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GENOTOXICITY AND CARCINOGENICITY

Role of human sulfotransferase 1A1 and *N*-acetyltransferase 2 in the metabolic activation of 16 heterocyclic amines and related heterocyclics to genotoxicants in recombinant V79 cells

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Abstract Heterocyclic aromatic amines (HAAs) are primarily produced during the heating of meat or fish. HAAs are mutagenic and carcinogenic, and their toxicity in model systems depend on metabolic activation. This activation is mediated by cytochrome P450 (CYP) enzymes, in particular CYP1A2. Some studies have indicated a role of human sulfotransferase (SULT) 1A1 and *N*-acetyltransferase (NAT) 2 in the terminal activation of HAAs. In this study, we conducted a metabolism/genotoxicity relationship analysis for 16 HAAs and related heterocyclics. We used the γH2AX genotoxicity assay in V79 cells (deficient in CYP, SULT and NAT) and V79-derived cell lines genetically engineered to express human CYP1A2 alone or in combination with human SULT1A1 or NAT2. Our data demonstrated genotoxic properties for 13 out of the 16 compounds tested. A clear relationship between metabolic bioactivation and genotoxicity allowed to distinguish four groups: (1) Trp-P-1 genotoxicity was linked to CYP1A2 bioactivation only—with negligible effects of phase II enzymes; (2) Glu-P-2, Glu-P-1, Trp-P-2, APNH, MeAαC and AαC were bioactivated by CYP1A2 in combination with either phase II enzyme tested (NAT2 or SULT1A1); (3) IQ, 4-MeIQ, IQx, 8-MeIQx, and 4,8-DiMeIQx required CYP1A2 in combination with NAT2 to be genotoxic, whereas SULT1A1 did not enhance their genotoxicity; (4) PhIP became genotoxic after CYP1A2 and SULT1A1 bioactivation—NAT2 had not effect. Our results corroborate some previous data regarding the genotoxic potency of seven HAAs and established the genotoxicity mechanism for five others HAAs. This study also permits to compare efficiently the genotoxic potential of these 13 HAAs.

Keywords Genotoxicity · H2AX · Heterocyclic aromatic amines · Metabolism · V79 cells

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Introduction

Heterocyclic aromatic amines (HAAs) are dietary carcinogens formed in some foodstuffs, but also arise in tobacco smoke (Turesky and Le Marchand 2011). HAAs are divided into two major classes. The pyrolysis HAAs (Fig. 1a) arise during high-temperature pyrolysis. These compounds contain a five-membered ring (imidazole or pyrrole) fused with two six-membered rings at separate sites. HAAs of the second class (Fig. 1b) contain a 2-aminoimidazo moiety fused to additional aromatic elements. They are formed in meats that are cooked at lower temperatures, more commonly used in household kitchens (150 °C) (Skog et al. 1998).

Several HAAs have been shown to be carcinogenic in rodents, inducing tumors in multiple organs and tissues (Kato et al. 1988; Ohgaki et al. 1991). Whereas the liver is a major target organ for the carcinogenicity of many HAAs in rodents, only PhIP was found to induce tumors at other sites. Based on current evidence, the International Agency for Research on Cancer (IARC) classified 2-amino-3-methyl-3H-imidazo[4,5-f]quinoline (IQ) as a probable human

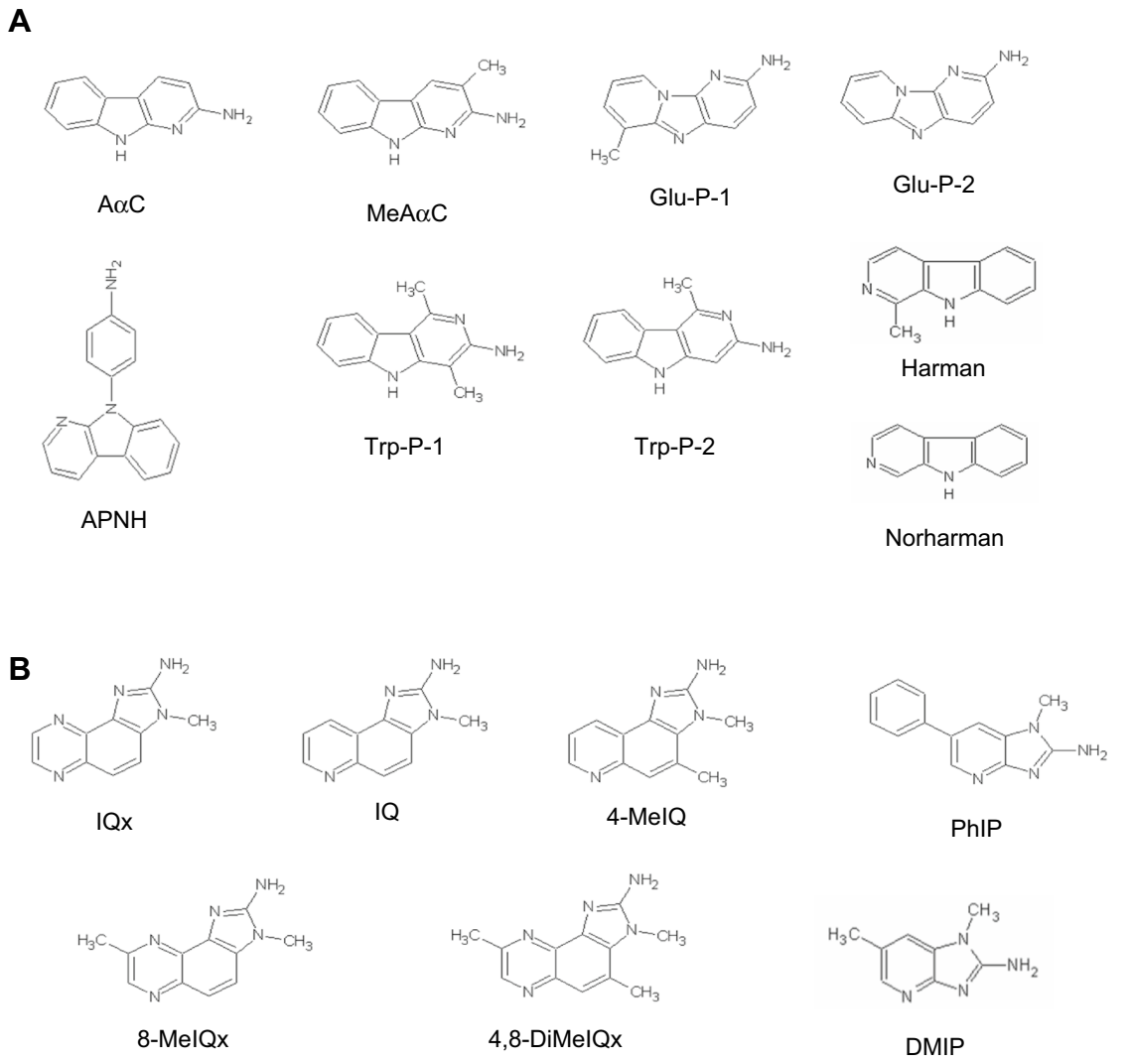

Fig. 1 Structures of the different heterocyclic aromatic amines (HAAs) tested in this study. **a** Pyrolysis HAAs, **b** aminoimidazoarene HAAs

carcinogen (class 2 A) and nine other HAAs as possible human carcinogens (class 2B) (IARC 2016). HAAs are pro-carcinogens and have to be metabolically activated to intermediates that form DNA adducts, leading to mutations and carcinogenesis. HAAs bioactivation pathway is initiated by the hydroxylation of the exocyclic amine group by cytochrome P450 enzymes (CYPs). In general, CYP1A2 is involved, but other CYPs are also able to *N*-hydroxylate some HAAs (Boobis et al. 1994; Hammons et al. 1997; Schut and Snyderwine 1999). *N*-Hydroxy-HAA metabolites are substrates for phase II enzymes, such as *N*-acetyltransferases (NAT) or sulfotransferases (SULT), and form unstable esters that can transfer a resonance stabilized arylnitrenium/carbonium ion to nucleophilic sites of DNA and other cellular molecules (Turesky and Le Marchand 2011).

The carcinogenicity of HAAs does not reflect the extraordinarily high mutagenicity of some congeners in the Ames test (Turesky and Le Marchand 2011). This discrepancy may be attributed in part to the observed interspecies differences in the metabolic fate of these compounds. The expression of biotransformation processes that can produce reactive intermediates varies widely among species (Martignoni et al. 2006), notably for HAAs (Turesky et al. 1999). On the other hand, the exceptionally efficient activation of some HAAs in the Ames test, owed to the expression of an acetyltransferase in the target cells, may be not reflecting the in vivo situation. In striking contrast to the Ames test, HAAs have often been found negative or only weakly positive in standard mutagenicity tests in mammalian cells in culture using external activation by S9 mix (Mizota et al. 2011; Westerink et al. 2010, 2011). This modest response in mammalian cell tests may be explained by the low expression, or lack, of NATs and SULTs in standard target cells. To better mimic the in vivo

situation, potential activating enzymes (CYPs and SULTs, NATs) were expressed in Chinese hamster V79 cells. Glatt and co-workers (Glatt et al. 2004; Glatt 2006) tested seven HAAs in these models for the induction of gene mutations at the *Hprt* locus (Glatt 2006; Glatt et al. 2004). Recently, we developed and validated a high-throughput genotoxicity assay, named γH2AX in-cell western (ICW), in human cells (Audebert et al. 2010). However, some human cells were unable to detect the genotoxicity of a limited number of genotoxic chemicals, notably IQ or PhIP. We suspected that these HAAs require specific bioactivation process not sufficiently covered by the cell lines used (Khoury et al. 2013, 2016a, b).

The aim of this study was to compare the genotoxic potential of 16 HAAs and related heterocyclics with the γH2AX ICW assay in parental V79 cells using three cell lines genetically engineered to express human CYP1A2 alone or together with human SULT1A1 or NAT2, as previously employed in the Hprt gene mutation assay (Glatt 2006; Glatt et al. 2004). The objective was to answer the following questions: (1) are HAAs tested positive using the Hprt assay also positive in the γH2AX ICW assay in V79-derived cell lines? (2) What is the relative sensitivity of Hprt and γH2AX ICW? (3) Are the enzymatic requirements for the individual HAAs the same in both cases? If γH2AX ICW gives credible answers for the seven HAAs for which Hprt mutagenicity data are available, then γH2AX ICW tests could be conducted with additional HAAs to which humans are exposed.

Materials and methods

Caution

Heterocyclic aromatic amines are potential human carcinogens, and they should be handled carefully.

Chemicals and reagents

All compounds used were of analytical grade. 9-(4′-aminophenyl)-9*H*-pyrido[3,4-*b*]indole (APNH), 2-amino-9*H*-pyrido[2,3-*b*]indole (AαC), 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole (MeAαC), 2-amino-6-methyldupyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1), 2-aminodipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-2), 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1), 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2), 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethyl-3*H*-imidazo[4,5-*f*]quinoline (4-MeIQ), 2-amino-3-methyl-3H-imidazo[4,5-*f*]quinoxaline (IQx), 2-amino-3,8-dimethyl-3*H*-imidazo[4,5-*f*]quinoxaline (8-MeIQx), 2-amino-3,4,8-trimethyl-3*H*-imidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx), 2-amino-1,6-dimethyl-1*H*-imidazo[4,5-*b*]pyridine (DMIP), 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), harman and norharman, were purchased from Toronto Research Chemicals (North York, ON, Canada). All stock solutions of the tests compounds were prepared in 100% dimethyl sulfoxide (DMSO). From the stocks, tenfold dilution series were prepared.

Penicillin, Streptomycin, trypsin, PBS, RNAse A, and Triton X-100 were purchased from Sigma–Aldrich (Saint Quentin Fallavier, France). The phosphatase inhibitor cocktail tablets ("PHOSSTOP") were purchased from Roche (France) and the blocking solution (MAXblock Blocking Medium) was purchased from Active Motif (Belgium). CF770 antibody and RedDot2 were purchased from Biotium (Hayward, CA, USA).

Cell culture

The V79 clone used in our laboratory (V79-Mz) has been investigated for many xenobiotic-metabolizing activities (Glatt et al. 1990). These cells do not show any endogenous CYP, SULT or NAT activities. V79-hCYP1A2, V79-hCYP1A2-hNAT2 and V79-hCYP1A2-hSULT1A1 cells were generated from V79-Mz cells by introduction of appropriate expression vectors as described previously (Glatt et al. 2004; Schmalix et al. 1993). Clones stably expressing the corresponding enzymes were selected. The expression of CYP1A2, measured by immunoblotting and mutagenicity tests with a compound whose activation only requires CYP activity (benzo[*a*]pyrene-*trans*-7,8-dihydrodiol), is known to be equal in V79-hCYP1A2, V79-hCYP1A2-hNAT2 and V79-hCYP1A2-hSULT1A1 cells (Glatt et al. 2004). The level of SULT1A1 protein in V79-hCYP1A2-hSULT1A1 cells is in the high hepatic physiological range, whereas the level of NAT2 in V79-hCYP1A2-hNAT2 is higher (20-fold above the hepatic in a subject with high expression) (Glatt et al. 2004). The cells were grown in DMEM medium supplemented with 5% fetal bovine serum, 100 U ml^{-1} penicillin and 100 µg ml^{-1} streptomycin (Glatt 2006; Glatt et al. 2004). Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37 °C and the medium was refreshed every 2–3 days during sub-culturing.

γH2AX in-cell western (ICW) assay

The γH2AX in-cell western technique was performed as previously described (Audebert et al. 2010, 2011, 2012; Graillot et al. 2012a, b; Jamin et al. 2013; Khoury et al. 2013, 2016a, b; Quesnot et al. 2016). Briefly, cells (3.2×10^4 cells per well) were grown in 96-well plates containing 200 µl of medium. Sixteen hours later, cells were

treated in duplicate with the model compounds or vehicle in serum free medium. For each plate, DMSO (0.2% v/v final dose) was used as negative control. The positive control used in each treatment was 1 µM etoposide. 24 h after the treatment, cells were analyzed by the ICW technique. For the determination of genotoxicity, relative fluorescent units for γH2AX per cell (as determined by γH2AX divided by DNA content) were divided by the respective controls (vehicle only) to determine the change in phosphorylation of H2AX level compared with the control cells. To determine cytotoxicity, the DNA content (related to the number of cells) recorded in the treated cells was compared to the DNA content in control cells. All experiments were performed at least in triplicate, independently.

Data analysis

Results are presented as mean ± SEM (standard error of the mean) of at least three separate experiments. Statistically significant increases in H2AX phosphorylation after treatment were compared with the vehicle (DMSO) control using two-sided Student's *t* test; $*p<0.05$; $**p<0.01$. Genotoxicity was considered positive when a compound induced a statistically significant 1.3-fold γH2AX histone phosphorylation at a level of cytotoxicity below 50% compared to the control DMSO. These parameters were based on our previous studies (Khoury et al. 2013, 2016a, b) and are similar to those used by other groups who use γH2AX quantification for genotoxicity determination (Ando et al. 2014; Bryce et al. 2014; Smart et al. 2011).

Results

In a first step, we performed the γH2AX ICW assay with 16 HAAs and related heterocyclics in the parental V79 cell line. None of these 16 compounds demonstrated any genotoxicity in this cell line devoid of functional human enzymatic activities (Fig. 2). Then we tested the same chemicals in three cell lines genetically engineered from V79 cells to express specific human xenobiotic-metabolizing enzymes (CYP1A2 alone, CYP1A2 plus SULT1A1 and CYP1A2 plus NAT2). Out of the 16 compounds tested, three (harman, norharman and DMIP) did not exert any genotoxicity whatever the cell line used and the concentration tested (data not shown).

For Trp-P-1, we observed almost the same concentration–response genotoxicity curves whatever the cell line used (V79-hCYP1A2, V79-hCYP1A2-hSULT1A1 and V79-hCYP1A2-hNAT2), with a LEC of 0.01 µM (Fig. 3; Table 1). AαC, Glu-P-1, MeAαC, Glu-P-2 and Trp-P-2 were not genotoxic in V79-hCYP1A2 cells (Figs. 2c, e, f, 4b, d). On the contrary, positive results were obtained with

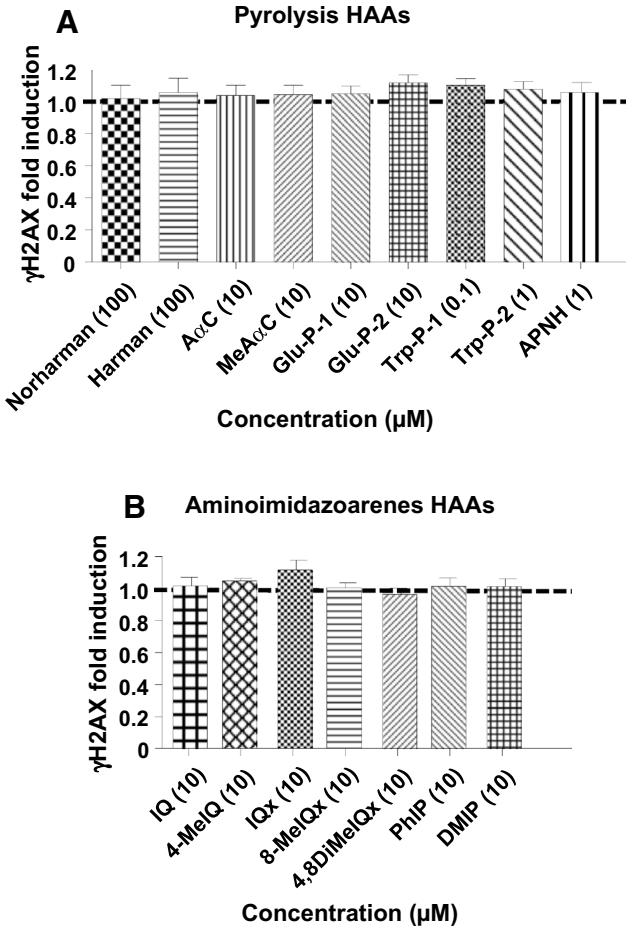

Fig. 2 In vitro genotoxicity tested with the γH2AX ICW assay in V79 cells. Each value represents the mean ± SEM ($n \geq 3$) after 24 h of treatment

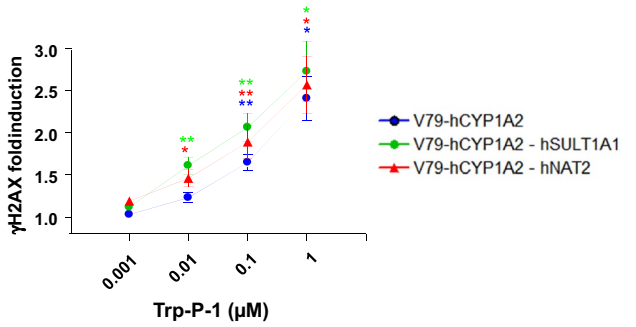

Fig. 3 In vitro genotoxicity of Trp-P-1 tested with the γH2AX ICW assay in V79-hCYP1A2, V79-hCYP1A2-hSULT1A1 and V79-hCYP1A2-hNAT2 cell lines. Each value represents the mean ± SEM ($n \geq 3$) after 24 h of treatment. Significant differences were observed between controls and matched group ($*p \leq 0.05$, $**p \leq 0.01$)

all these HAAs in V79-hCYP1A2-hSULT1A1 as well as in V79-hCYP1A2-hNAT2 cells with a LEC of 0.1 µM for AαC, Glu-P-1 and MeAαC, 1 µM for Trp-P-2, respectively,

Table 1 Comparative genotoxicity of HAAs in V79 cells engineered for the expression of specific human xenobiotic-metabolizing enzymes, with the Hprt (Glatt et al. 2004; Glatt 2006) or γH2AX ICW (this study) assays

Substances	V79-hCYP1A2		V79-hCYP1A2-hNAT2		V79-hCYP1A2-hSULT1A1	
	Hprt	γH2AX	Hprt	γH2AX	Hprt	γH2AX
IQ	−(30)	+(10)	+(0.01)	+(0.01)	−(30)	+(1)
4-MeIQ	nd	+(0.1)	nd	+(0.01)	nd	+(1)
IQx	nd	−(10)	nd	+(0.1)	nd	−(10)
8-MeIQx	−(100)	−(1)	+(0.1)	+(0.01)	−(30)	+(1)
4,8-DiMeIQx	nd	−(10)	nd	+(0.1)	nd	−(10)
PhIP	−(30)	+(1)	+(10)	+(1)	+(0.3)	+(0.1)
AαC	−(30)	−(10)	−(30)	+(1)	+(0.1)	+(0.1)
MeAαC	−(10)	−(100)	−(10)	+(0.1)	+(0.1)	+(0.1)
Glu-P-1	−(100)	−(10)	+(0.3)	+(0.1)	−(30)	+(0.1)
Glu-P-2	nd	−(100)	nd	+(10)	nd	+(10)
Trp-P-1	nd	+(0.1)	nd	+(0.01)	nd	+(0.01)
Trp-P-2	−(3)	−(10)	−(3)	+(1)	−(3)	+(10)
APNH	nd	+(0.1)	nd	+(0.001)	nd	+(0.001)

Concentrations in micromolar units correspond to the lowest effective concentration (LEC) observed (positive results) or the highest concentration that could be adequately tested (negative results)

nd, not determined; +, tested positive; − tested negative in this assay

and a LEC of 10 µM for Glu-P-2. APNH was tested positive in V79-hCYP1A2 cells at the 0.1 and 1 µM concentrations, but its effects were drastically enhanced, with a LEC of 0.001 µM, when SULT1A1 or NAT2 were co-expressed with CYP1A2 (Fig. 4a). The concentration–response curves were found to be nearly equal in the latter cell lines for all these HAAs.

For the five other compounds (IQ, IQx, 4-MeIQ, 8-MeIQx and 4,8-DiMeIQx) we observed only a minimal genotoxicity when using V79-hCYP1A2 or V79-hCYP1A2-hSULT1A1 cell lines, and this only for the two highest concentrations tested (1 and 10 µM, Fig. 5). Conversely, these chemicals were found to be strongly genotoxic in V79-hCYP1A2-hNAT2 cells, with a LEC of 0.01 µM for IQ, 4-MeIQ and 8-MeIQx, and a LEC of 0.1 µM for IQx and 4,8-DiMeIQx. For PhIP (Fig. 6), we observed similar genotoxicity results in V79-hCYP1A2 and V79-hCYP1A2-hNAT2 cells, with a LEC of 1 µM. Nevertheless, the genotoxicity of PhIP was increased by a factor of 10 in the V79-hCYP1A2-hSULT1A1 cell line, with a LEC of 0.1 µM.

Discussion

We investigated 16 HAAs and heterocyclics in four cell lines (V79 and three V79 derived cell lines engineered for expressing specific human enzymes) with the γH2AX ICW genotoxicity assay. This experimental work required approximately six person-months, much less than Hprt assays in the same cell lines (over 12 person-months for only seven compounds) (Glatt et al. 2004; Glatt 2006). For seven HAAs it is possible to compare the results of the γH2AX ICW assay (current study) with those of the Hprt mutagenicity assay previously performed in the same cell lines (Table 1). None of these seven HAAs was found to be genotoxic with either assays in parental V79 cells. The remaining 21 situations (seven compounds in three cell lines) can be subdivided as follows: five HAAs were negative in parental V79 cells with both assays; in three situations, both endpoints gave positive test results with a similar LEC (AαC and MeAαC in V79-hCYP1A2-hSULT1A1; IQ in V79-hCYP1A2-hNAT2); in five situations both assays gave positive test results, but the LEC was lower for γH2AX ICW than for gene mutations (8-MeIQx, PhIP, AαC Glu-P-1 in hCYP1A2-hNAT2; PhIP in V79-hCYP1A2-hSULT1A1); in eight situations, positive results were only obtained when using the γH2AX ICW assay (IQ and PhIP in V79-hCYP1A2; Trp-P-2 and MeAαC in V79-hCYP1A2-hNAT2; IQ, in V79-hCYP1A2-hSULT1A1 and Glu-P-1 in V79-hCYP1A2-hSULT1A1). This comparison demonstrates that γH2AX ICW was more sensitive than the gene mutation assay. However, some differences cannot be solely explained by differences in sensitivity. NAT2 enhanced the mutagenicity of PhIP, but not the γH2AX response in CYP1A2 expressing cells; NAT2, unlike SULT1A1, failed to enhance the mutagenicity of *N*-hydroxy-PhIP in *S. typhimurium* TA1538/1,8-DNP-derived strains (devoid of endogenous acetyltransferase) (Muckel et al. 2002). Conversely, NAT2 enhanced the γH2AX response to MeAαC similar to SULT1A1, whereas SULT1A1 was required for a mutagenic effect

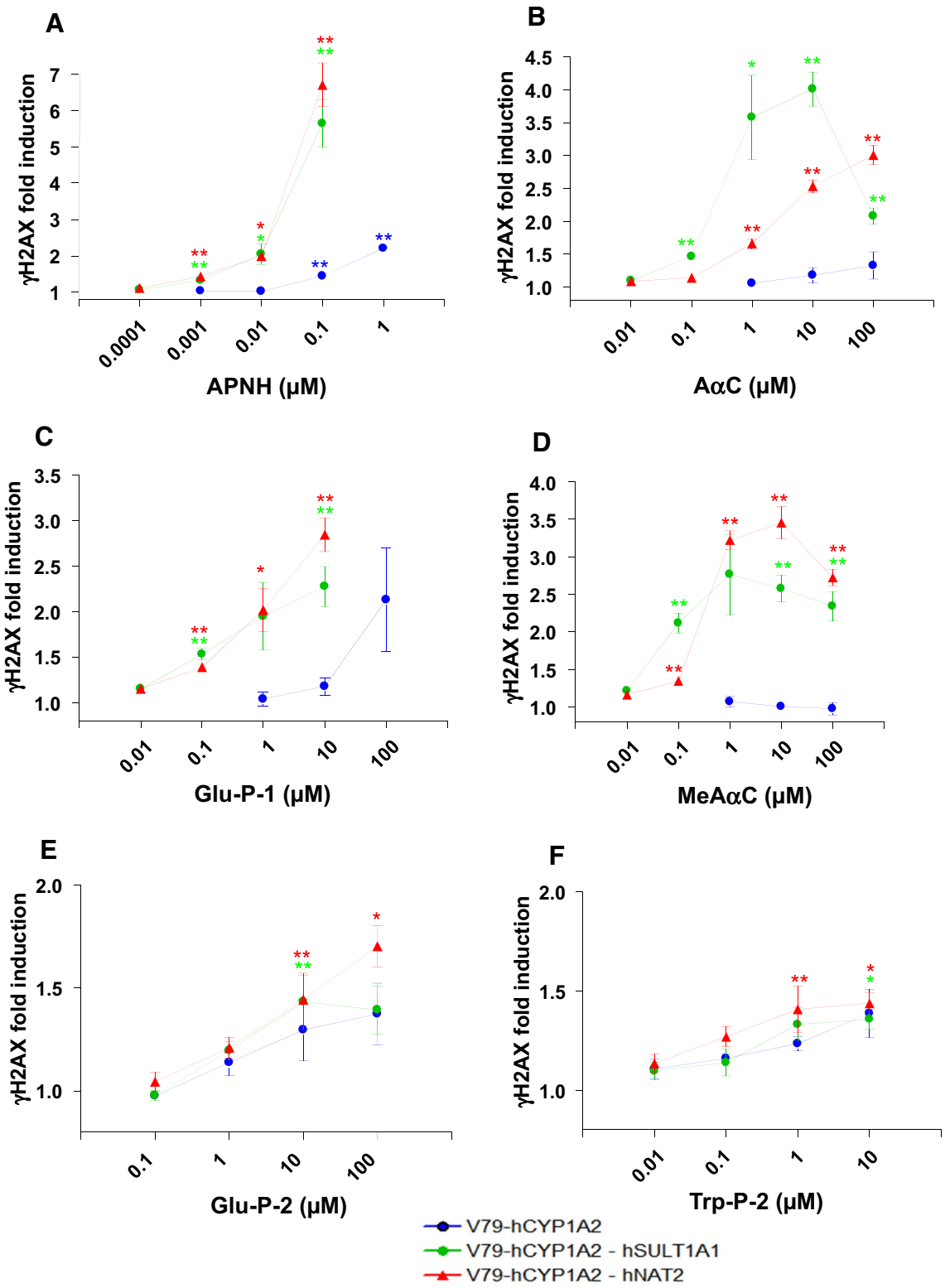

Fig. 4 In vitro genotoxicity of APNH, AαC, MeAαC, Trp-P-2, Glu-P-2 and Glu-P-1 tested with the γH2AX ICW assay in V79-hCYP1A2, V79-hCYP1A2-hSULT1A1 and V79-hCYP1A2-hNAT2 cell lines. **a** APNH, **b** AαC, **c** Glu-P-1, **d** MeAαC, **e** Glu-P-2, **f** Trp-P-2. Each value represents the mean ± SEM ($n \geq 3$) after 24 h of treatment. Significant differences were observed between controls and matched group (*$p \leq 0.05$, **$p \leq 0.01$)

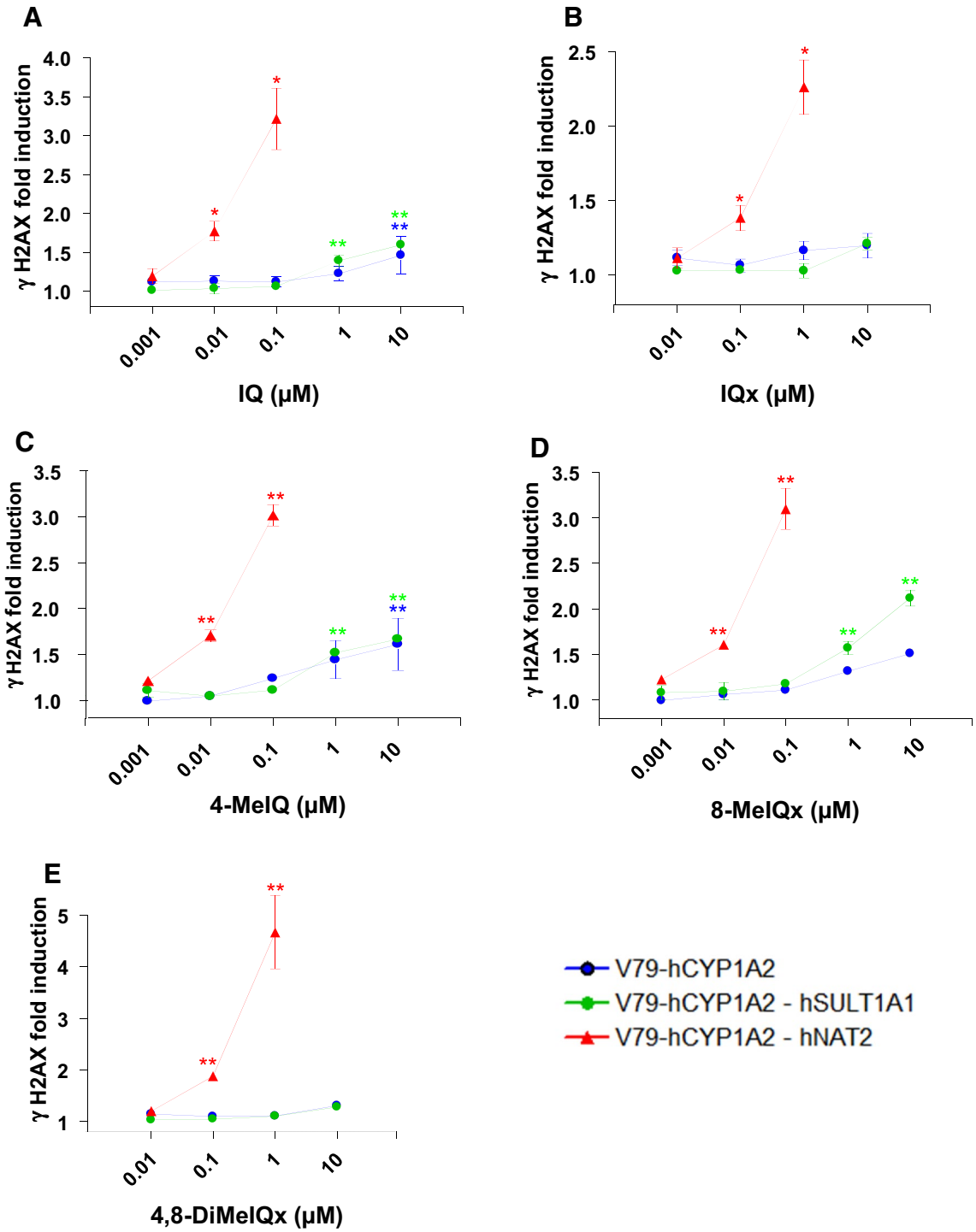

Fig. 5 In vitro genotoxicity of IQ, IQx, 4-MeIQ, 8-MeIQx and 4,8-diMeIQx tested with the γH2AX ICW assay in V79-hCYP1A2, V79-hCYP1A2-hSULT1A1 and V79-hCYP1A2-hNAT2 cell lines. **a** IQ, **b** IQx, **c** 4-MeIQ, **d** 8-MeIQx, **e** 4,8-diMeIQx. Each value represents the mean ± SEM ($n \geq 3$) after 24 h of treatment. Significant differences were observed between controls and matched group (*$p \leq 0.05$, **$p \leq 0.01$)

in the Hprt assay. Finally, SULT1A1 enhanced the mutagenicity of MeAαC and *N*-hydroxy-MeAαC in *S. typhimurium* TA1538/1,8-DNP-derived strains, although not as strongly as NAT2 (Glatt et al. 2004). Thus, few results obtained with the γH2AX ICW assay in recombinant V79 cells do not exactly match Hprt results in the same cells,

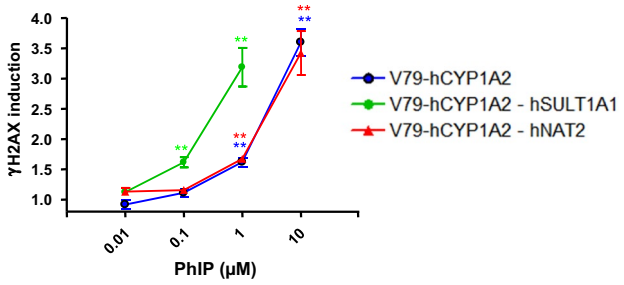

Fig. 6 In vitro genotoxicity of PhIP tested with the γH2AX ICW assay in V79-hCYP1A2, V79-hCYP1A2-hSULT1A1 and V79-hCYP1A2-hNAT2 cell lines. Each value represents the mean ± SEM ($n \geq 3$) after 24 h of treatment. Significant differences were observed between controls and matched group ($*p \leq 0.05$, $**p \leq 0.01$)

but are consistent with mutagenicity findings in recombinant *S. typhimurium* strains. Incubation conditions (such as cell density) may affect the expression levels and impact of metabolizing enzymes. Putting aside these subtle differences, our findings regarding the role of phase II enzymes in the response returned by the γH2AX ICW test clearly match the results returned by the Hprt assay for the same cellular model, as well as that of various other experimental models (Glatt 2006; Glatt et al. 2004; Muckel et al. 2002; Schut and Snyderwine 1999; Turesky and Le Marchand 2011; Wild et al. 1995). All these data may be helpful to improve structure–mutagenicity relationship for HAAs (Ripa et al. 2014; Shamovsky et al. 2011, 2012).

Selective or preferential terminal activation by either SULT1A1 or NAT2 may be owed to two factors. The first factor is the substrate specificity of enzymes. This is illustrated by the observation that mouse's Sult1a1, unlike its human orthologue, is not able to activate PhIP (in genetically recombinant *S. typhimurium* strains as well as genetically modified mouse lines) and that rat Nat1 (the orthologue of human NAT2), unlike human NAT1 and NAT2, expressed in *S. typhimurium*, is capable of activating *N*-hydroxy-PhIP (Meinl et al. manuscript in preparation), although much less efficiently than SULT enzymes. The second factor is the chemical reactivity of acetic acid versus sulfuric acid esters. Sulfate is a better leaving group than acetate, as also reflected by the higher acidity of sulfuric acid as compared to acetic acid. There is no information available so far on the respective half-life times of acetic and sulfuric esters derived from heterocyclic amines. Yet, it has been shown that 1-acetoxymethylpyrene is only marginally hydrolyzed after a 70 h stay in water at 37 °C, whereas 1-sulfooxymethylpyrene exhibits a half-life time of 2.8 min under the same conditions (Landsiedel et al. 1996).

Three out of the 16 compounds tested did not exert any genotoxicity in the recombinant cells. Two of these compounds, harman and norharman, are heterocyclics lacking an exocyclic amino group. All previous genotoxicity tests experiments with these compounds have returned negative results (Chang et al. 1978; Holme et al. 1985). These negative findings underline the importance of the exocyclic amino group in the biological activity of HAAs. DMIP was the only HAA showing no genotoxic activity in this study, whatever the cellular model used. It differs from PhIP by the substitution of a methyl group for a phenyl group (Fig. 1). Therefore, the resonance stabilization of a nitrenium/carbonium ion formed from DMIP is expected to be weaker, as compared to PhIP. Then, even an excellent leaving group, such as sulfate, may not provide enough reactivity to produce a genotoxic effect, simply because the intermediate metabolite is not stable enough. APNH was found to be the most genotoxic HAA among the congeners tested in this study, exhibiting a LEC of 1 nM. Interestingly, in previous long-term carcinogenicity studies, lower in vivo doses were required to observe a carcinogenic effect of APNH (Husain et al. 2007) than of other HAAs (Sugimura et al. 2004). APNH differs from the other HAAs tested by the presence of a fourth aromatic ring. This additional ring may enhance the resonance stabilization of nitrenium/carbonium ions, and consequently, the chemical reactivity of *N*-sulfoxy and *N*-acetoxy metabolites. Furthermore, we found that both human NAT2 and SULT1A1 were able to strongly enhance the genotoxic activity of APNH. Taken together, these findings may suggest a high carcinogenic activity of APNH in humans. Further studies, like APNH quantification in human urine (Nishigaki et al. 2007) should be carried out to clarify the extent of human exposure to APNH. As well, the conditions in which APNH is formed should be further investigated.

Only Trp-P-1 demonstrated a high genotoxic activity linked with CYP1A2 bioactivation, independently of the expression of SULT1A1 and NAT2 enzymes. Yamazoe et al. reported that seryl-tRNA synthetase from yeast and prolylyl-tRNA synthetase from rat are able to stimulate the covalent binding of *N*-OH-Trp-P-2 to DNA in cell-free systems (Yamazoe et al. 1981, 1985). Likewise, Saito et al. observed the formation of a semistable glutathione conjugate from *N*-OH-Trp-P-2, which exhibited higher mutagenic activity in *S. typhimurium* than *N*-OH-Trp-P-2 itself. Thus, unusual conjugation reactions may be involved in the activation of Trp-P-2 (Saito and Kato 1984).

In animal models, the main target organs of carcinogenesis differ between HAAs. It is probable that differences in bioactivation requirements are important factors underlying these organotropisms and species-dependent differences. In this study, we demonstrated that the final activation step of many HAAs is highly dependent upon conjugation reactions. One might expect that tissues expressing high levels of the appropriate phase II enzyme(s) are potential targets for adverse effects of HAAs, provided they are sufficiently

exposed to *N*-hydroxy-HAAs, via local activation of HAAs by CYPs or via the circulation. In humans, SULT1A1 is expressed in many different tissues, the levels being particularly high in liver and gut (Teubner et al. 2007). It appears that few human tissues express NAT2, primarily the large intestine (Husain et al. 2007). It has to be emphasized that expression sites and substrate specificity of orthologous enzymes can substantially vary between species. This is true in particular for SULTs (Dobbernack et al. 2011; Glatt et al. 1998; Meinl et al. 2013). The tissue distribution of DNA adduct formation by PhIP in wild-type mice and in mice transgenic for the human *SULT1A1–SULT1A2* gene cluster was investigated by in animals orally exposed to PhIP (Dobbernack et al. 2011; Hoie et al. 2016). Whereas the liver demonstrated the lowest level of DNA adducts in wild-type mice, it was the tissue exhibiting the highest adduct levels in transgenic mice. This selective influence of transgenic SULT1A1–SULT1A2 in the liver contrasted with the high expression of the transgene in many extrahepatic tissues. We suspect that PhIP is primarily converted to *N*-hydroxy-PhIP in the liver; in transgenic mice, it is expected that this metabolite is immediately converted into a DNA reactive, short-lived ester by human SULT1A1 and SULT1A2, explaining the high hepatic adduct levels. Since in wild-type mouse, SULT1A1 does not activate *N*-hydroxy-PhIP, much *N*-hydroxy-PhIP may escape the liver and be further activated in other tissues (Meinl et al. manuscript in preparation). This example illustrates that knowledge of critical phase II enzymes may be useful to explain target sites of HAAs. Nevertheless, broad knowledge on the toxicokinetics, including the role of many phase I and phase II enzymes, will be required for the prediction of target sites in humans. High-throughput genotoxicity assays, such as γH2AX ICW, in combination with recombinant cell lines should be useful tools for obtaining the relevant information.

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