalfont, UK). β-Actin detection was used to verify equal loading.

2.9. Detection of apoptosis

The apoptosis detection was based on morphological criteria (fragmentation of nuclei) as described previously (Chramostová et al., 2004). Briefly, the cell incubated with the test compounds as described above were fixed and stained with DAPI (1 μg/ml methanol) for 30 min at room temperature. After the incubation, the cells were centrifuged and mounted in Mowiol solution (10% Mowiol 4-88 was prepared in 25% glycerol, 100 mM Tris–HCl, pH 8.5). The slides were evaluated with a fluorescence microscope Olympus IX70 (Tokyo, Japan). A minimum of 200 DAPI-stained nuclei were counted per sample.

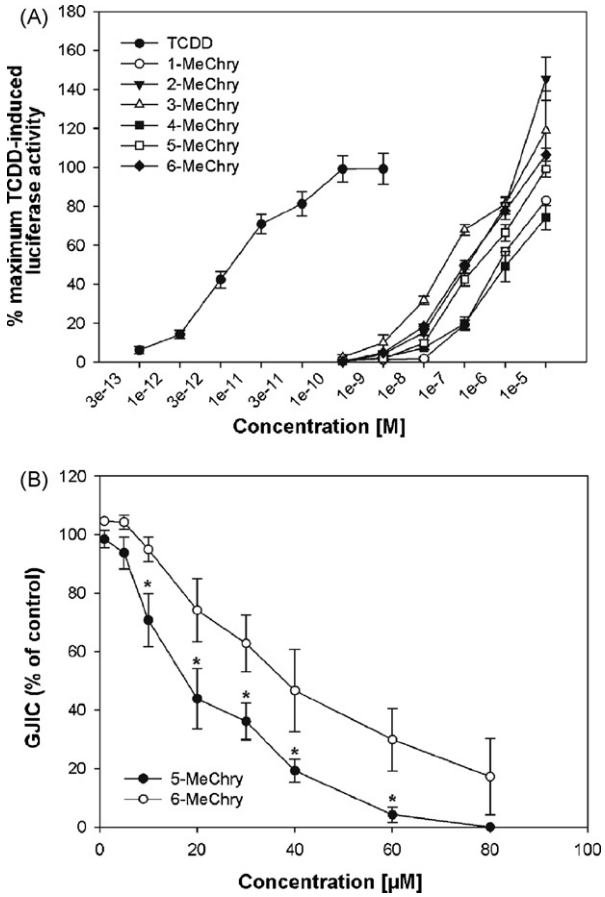

Fig. 1. Effects of methylated chrysenes on AhR activation and GJIC inhibition. (A) AhR-mediated induction of luciferase reporter gene in H4IIEpGudLuc1.1 rat hepatoma cell line by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (positive control) and methylated chrysenes after 24-h exposure. The data shown here are representative of means ± S.D. of three independent experiments. (B) Inhibition of gap junctional intercellular communication (GJIC) induced by 5- and 6-MeChry in WB-F344 cells, as determined by modified scrape loading/dye transfer method using the Lucifer Yellow fluorescent dye after 60 min exposure to tested compounds. The results represent means ± S.D. of three independent experiments. *A significant difference between dye migration in cells treated with 5-MeChry and 6-MeChry ($p < 0.05$).

2.10. Statistical analysis

For each compound tested, relative AhR-inducing potencies were defined as the ability to induce luciferase activity using concentration–response curves. Their relative induction equivalency factors (IEFs) were estimated as described previously (Machala et al., 2001). The ratio of GJIC inhibition related to the negative control was evaluated and expressed in percentage (fraction of control) (Bláha et al., 2002). Differences between individual compounds were evaluated using Mann–Whitney *U*-test. Proliferation data were expressed as means ± S.D. and analyzed by ANOVA followed by Tukey range test (cell numbers) or Student's *t*-test (percentage of cells in S-phase). When the variances were not homogenous, non-parametric Mann–Whitney *U*-test was used. A *p*-value of less than 0.05 was considered significant.

3. Results

3.1. Effects of MeChry on AhR activation and GJIC inhibition

We first examined the ability of MeChry to activate AhR or to inhibit GJIC using rat hepatoma H4IIE cells stably transfected

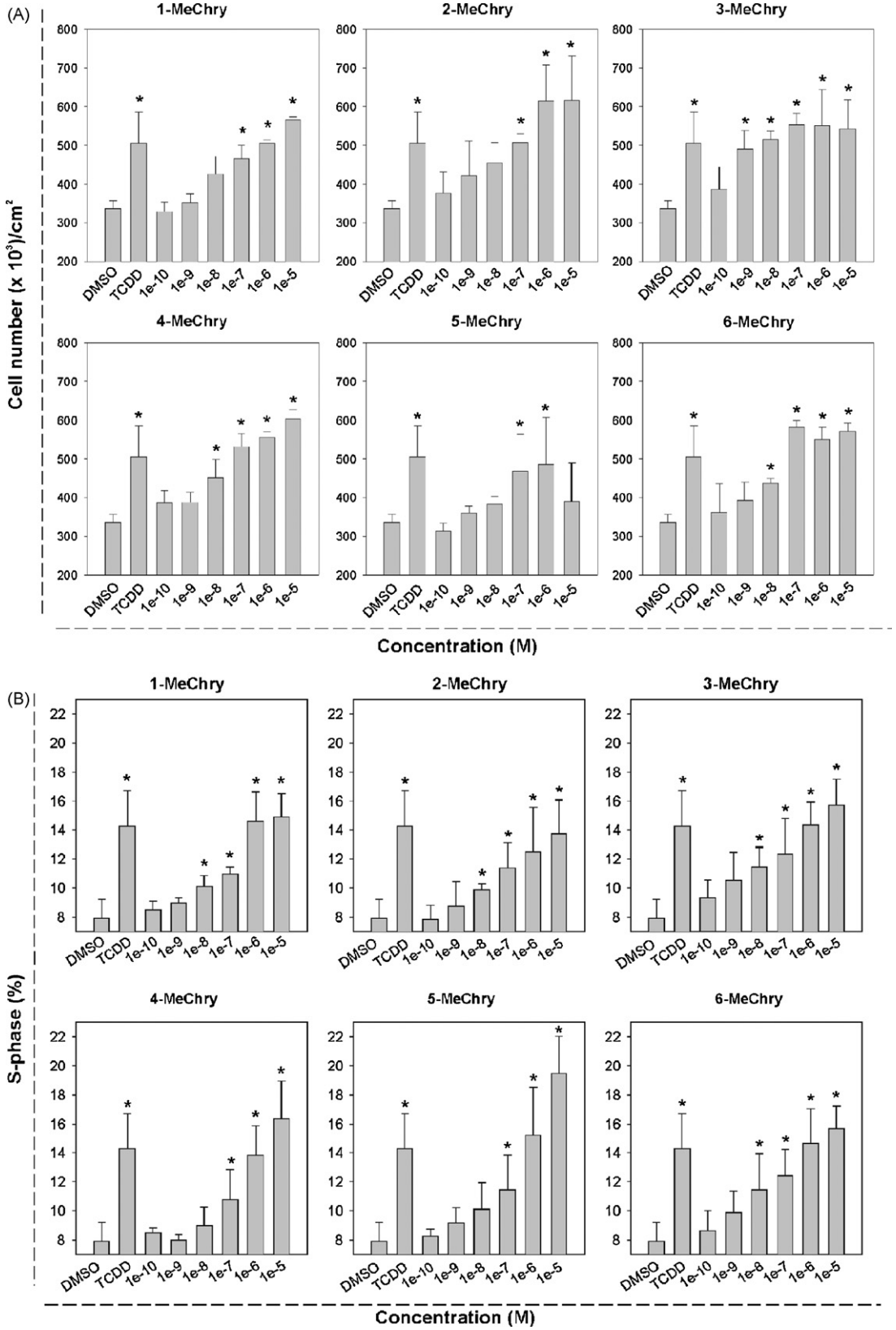

Fig. 2. Modulation of cell proliferation by methylated chrysenes in WB-F344 cells, assessed by counting cell numbers (A) and measuring percentage of cells in S-phase of cell cycle (B). Cells were cultured for 72 h prior to exposition, and then treated for another 72 h with test compounds at concentration range 100 pM to 10 μM; 5 nM TCDD: positive control; 0.1% DMSO: negative control. Cells were then trypsinized, counted and fixed in ethanol for a subsequent flow cytometric analysis of DNA content. All data are expressed as mean ± S.D. of three independent experiments ran in duplicates. *A significant difference between control (0.1% DMSO) and treated samples ($p < 0.05$).

Table 1
Effects of MeChry on AhR-medited activity and GJIC inhibition

	AhR-IEFs[a]	GJIC inhibition IC_{50}[b]
TPA	n.a.	7.6 nM
TCDD	1[a]	n.a.
1-MeChry	1.24×10^{-5}	n.i.
2-MeChry	7.73×10^{-5}	n.i.
3-MeChry	48.9×10^{-5}	n.i.
4-MeChry	8.28×10^{-5}	n.i
5-MeChry	6.38×10^{-5}	18.1 μM
6-MeChry	11.5×10^{-5}	37.0 μM

[a] Relative potencies of MeChry to activate AhR (DR-CALUX® assay) after 24 h of exposure. The numbers represent induction equivalency factors (IEFs), calculated as the ratio between the 25% effective concentration (EC25) of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and concentration of selected compound inducing 25% of maximum TCDD-induced luciferase activity.

[b] Inhibition of GJIC in WB-F344 cells after in vitro exposure to model tumor promoter phorbol-12-myristate-13-acetate (TPA) or MeChry. IC_{50} – a concentration causing 50% inhibition of GJIC after 60 min exposure calculated as average value from three independent experiments run in triplicates. n.a.: not analyzed; n.i.: not inhibiting GJIC up to concentration 80 μM.

with luciferase reporter under control of dioxin responsive elements for determination of AhR activation (DR-CALUX® assay) and rat liver epithelial cell line WB-F344 for assessment of GJIC inhibition (Lucifer Yellow dye transfer/scrape loading assay). The results are summarized in Fig. 1 and Table 1. All six MeChry induced maximum levels of luciferase activity similar to model AhR ligand TCDD as shown in Fig. 1. The weakest AhR ligand was 1-MeChry, its IEF being 1.24×10^{-5}. The IEF of the most potent AhR ligand, 3-MeChry, was 48.9×10^{-5}, more than 40 times higher. The remaining compounds had all very similar relative potencies to activate AhR, close to 1×10^{-4}. In contrast, only 5- and 6-MeChry were found to inhibit GJIC in WB-F344 cells. Both compounds were able to elicit a full inhibition of GJIC in a dose-dependent manner (Fig. 1), while 1-, 2-, 3- and 4-MeChry had no effect on GJIC at concentrations up to 80 μM. 5-MeChry was approximately twice more potent than 6-MeChry, with IC_{50} 18.1 μM (Table 1). These results confirmed that the position of methyl group is a crucial determinant of GJIC inhibitory activity of methylated PAHs.

3.2. MeChry disrupt contact inhibition in confluent WB-F344 cells

Using *in vitro* model of rat liver progenitor cells, WB-F344 cell line, it has been previously shown that PAHs may disrupt cell cycle control in the AhR-dependent manner, which leads to increased proliferative rate of contact-inhibited cells (Andrysík et al., 2007; Chramostová et al., 2004). As all studied MeChry were found to activate AhR, we next examined their ability to induce cell proliferation in contact-inhibited WB-F344 cells. As outlined in Fig. 2A, all six MeChry induced cell proliferation of contact-inhibited WB-F344 cells in a dose-dependent manner. Their proliferative effects were similar with two notable exceptions. 3-MeChry was the most potent inducer of cell proliferation, which corresponds with its high AhR-mediated activity (Table 1). In case of 5-MeChry, the proliferative effect was not significant at the highest used 10 μM concentration. This could be due to its toxic effects at higher concentrations, as will be shown below. The results of cell cycle analysis corresponded with the cell numbers data – all six studied compounds induce a significant increase of percentage of cells in S-phase in a dose-dependent manner (Fig. 2B). The highest increase in S-phase percentage was observed in case of 10 μM 5-MeChry, which might be attributed to its genotoxic properties (see below).

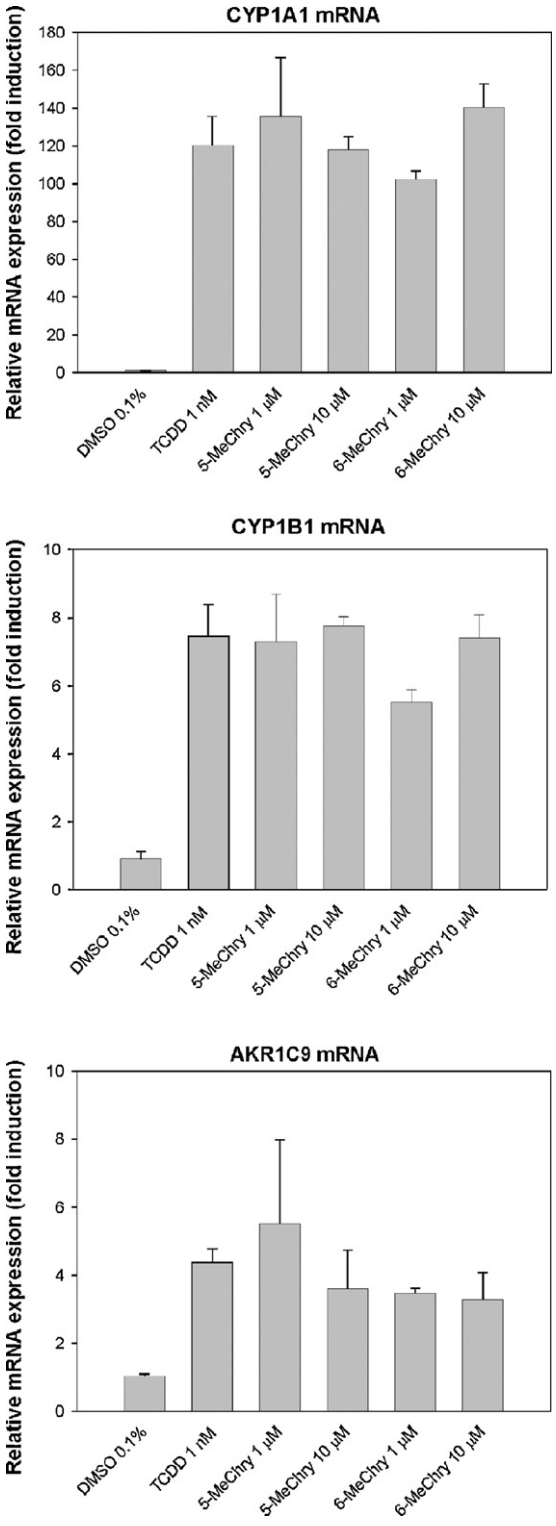

Fig. 3. Induction of CYP1A1, CYP1B1 and AKR1C9 mRNA after 24 h exposure to 5- and 6-MeChry, as compared to 1 nM TCDD. Isolation of total mRNA and quantitative real-time RT-PCR was performed as described in Section 2. The results are expressed as mean ± S.D. of three independent experiments.

3.3. Effects of 5- and 6-MeChry on induction of enzymes involved in their metabolic activation, formation of covalent DNA adducts and related genotoxic events

5-MeChry and 6-MeChry have been often used as model compounds in studies attempting to characterize the role of methyl

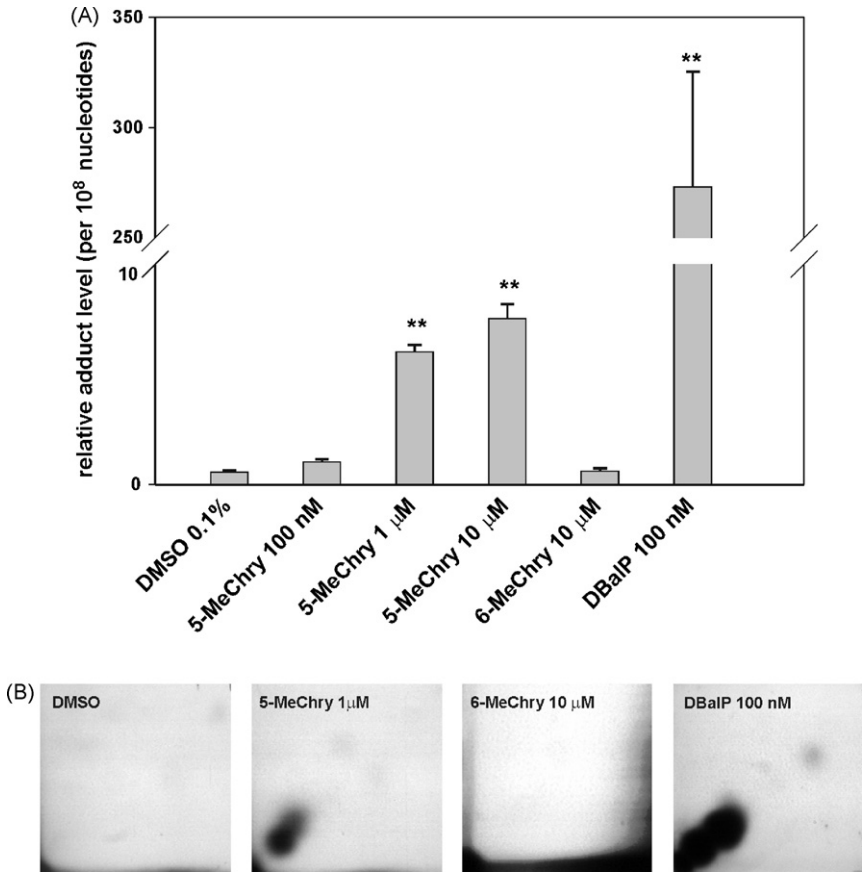

Fig. 4. 5-MeChry induces formation of DNA adducts in WB-F344 cells. (A) DNA-PAH adducts formed in WB-F344 cells as detected by ^{32}P-postlabelling. Cells were exposed to DMSO (solvent control), 5-MeChry, 6-MeChry or dibenzo[*a,l*]pyrene (DBalP-positive control) for 24 h. Total DNA adduct levels were expressed as a number of adducts per 10^8 nucleotides. All data represent means ± S.D. of three independent experiments. **A significant difference between control (0.1% DMSO) and treated samples ($p < 0.01$). (B) Representative autoradiograms of ^{32}P-labelled DNA isolated from WB-F344 cells treated with DMSO (0.1%, solvent control), 5-MeChry, 6-MeChry or DBalP (positive control) for 24 h. The duration of screen-enhanced autoradiography was 24 h and the presented autoradiograms are representative of three independent experiments.

position in carcinogenic effects of methylated chrysenes, as only one of them, 5-MeChry, is a potent chemical carcinogen in experimental animals (Hecht et al., 1987). However, a major part of these studies concentrated on skin cancer models and little is known about their genotoxic effects in other types of epithelial cells. Therefore, we next compared effects of MeChry, which could be related to their genotoxicity, in the model rat liver WB-F344 progenitor cell line.

As outlined in Fig. 3, both compounds exerted similar effects on expression of enzymes involved either in formation of ultimate mutagenic dihydrodiol epoxides – CYP1A1 and CYP1B1 – or in formation of *ortho*-quinones contributing both to direct DNA damage and oxidative stress—aldo-keto reductase 1C9 (AKR1C9) (Penning et al., 1999; Xue and Warshawsky, 2005). Their effects were similar to model AhR ligand – TCDD, thus suggesting that induction of xenobiotic metabolising enzymes does not present a limiting step explaining different toxic properties of 5- and 6-MeChry. However, when we compared their ability to form stable DNA adducts, only 5-MeChry induced formation of DNA adducts in a dose-dependent manner (Fig. 4A). The level of DNA adducts, as detected by ^{32}P-postlabelling method, in cells treated with highest used dose of 6-MeChry (10 μM) was on the background level (Fig. 4B).

We have previously shown that in WB-F344 cells, potent genotoxins, forming large amounts of DNA adducts, induce DNA-damage-related apoptosis and phosphorylation of p53 tumor suppressor in reaction to DNA lesions (Topinka et al., 2008). Therefore, we next examined effects of MeChry on these events. However, none of the compounds induced p53 phosphorylation at Ser-15 residue or apoptosis in a manner similar to DBalP, a model genotoxin (Fig. 5). The lack of effect of 5-MeChry on these endpoints might be related to relatively low levels of DNA adducts formed in WB-F344 cells by this compound. This was one or two orders of magnitude lower that in case of potent genotoxins, such as BaP, DBalP or 7,12-dimethylbenz[*a*]anthracene, in our previous studies (Marvanová et al., 2008; Topinka et al., 2008).

4. Discussion

Monomethylated chrysenes have been intensively studied for many years, in order to understand the structural requirements underlying the carcinogenicity of 5-MeChry. Based on the available data, both 5-MeChry and Chry are currently classified as Group 2B carcinogens by the IARC (IARC Monographs, vol. 92 – in preparation). Almost all studies so far concentrated on effects of 5-MeChry associated with tumor initiation, especially on its metabolism and production of mutagenic DNA-binding metabolites in few selected tissues, such as in skin. Toxic effects of all MeChry in other types of cells have so far received very little attention. However, our knowledge about their nongenotoxic effects possibly associated with tumor promotion is only limited. Although we know that MeChry bind to AhR (Cheung et al., 1993) or that 5-MeChry may inhibit GJIC (Bláha et al., 2002), there are no data available on either the AhR-mediated activity or GJIC inhibition, which would allow us to analyze the impact of methyl group position on these types of

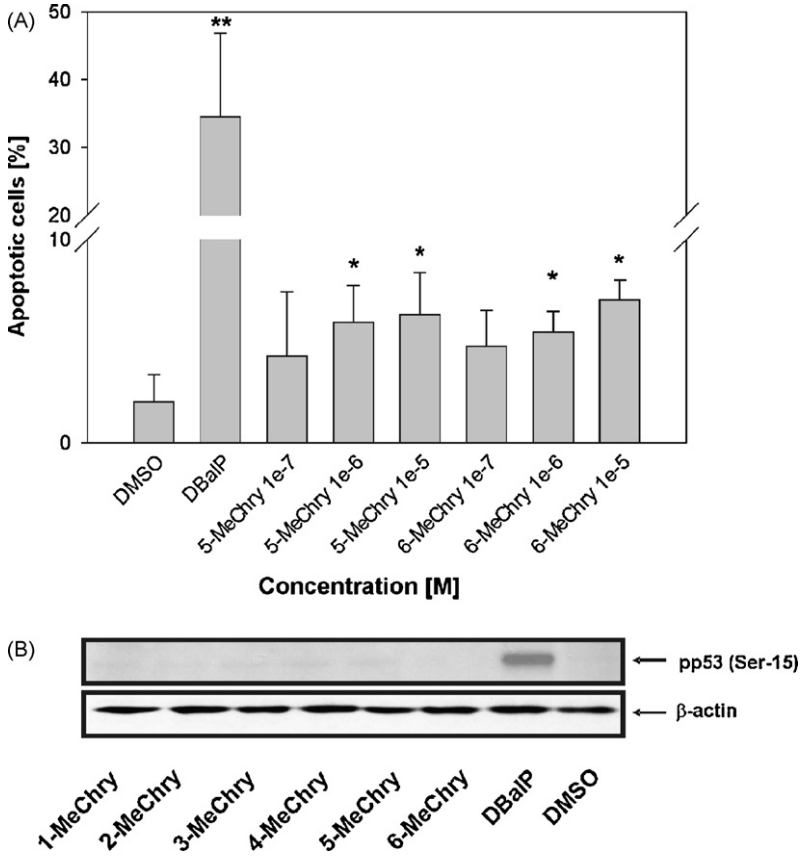

Fig. 5. Effects of methylated chrysenes on induction of apoptosis and phosphorylation of p53 protein in WB-F344 cells. (A) For detection of apoptosis, both floating and adherent cells were collected and stained with DAPI, and studied by fluorescent microscopy. A minimum of 200 cells were counted per each sample. Cells were exposed to 0.1, 1 and 10 μM concentrations of 5- and 6-MeChry, 0.1% DMSO, or 100 nM dibenzo[*a*,*l*]pyrene (DBalP) as a positive control. (B) Induction of p53 phosphorylation at Ser15 in cells treated with the indicated concentrations of test compound for 24 h. Cells were exposed to 10 μM concentrations of methylated chrysenes, DMSO 0.1% (control) or to 100 nM DBalP as a positive control. Cell lysates were prepared and Western blotting was performed as described in Section 2. β-Actin was used to control equal loading. The results shown here are representative of three independent experiments. *A significant difference between control (DMSO) and the respective treatment ($p < 0.05$). **A significant difference between control (DMSO) and the respective treatment ($p < 0.01$).

toxic activities. Therefore, the basic aim of the present study was to provide a comprehensive analysis of these toxic effects of MeChry in model rat liver cells. Based on these data, we further investigated the effects of MeChry on induction of xenobiotic metabolizing enzymes, cell cycle regulation, DNA damage, tumor suppressor p53 activation and apoptosis in the same cellular model.

The AhR activation is one of the principal effects of many carcinogenic PAHs (Machala et al., 2001). Activity of this transcription factor has been shown to be related not only to upregulation of expression of many enzymes involved in metabolic activation of PAHs, but also to tumor promotion (Sjögren et al., 1996; Nebert and Dalton, 2006). Many PAHs enhance their own activation through binding to the Ah receptor, which mediates the selective overexpression of particular drug metabolizing enzymes, which might also lead to PAH detoxification (Nebert and Dalton, 2006). On the other hand, activation of AhR has been repeatedly shown to induce numerous effects related to deregulation of cell proliferation, differentiation and apoptosis, thus suggesting that the AhR-mediated activity of PAHs might have consequences beyond the simple regulation of metabolic enzymes (Bock and Köhle, 2005; Marlowe and Puga, 2005). A previous study has suggested that the AhR binding capacities of individual MeChry are very similar (Cheung et al., 1993). On the other hand, it is known that AhR binding does not always fully predict the AhR-mediated activity of a given compound. For example, although the AhR-binding affinities of BaP and benzo[*a*]anthracene have been reported to be almost identical (Piskorska-Pliszczynska et al., 1986), BaP is a more potent inducer of AhR-mediated activity, detected either in DR-CALUX® assay or as induction of ethoxyresorufin-*O*-deethylase activity (Till et al., 1999; Machala et al., 2001). The present results indeed suggest that there is more than 40-fold difference between the AhR-mediated activities of 1- and 3-MeChry, the latter one being the most potent AhR ligand identified in the present study. The AhR-mediated activities of remaining MeChry fell within this range of relative potencies (approximately 1×10^{-5} to 5×10^{-4}), when expressed relative to TCDD. These values were similar to relative potencies of Chry or other carcinogenic PAHs, such as BaP or benzo[*b*]fluoranthene (Machala et al., 2001). The high AhR activating capacity of 3-MeChry is in line with the evidence that it is a more efficient inducer of EROD and CYP1 expression than 5-MeChry in liver of C57BL/6J mice (Shimada et al., 2003).

The high AhR-mediated activity of 3-MeChry was also confirmed when we analyzed effects of MeChry on cell cycle distribution and proliferation of confluent WB-F344 cells. It has been previously shown that disruption of contact inhibition in this cell line depends on AhR activation by PAHs (Andrysík et al., 2007) or TCDD (Weiss et al., 2008). Disruption of cell proliferation control has been suggested to contribute to carcinogenic effects of tumor promoters (Oesch et al., 1988; Dietrich et al., 2002). All six MeChry increased cell proliferation in contact-inhibited WB-F344 cells in a dose-dependent manner starting from nanomolar concentrations.

In contrast to all six MeCHry activating the AhR-dependent reporter gene expression, only 6-MeChry was found to inhibit GJIC in a manner similar to 5-MeChry. All remaining compounds were completely inactive, thus confirming that the position of methyl group is a critical requirement for this type of activity, as it has been shown, e.g. for methylated anthracenes (Upham et al., 1996) or benz[*a*]anthracenes (Marvanová et al., 2008). As many other carcinogenic genotoxic PAHs do not posses the GJIC inhibitory activity, it seems to be a unique property of compounds such as DMBA or 5-MeChry, which might perhaps further contribute to their tumorigenicity. This hypothesis is based on the evidence that disruption of GJIC is a characteristic feature of tumor cells. A majority of tumor promoters are known to inhibit GJIC, suggesting that this effect may contribute to tumor promotion (Rosenkranz et al., 2000; Trosko and Upham, 2005).

5-MeChry has been repeatedly shown to be the most potent carcinogenic MeChry (Hecht et al., 1974; Hoffmann et al., 1974). On the other hand, other MeChry were found to be potent mutagens in bacterial mutagenicity assays (Coombs et al., 1976; Cheung et al., 1993). The unique carcinogenic properties of 5-MeChry have been therefore hypothetically attributed to high levels of persistent DNA adducts formed specifically by this compound, which might be due to limited DNA repair of these adducts or decreased detoxification of 5-MCDE (Amin et al., 1985; Melikian et al., 1991; Cheung et al., 1993). Induction of CYP enzymes contributing to metabolic activation of MeChry is therefore probably not the limiting factor in genotoxicity, as shown both here, and in previous studies (Cheung et al., 1993). Because AKR enzymes have been also suggested to contribute to DNA damage elicited by carcinogenic PAHs (Penning et al., 1999), we also compared inducibility of AKR1C9 enzyme and found no difference between 5- and 6-MeChry effects.

Therefore, we also directly compared genotoxic effects of 5- and 6-MeChry in WB-F344 cells. We found that only 5-MeChry was able to induce significant levels of DNA adducts in this model of rat liver progenitor cells. As it has been previously shown that cells, in which DNA is modified by 5-MCDE, may evade G1 arrest and accumulate preferentially in S-phase of cell cycle (Khan et al., 1999), we analyzed effects of MeChry on cell cycle. We found that in WB-F344 cells, 5-MeChry induced a higher S-phase percentage than any other MeChry, thus again suggesting that other MeChry, like 6-MeChry, do not form significant levels of covalent DNA adducts in WB-F344 cells. This might explain also the lack of proliferative effects of high concentration of 5-MeChry in confluent WB-F344 cells. Nevertheless, in comparison with another strong mutagen, DBalP, 5-MeChry failed to induce significant amount of apoptosis or p53 phosphorylation, both being related to formation of high levels of DNA adducts in rat liver epithelial cells (Topinka et al., 2008). The level of DNA damage induced by 5-MeChry in WB-F344 cells might not be sufficient to activate p53 or to trigger apoptosis.

Taken together, the results of the present study seem to suggest that 5-MeChry might exert a unique combination of toxic effects in model epithelial cells. It is a single MeChry, which combines induction of DNA damage, relatively high AhR-mediated activity and inhibition of GJIC. On the other hand, other MeChry should be also taken into account, when analyzing toxic impact of complex environmental mixtures of pollutants or cigarette smoke, as they are all relatively efficient AhR ligands, and similar to 5-MeChry, 6-MeChry is a compound inhibiting GJIC. However, more information on both environmental and tissue MeChry levels, and toxicokinetics of MeChry in humans, are necessary in order to evaluate possible health hazards resulting from the exposure to these compounds.

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