Comparative genotoxic potential of 27 polycyclic aromatic hydrocarbons in three human cell lines

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Abstract

Polycyclic Aromatic Hydrocarbons (PAHs) form a family of compounds that are generally found in complex mixtures. PAHs can lead to the development of carcinogenesis. The Toxicity Equivalent Factor (TEF) approach has been suggested for estimating the toxicity of PAHs, however, due to the relative weakness of available data, TEF have not been applied for the risk characterization of PAHs as food contaminants in Europe. The determination of new TEFs for a large number of PAHs could overcome some limitations of the current method and improve cancer risk assessment. The present investigation aimed at deriving new TEFs for PAHs, based on their genotoxic effect measured *in vitro* and analyzed with mathematical models. For this purpose, we used a genotoxicity assay (γH2AX) with three human cell lines to analyze the genotoxic properties of 27 selected PAHs after 24 h treatment. For 11 compounds, we did not detect any genotoxic potential. For the remaining 16 PAHs, the concentration-response for genotoxic effect was modelled with the Hill equation; equivalency between PAHs at low dose was assessed by applying constraints to the model parameters. We developed for each compound, in each cell line, Genotoxic Equivalent Factor (GEF). Calculated GEF for the tested PAHs were similar in all cell lines and generally higher than the TEF usually used. These new equivalent factors for PAHs should improve cancer risk assessment.

Keywords: Polycyclic aromatic hydrocarbons; Modelization; Toxic Equivalent Factor; genotoxicity; H2AX; in vitro.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a family of substances containing two or more aromatic rings that includes a few hundred compounds. They occur naturally in coal and crude oils and are present in products made from fossil fuels (Harvey, 1991). They can also result from the combustion or pyrolysis of various organic materials, such as coal, petroleum and wood (Lima et al., 2005). Pyrogenic sources include among others forest fires, volcanic eruptions, industrial facilities and power plants using fossil fuels, smelting, waste incinerators, and combustion-powered vehicles. PAHs are widely distributed in the environment (air, water, soil) and are also present in food (Purcaro et al., 2013). Smoking and grilling food, particularly meat and fish, may contribute substantially to the formation and the intake of PAHs. In the non-smoking general population, the main route of exposure to PAHs is *via* food consumption (EFSA, 2008; IARC, 2010). Although processed fish, food supplement, mollusks, barbequed and grilled meat were reported to be the most contaminated food categories in the European Union, the highest contributors to the dietary exposure were cereals and cereal products, due to the high consumption of these products (EFSA, 2008). Sixteen priority PAHs were recommended by different international agencies for the analysis of environmental and/or food samples (EFSA, 2008; EU, 2002; FAO/WHO, 2005; US-EPA, 1993).

A number of PAHs tested individually or in complex mixtures, have been shown to be genotoxic *in vitro* and *in vivo* and to be carcinogens in laboratory animals. The International Agency for Research on Cancer (IARC) evaluated the carcinogenicity to humans of 60 PAHs (IARC, 2010). The agency classified benzo[a]pyrene as carcinogenic to humans (Group 1), cyclopenta[c,d]pyrene, dibenz[a,h]anthracene and dibenzo[a,l]pyrene as probably carcinogenic to humans (Group 2A) and benz[j]aceanthrylene, benz[a]anthracene, benzo[b]fluoranthene, benzo[j]fluoranthene, benzo[k]fluoranthene, benzo[c]phenanthrene,

chrysene, dibenzo[a,h]pyrene, dibenzo[a,i]pyrene, indeno[1,2,3-cd]pyrene and 5-methylchrysene as possibly carcinogenic to humans (Group 2B). It has been clearly demonstrated that tumorigenic PAHs require metabolic activation to electrophilic intermediates and to ultimate carcinogens (Moorthy et al., 2015). As a first step, PAHs are biotransformed by phase I enzymes and peroxidases, giving rise to DNA-reactive metabolites. Then, phase I metabolites can be conjugated to polar metabolites by phase II enzymes. Specific cytochrome P450 isozymes (mainly 1A1, 1A2 and 1B1) and epoxide hydrolase are responsible for the oxidation of many PAHs to form phenols, diols and diol epoxides that are ultimate carcinogenic metabolites. Other bioactivation pathways, such as the formation of radical cations or quinones are known to lead ultimate carcinogenic metabolites.

For some chemical mixtures, the risk assessment can be based on an index compound, which is often the compound for which the toxicological data are most robust (Committee et al., 2019). The concept of Toxic Equivalency Factor (TEF), defined as an estimate of the toxicity of a chemical related to that of a reference compound and also referred to as relative potency factor (RPF) was proposed for PAHs by toxicologists and risk assessors (Bostrom et al., 2002; Collins et al., 1998; Delistraty, 1997; Petry et al., 1996; US-EPA, 1993). These factors are used to relate the carcinogenic potential of other PAHs to that of B(a)P. In some cases, however, the concept of TEFs for PAHs is not accepted by risk assessors. In exploring whether a TEF approach of the PAH mixtures in food was appropriate for risk characterisation, the EFSA CONTAM Panel concluded that this approach was not valid because of (1) the lack of data from oral carcinogenicity studies on individual PAHs, (2) their different modes of action and (3) the evidence of poor predictivity of the carcinogenic potency of PAH mixtures based on the currently proposed TEF values. The last two objections lead us to consider the estimation of TEFs based on genotoxic events such as those reflected by the induction of the phosphorylated histone H2AX (γH2AX). Based on γH2AX

genotoxicity testing, we compared recently the genotoxicity of 13 PAHs and derived corresponding TEFs called genotoxic equivalent factors (GEFs) (Audebert et al., 2012).

In the present study, we analysed the genotoxic potential of 27 PAHs comprising the 16 priority PAHs. First, the genotoxicity of the 27 selected compounds were tested in three human cell lines representative of PAHs target: hepatoma cell line Hep3B, epithelial colorectal adenocarcinoma cells LS-174T and in bronchioalveolar carcinoma NCI-H358 cell line. The genotoxic potential of each PAHs was tested with the γH2AX global genotoxic endpoint (Kopp et al., 2019). This assay relies on the detection of the phosphorylation of the histone H2AX that reflects a global genotoxic insult resulting from diverse type of DNA damage, notably DNA adducts and oxidative lesions (Khoury et al., 2013, 2016a, b; Kopp et al., 2019). Using this genotoxicity assay, we previously demonstrated that Hep3B liver cell line was more sensitive than HepG2 to detect PAHs genotoxicity (Khoury et al., 2016b). In a second step, the concentration-response relationship for genotoxic effects of each compound in the three cell lines was modelled and analysed with the Hill equation. This equation is one of the most often applied to dose-response relationships (Walker and Yang, 2005), because of its ability to reflect the binding of ligands to target molecules. Based on this approach, and using a complete dose-response curve covering low dose effects for each PAH, we derived GEFs for PAHs. The GEF values we estimated for 16 PAHs would likely improve the relevance of cancer risk assessment for humans exposed to PAHs.

Material and Methods

Caution: PAHs are hazardous compounds and should be handled with care in accordance with the NIH guidelines for the laboratory use of chemical carcinogens.

Chemicals and Reagents

5-Methylchrysene (5MC), 7,12-Dimethyl-benz[*a*]anthracene (DMBA), acenaphthene (ACT), acenaphthylene (ACTY), anthracene (ANT), benz[*a*]anthracene (BaA), benzo[*c*]fluorene (BcF), benzo[*a*]pyrene (BaP), benzo[*b*]fluoranthene (BbF), benzo[*e*]pyrene (BeP), benzo[*ghi*]perylene (BghiP), benzo[*j*]fluoranthene (BjF), benzo[*k*]fluoranthene (BkF), chrysene (CHR), coronene (COR), cyclopenta[*c,d*]pyrene (CPP), dibenz[*a,h*]anthracene (DBahA), dibenzo[*a,h*]pyrene (DBahP), dibenzo[*a,i*]pyrene (DBaiP), dibenzo[*a,l*]pyrene (DBalP), dibenzo[*a,e*]pyrene (DBaeP), fluoranthene (FLA), fluorene (FLE), indeno[1,2,3-*cd*]pyrene (IPY), naphtalene (NAP), phenanthene (PHE), pyrene (PYR) (with chemical purity > 97%) were obtained from AccuStandard (New Haven, USA) and Dr. Ehrenstorfer (CIL, France) (Fig 1.). All compounds were diluted in dimethyl sulfoxide (DMSO) obtained from Sigma-Aldrich (Saint Quentin Fallavier, France). Concentration of stock solutions was 5 mM. Cells were exposed to 0.2% (v/v) DMSO in culture medium.

Cell Lines and cultures

Hep3B human hepatoblastoma cells (ATCC HB-8064) and LS-174T human epithelial colorectal adenocarcinoma cells (ATCC CL-188**)** were cultured in αMEM medium, 10% fetal calf serum v/v, penicillin (100 U ml^{-1}), streptomycin (100 µg ml^{-1}), in a 5% CO_2 atmosphere at 37°C. NCI-H358 human bronchioalveolar carcinoma cells (ATCC N° CRL-5807) were

cultured in RPMI-1640 medium, 10% fetal calf serum v/v, penicillin (100 U ml^{-1}), streptomycin (100 µg ml^{-1}), in a 5% CO_2 atmosphere at 37°C.

γH2AX In-Cell Western (ICW) assay

The γH2AX In-Cell Western technique was performed as previously described (Audebert et al., 2010; Audebert et al., 2012). This genotoxicity assay is based on histone H2AX phosphorylation (named γH2AX) quantification. This biomarker has been linked to different type of DNA damage including DNA bulk adducts as with PAHs exposure (Kopp et al., 2019). To determine cytotoxicity, the DNA content (as a surrogated to the number of cells) recorded in the different treated cells was compared to the DNA content in control cells and was expressed as relative cell count (% RCC).

Modelling genotoxicity of individual PAHs

The toxic loads of chemicals with a similar mode of action can be expressed relative to one reference compound by means of Toxic Units (Sprague, 1970) and Toxic Equivalency Factors, often based on EC_{50} ratios. This concept is only valid when all compounds can elicit the same responses. An important feature of our data is that the maximum response levels depend on the PAH studied; Toxic Units could therefore only be calculated for a limited range of responses. Specific methods have been developed to deal with the analysis of partial agonists, often encountered in the field of pharmacology in receptor-ligand relationships (Dinse and Umbach, 2011; Dinse and Umbach, 2012; Gennings et al., 2004). In gene expression data, supramaximal responses (also called superinduction) are often observed, for example in oestrogenicity assays where the underlying mechanisms of superinduction are not

yet fully understood, and it is unclear whether this mechanism is relevant *in vivo* (Montano et al., 2010; Sotoca et al., 2010). According to Dardenne et al. (2008), in gene induction data, the maximum induction level for every gene reporter is not only determined by its intrinsic regulatory mechanism but also is dependent on the nature of the inducing compound and on the cytotoxicity. The EC_{50} therefore do not have any true biological meaning and toxic equivalents cannot be based on the EC_{50}. This is a major impediment to the analysis of gene expression data. We analyse genotoxicity using all dose-response data available, including high response levels, and compare PAH genotoxicity based on the slope at low doses.

In the present paper, genotoxicity was represented as the fold-induction relative to the mean induction in the control on the plate. Read-outs from duplicate wells were averaged. The log-fold-induction was used to avoid heteroscedasticity due to larger variance of the higher responses (Charles et al., 2002; Mumtaz et al., 2002) and has been used in oestrogen receptor (ER) transcriptional activation assay result analysis (Gennings et al., 2003) and in micro-array data analysis (Huber et al., 2002; McCarthy and Smyth, 2009).

The log- fold-induction dose-response relationship for individual chemicals was modelled by least squares using a 3-parameter Hill (or log-logistic) model which is commonly used to model dose–response relationships (Khinkis et al., 2003):

$$\Phi(x) = \frac{Max}{1+\left(\frac{x}{EC50}\right)^{\beta}}$$

where *Max* is the maximal response, *EC50* is the concentration that induces a 50% effect, β is the Hill slope, hereafter referred to as the Hill constant, and *x* is the PAH concentration.

At low doses, the Hill model simplifies into:

$\Phi(x) = ax^{-\beta}$ where *a* is the slope at low doses, $a = \frac{Max}{EC^{-\beta}}$ (Audebert et al., 2012).

Statistical tests were used to identify which PAHs were genotoxic and whether the model was adequate. Genotoxic PAHs were identified as showing a significant difference in fit between the 3-parameter Hill model and a Hill model with *Max* or β equal to 0, using an F-test. The goodness of fit of the 3-parameter Hill model (full model) was assessed with a lack-of-fit test, an approximate F-test which compares the dose-response model to the ANOVA model with a separate level for each dose (Bates and Watts, 1988; Ritz and Martinussen, 2011). Adequacy of a common Hill constant was checked by comparing the goodness of fit of the full model with that of the reduced model with a common Hill constant β for all genotoxic PAHs. The comparison was performed with an F-test analogous to the one used in nested models:

$$F = \frac{(SS_{M1} - SS_{M2})/(p_{M2} - p_{M1})}{SS_{M2}/(n-1-p_{M2})}$$

where *SS* is the residual sum of squares, *p* is the number of model parameters, and *n* is the number of observations. M1 refers to the reduced model and M2 refers to the full model.

Once genotoxicity of each compound had been assessed, genotoxicity equivalent factors (GEF) of each PAH compared to BaP were then calculated as in (Audebert et al., 2012) by first estimating a common value of the Hill constant β for all genotoxic PAHs and then calculating the β-root of the ratios of *a* values. Finally, the differences in GEFs between the three cell lines were analysed by calculating correlation coefficients between the logarithm of the GEFs.

Experimental designs

Individual dose-responses were tested at a minimum of three geometrically-spaced concentrations on independent plates, with at least three concentrations per plate. The concentrations were selected based on previous range-finding experiments. With LS-174T and NCI-H358 cell lines the highest concentration tested was at most 10µM. When high

cytotoxicity was observed (more than 50%), data was discarded from analysis. In LS-174T cells, the number of independent experiments for each compound varied from 8 to 56, most experiments being performed with 4 concentrations. With Hep3B and NCI-H358 cell lines, the number of experiments varied from 3 to 7.

Uncertainty estimation

The uncertainty around the slopes at low doses was estimated by a 2-level bootstrap: at each iteration, for both the genotoxic PAH and BaP, plates were sampled with replacement, and for each of the sampled plates, at each concentration, the two wells were sampled with replacement. The geometric mean of the two wells was modelled with a Hill model with a fixed Hill constant: the final points could be either one of the two original wells or the mean of the two. The GEF was calculated at each iteration based upon the slopes at low concentrations for the genotoxic PAH and BaP. The 95% confidence interval was estimated using the 2.5% and 97.5% quantiles.

Results

The sensitive genotoxic γH2AX assay was used to determine the genotoxicity of 27 different PAHs (Fig. 1) in three human cell lines (Hep3B, LS-174T and NCI-H358). All PAHs were analyzed for cytotoxicity and genotoxicity after 24 h treatment at different concentrations covering a four order of magnitude range. Only no and low-cytotoxic concentrations (cell viability > 50%) were analyzed for genotoxicity. Eleven compounds (ACT, ACTY, ANT, BghiP, COR, BeP, FLA, FLE, NAP, PHE and PYR) were found to be non-genotoxic, whatever the cell line tested (F- test, p>0.01) (Fig, S1-S3). In all cell lines, the most genotoxic tested compound was DBalP, with a genotoxic potential at concentrations 10^{-9} M and upwards in NCI-H358 and Hep3B cells and at concentrations 10^{-10} M and upwards in LS-174T cell line (Table 1). A clear genotoxic dose-response effect was observed for 16 PAHs tested in LS-174T cells (Table 1; Fig. S4). The estimated common Hill constant for the 16 genotoxic compounds was equal to -0.678; using this common slope resulted in a decrease in goodness of fit only for DBaeP and DBalP at a 1% significance threshold. The GEFs are reported together with the dose-response parameters in Table 2.

Unlike in LS-174 cells, BcF did not demonstrate genotoxic potential in the two other cell lines. CPP was not genotoxic in the NCI-H358 cell line and CPP and IPY were not genotoxic in the Hep3B cell line (Table 1). With the Hep3B cell line, for 6 compounds (5MC, BaP, DBaeP, DBahP, DBalP, and DMBA), the 2-parameter exponential model did not fit as well as the 3-parameter Hill model (Fig. S5). The estimated common Hill constant for the 13 genotoxic compounds in Hep3B was equal to -1.86 and was acceptable for all genotoxic PAHs (F-test, p>1%) (Table 2).

With the NCI-H358 cell line, for 8 compounds 5MC, BaA, BbF, CHR, DBaeP, DBahP, DBalP, and IPY), the 3-parameter Hill model was selected (Fig. S6). The estimated common

Hill constant for the 15 genotoxic compounds was equal to -1.01 and was acceptable for all genotoxic PAHs. The dose-response parameters and GEF are reported in Table 2.

We noted that for most compounds, GEFs calculated in LS-174T cells were lower than the GEFs calculated for the two other cell lines (Table 2). The correlation coefficients between the logarithms of the GEFs for genotoxic PAHs in all cell lines shows that Hep3B and NCI-H358 cell lines produce very similar GEFs (r=0.95) (Table 3). The LS-174T cell line produces slightly different GEFs (approximately r=0.8 on logGEFs of the two other cell lines) still demonstrating a good agreement between the results from the three cell lines. This good correlation between GEFs and between cell lines, demonstrated that despite different levels of GEFs in each cell line, partly due to variation in potency of the reference compound, relative genotoxicity of each tested PAH was conserved between cell lines. The greatest differences in relative genotoxicity are observed for DBaiP and BkF.

Discussion

In this study, we reported a detailed analysis of the genotoxicity of 27 different PAHs in three human cell lines derived from target organs of PAHs. The LS-174T intestinal cell line was on average more sensitive to the genotoxicity of PAHs than hepatoma Hep3B or lung NCI-H358 cell lines. This difference of PAHs genotoxicity between organs have also be observed *in vivo* (Long et al., 2016). PAHs are genotoxic after metabolization by some cytochrome P450 isozymes or aldo-keto reductases (Xue and Warshawsky, 2005). The difference, in terms of genotoxic sensitivity, between the three cell lines tested may be explained by the difference in CYP expression and induction (Iwanari et al., 2002; Li et al., 1998). Iwanari and co-workers found that in LS180 cells (parent cells from which LS-174T are derived), the constitutive expression levels of CYP1A1, 1A2, and 1B1, the main CYPs involved in PAHs metabolism, are expressed at higher levels than in other human-tissue derived cells lines such as MCF7, HepG2, ACHN or Hela. In addition, the induction of CYP1 family by PAHs was found to occur at a higher extent in LS180 cells compared to the other cell lines tested (Iwanari et al., 2002).

In our study, no statistically significant difference to control was shown for 11 PAHs (ACT, ACTY, ANT, BeP, BghiP, COR, FLA, FLE, NAP, PHE and PYR) on any of the three human cell lines tested. These data are in accordance with other studies showing that most of them were neither mutagenic nor genotoxic (Vaca et al., 1992, Durant et al. 1996; Sjogren et al. 1996; Long et al., 2016). Although the carcinogenic potential of some of them was considered as equivocal (Stocker et al., 1996; Wang and Busby, 1993; Wang et al., 1995), most of the published data do not support a carcinogenic effect of these PAHs (Table 4) (IARC, 2010). ACTY was not classified by IARC. ACT, ANT, BeP, BghiP, COR, FLA, FLE, PHE and PYR were classified 3 “not classifiable as to its carcinogenicity to humans” by IARC. Only NAP was classified 2B “possibly carcinogenic to humans”. However, different reviews on the

genotoxicity of NAP have concluded that this PAH is not mutagenic in bacterial or mammalian cell culture systems and is not genotoxic *in vivo* in either the liver or the bone marrow (Recio et al., 2012). In contrast, most of the 16 PAHs showing a genotoxic potential in our study were classified as 2A (CPP, DBahA and DBalP) or 2B (5MC, BaA, BbF, BjF, BkF, CHR, DBahP, DBaiP and IPY) by IARC (Table 4). The only exceptions were BcF and DBaeP that were classified in group 3. DMBA was not classified by IARC (IARC, 2010).

Formation of DNA adducts by genotoxic carcinogens and subsequent repair were expected to be proportional to dose as long as the rates of the enzymatic and non-enzymatic activation and inactivation reactions are all proportional to the substrate concentration. At low doses, this is modelled with a Hill constant equal to 1 or -1. With LS-174T cells, the Hill constant equal to -0.678 indicates supralinearity at low doses; with Hep3B cells, -1.86 indicates sublinearity at low doses whereas with NCI-H358 cells, the constant is close to -1, indicating linearity (Figure S7).

The GEFs calculated in this paper based on LS-174T cell line experimentations differ from those published in 2012 by a factor 3.6 on average (Table 4). This difference is due to the fact that here we modelled all concentration-responses with the same model whereas, in our previous study (Audebert et al., 2012), BaP and DBalP were modelled directly with the 2-parameter exponential model. With BaP, the reference for the calculation of the GEFs, the 2-parameter exponential function overpredicted the response for the three lowest concentrations thus yielding a lower estimate of the slope at low concentrations for BaP and consequently higher GEFs for the other PAHs. The rather high within-treatment variability of the data had been taken into account in the statistical test therefore leading to the conclusion that the goodness of fit of the 3-parameter Hill model was not significantly different from that of the 2-parameter model. The choice of concentration-responses model is critical for the estimation of the slope at low concentrations, far more than for the estimation of an EC_{50} in the case of

sigmoidal concentration-responses. With most PAHs a sigmoidal concentration-response relationship with a plateau at high concentrations could not be observed due to cytotoxicity at high concentrations. On the other hand, the inflexion point (*EC50*) was often readily observed, with some uncertainty. We therefore settled on the use of a common model for all PAHs with only the *Max* and *EC50* allowed varying from one to another. The slight mispredictions that can be made with the 2-parameter exponential model on one compound are likely to occur on other PAHs; the choice of a common model ensures that the same bias is obtained on all concentration-responses if the actual concentration-responses are similar. Another solution would consist in testing a large number of different models and selecting the best fitting model (Scholze et al., 2001). In our application, this could impair the calculation of the slope at low concentrations which, if estimable, would possibly not be valid for the same range of response levels. The fact that we modelled log fold-inductions without adjusting for heteroscedascity rather than fold-inductions taking heteroscedasticity into account only affects the GEF to a small extent although the concentration-response parameter estimates are different. The discrepancy for the BcF, pinpointed by our 2012 paper as being more genotoxic than expected, was likely due to a lack of purity of the commercial solution in this previous study: the present LS-174T GEFs are based on a larger amount of data.

The method we propose can be used to calculate Equivalency Factors even when the plateau of the concentration-response relationships is not observed. TEFs are calculated as a ratio of EC50. They are based on the assumption that the concentration-response curves are similar (same EC_{50}, same Max) and their values are independent of the response level chosen. GEFs on the other are not equivalent to TEFs based on a low proportion (e.g. 1%) of maximal response, as they are calculated as a ratio of slopes at low concentrations, the concentration-response curves being modelled with a common Hill constant estimated separately for each cell line. The GEFs are actually similar to TEFs based on a low response level (rather than a

proportion of the maximal response). The correlation coefficient between the logarithm of the GEF and the log-concentration producing a 0.02 log-fold-induction (1.05 fold induction) is 0.9996. The advantage of our method is that there is no need for a user-defined response level as we rely upon function approximation. Our method could be applied by only using data from the low dose area of the concentration-response curve, with more closely spaced dilutions. In this case, the 2-parameter function could be used directly. The experimental design would however require a more precise design prior knowledge of the location of the low concentration area or of the "point of departure" and may therefore require a higher experimental effort.

The LS-174T cell line was the most responsive one as 16 PAHs demonstrated genotoxicity, compared to 14 in NCI-H358 and 13 in Hep3B cell lines. The LS-174T cells were also the only ones to show supralinearity at low concentrations, thus making it a more suitable biological model for studying low dose effects. The important cytotoxicity observed in Hep3B at high concentrations makes it unsuitable for a reliable analysis of the concentration-response of the least genotoxic compounds at high concentrations. On this basis, LS-174T cell line appears as a good candidate for further study of the genotoxicity of PAH, notably in mixtures. Table 4 shows the GEFs together with their confidence interval determined by bootstrap for each compounds in each cell line. Calculated GEF for the tested PAHs were quite similar in all cell lines with in most case an overlap of the confidence interval (Table 4). Generally, GEFs were higher than the TEF usually used, notably for BaA, BbF and CHR (Nisbet and LaGoy, 1992).

In a previous study, we started to model the GEFs for a limited number of PAHs, tested for genotoxicity in two different cell lines, HepG2 and LS-174T (Audebert et al., 2012). In the current article, the approach was expanded to a larger number of compounds and the genotoxicity was tested on two different cell lines, in addition to LS-174T cells. As reported

by (Delistraty, 1997; Nisbet and LaGoy, 1992), our calculation is based on cancer-related endpoint data, and our approach assumes that carcinogenic PAHs have a toxicological mode of action similar to the model compound BaP. In addition to the fact that the data we have produced are based on metabolically active human cell lines derived from the main PAH target tissues and that γH2AX assay allows genotoxicity testing at low concentrations, these data offer the advantage of resulting from identical bioassay systems and from the same mathematical treatments of the set of concentration-response curves, which makes the results highly comparable. These methodological advances as well as the large number of PAHs tested largely respond to the uncertainties highlighted by EFSA regarding the TEFs reported so far in the literature (EFSA, 2008). The values we have obtained are all based on the genotoxicity of PAHs and can be considered as predictive of the carcinogenic potential of these substances. As recommended by the US EPA (1993), these values should therefore only be used in relation to the carcinogenicity of PAHs, which is, however, the most sensitive effect observed for these compounds. Conversely, the approach used by EFSA in its 2008 risk assessment has significant uncertainties (EFSA, 2008). The EFSA CONTAM Panel based the risk characterization on a two years experimental study carried out in rats orally exposed to a complex PAH mixture (coal tar) or to B(a)P (Culp et al., 1998). In addition to the limitations related to animal-human extrapolations, the CONTAM Panel assumed that the PAH mixture tested by Culp and co-workers was representative of the dietary exposure of consumers to PAHs, but it is likely that the data provided Culp et al. (1998) are specific to the mixtures tested. In order to improve the consistency of GEF approach in risk assessment, it is obvious, that toxicokinetic adjustments are needed to account for both routes of exposure and relative concentrations in target tissues in the calculation of our TEFs.

In addition to the incorporation of toxicokinetic descriptors, the next step of this work will be to investigate the genotoxicity of these PAHs as binary or complex mixtures, in order to

determine how interactions between different hydrocarbons can affect the cancer-related endpoint responses.

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Conflict of interest statement

The authors are not aware of any conflicts of interest.

Author contributions

T.F. and G.V. performed all of the genotoxicity screening experiments. Data analysis and mathematical modelling were performed by T.C. Z.F. and P.A. M.A. and C.JP. planned and supervised the project. The manuscript was written by M.A. and C.JP. All the authors discussed the results and contributed to editing of the manuscript.

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Table 1: Lowest effective concentration (in µM) for γH2AX induction for each genotoxic PAH in the three cell lines tested.

Compound	CAS number	NCI-H358	Hep3B	LS-174T
5-Methylchrysene (5MC)	3697-24-3	10^{-7}	10^{-7}	10^{-7}
7,12-Dimethylbenz(*a*)anthracene (DMBA)	57-97-6	10^{-7}	10^{-7}	10^{-8}
Benz(*a*)anthracene (BaA)	56-55-3	10^{-6}	10^{-6}	10^{-7}
Benzo(*a*)pyrene (BaP)	50-32-8	10^{-7}	10^{-7}	3.10^{-8}
Benzo(*b*)fluoranthene (BbF)	205-99-2	10^{-7}	10^{-7}	10^{-7}
Benzo(*c*)fluorene (BcF)	205-12-9	nd	nd	10^{-6}
Benzo(*j*)fluoranthene (BjF)	205-82-3	10^{-6}	10^{-6}	10^{-6}
Benzo(*k*)fluoranthene (BkF)	207-08-9	10^{-6}	10^{-6}	10^{-6}
Chrysene (CHR)	218-01-9	10^{-6}	10^{-6}	10^{-7}
Cyclopenta(*c,d*)pyrene (CPP)	27208-37-3	nd	nd	10^{-6}
Dibenz(*a,h*)anthracene (DBahA)	53-70-3	10^{-7}	10^{-7}	10^{-7}
Dibenzo(*a,e*)pyrene (DBaeP)	192-65-4	10^{-7}	10^{-7}	10^{-8}
Dibenzo(*a,h*)pyrene (DBahP)	189-64-0	10^{-8}	10^{-7}	10^{-8}
Dibenzo(*a,i*)pyrene (DBaiP)	189-55-9	10^{-8}	10^{-7}	10^{-6}
Dibenzo(*a,l*)pyrene (DBalP)	191-30-0	10^{-9}	10^{-9}	10^{-10}
Indeno(*1,2,3-c,d*)pyrene (IPY)	193-39-5	10^{-6}	nd	10^{-6}

Table 2: Parameters of individual PAH dose-responses with a common Hill constant.

	LS-174T				Hep3B				NCI-H358			
PAH	Max	EC50	slope at low dose	GEF	Max	EC50	slope at low dose	GEF	Max	EC50	slope at low dose	GEF
5MC	0.726	0.635	0.988	0.48	0.748	0.129	33.6	1.83	0.38	0.116	3.33	1.95
BaA	0.495	0.394	0.930	0.44	0.339	0.197	6.93	0.78	0.259	0.187	1.41	0.83
BaP	0.511	0.180	1.63	1	0.846	0.253	10.9	1	0.632	0.376	1.69	1
BbF	0.647	0.371	1.27	0.69	0.496	0.13	22.2	1.46	0.366	0.171	2.17	1.28
BcF	0.572	2.36	0.320	0.09	0	Inf	0	0	0	Inf	0	0
BjF	0.474	0.628	0.649	0.25	44.1	14.6	0.3	0.15	0.181	0.367	0.496	0.30
BkF	0.351	1.37	0.284	0.07	0.237	0.211	4.29	0.61	0.208	0.328	0.638	0.38
CHR	0.530	0.646	0.712	0.29	2.38	2.98	0.31	0.15	0.257	0.429	0.605	0.36
CPP	0.135	0.293	0.311	0.08	0	Inf	0	0.93	0	Inf	0	0
DBaeP	0.678	0.090	3.47	3.04	0.543	0.069	78.2	2.89	0.242	0.052	4.73	2.77
DBahA	0.555	0.403	1.03	0.50	0.37	0.086	35	1.87	0.329	0.071	4.72	2.77
DBahP	0.335	0.025	4.1	3.89	0.362	0.026	325	6.21	0.385	0.010	39.5	22.74
DBaiP	0.182	0.113	0.799	0.35	0.245	0.032	147	4.05	0.515	0.026	20.1	11.64
DBalP	1.18	0.027	13.6	22.9	0.83	0.008	6220	30.35	0.708	0.003	244	139
DMBA	0.71	0.101	3.36	2.90	0.59	0.085	57.1	2.44	0.36	0.057	6.41	3.75
IPY	0.303	0.362	0.602	0.23	0	Inf	0	0	0.198	0.33	0.606	0.36

Inf: Infinite

Table 3: Pearson correlation coefficients between the logarithms of the GEFs calculated on the three cell lines.

	Hep3B	NCI-H358	LS-174T
Hep3B	1		
NCI-H358	0.95	1	
LS-174T	0.78	0.81	1

Table 4: Classification and toxicity relative to benzo(a)pyrene of the tested PAHs.

Compound	CAS number	IARC	TEF[a]	TEF[b]	GEF in LS-174T[c]	GEF in Hep3B[c]	GEF in NCI-H358[c]
5-Methylchrysene	3697-24-3	2B	nd	nd	0.48 [0.14; 0.56]	1.83 [1.4 ; 2]	1.95 [0.56; 2.9]
7,12-Dimethylbenz(*a*)anthracene	57-97-6	nd	nd	nd	2.9 [1.8; 3.8]	2.44 [1.9; 2.6]	3.75 [0.95; 6.0]
Acenaphthene	83-32-9	3	0.001	nd	0	0	0
Acenaphthylene	208-96-8	nd	0.001	nd	0	0	0
Anthracene	120-12-7	3	0.01	nd	0	0	0
Benz(*a*)anthracene	56-55-3	2B	0.1	0.145	0.44 [0.3; 0.71]	0.78 [0.27; 1.1]	0.83 [0.26; 1.2]
Benzo(*a*)pyrene	50-32-8	1	1	1	1	1	1
Benzo(*b*)fluoranthene	205-99-2	2B	0.1	0.140	0.69 [0.31; 0.74]	1.46 [0.17; 2.2]	1.28 [0.37; 2.1]
Benzo(*c*)fluorene	205-12-9	3	nd	nd	0.09 [0.06 ; 0.18]	0	0
Benzo(*e*)pyrene	192-97-2	3	nd	0.004	0	0	0
Benzo(*ghi*)perylene	191-24-2	3	0.01	0.022	0	0	0
Benzo(*j*)fluoranthene	205-82-3	2B	nd	0.061	0.26 [0.15; 0.53]	0.15 [0.12; 0.22]	0.30 [0.068; 0.42]
Benzo(*k*)fluoranthene	207-08-9	2B	0.1	0.066	0.076 [0.058; 0.24]	0.61 [0.10; 1.1]	0.38 [0.089; 0.74]
Chrysene	218-01-9	2B	0.01	0.0044	0.29 [0.12; 0.45]	0.15 [0.11; 1.2]	0.36 [0.10; 0.52]
Coronene	191-07-1	3	0.001	nd	0	0	0
Cyclopenta(*c,d*)pyrene	27208-37-3	2A	0.1	0.023	0.086 [0.024; 0.13]	0	0
Dibenz(*a,h*)anthracene	53-70-3	2A	5	1.11	0.50 [0.29; 0.74]	1.87 [1.1; 3.4]	2.77 [0.65; 4.4]
Dibenzo(*a,e*)pyrene	192-65-4	3	nd	nd	3.0 [2.6; 6.6]	2.89 [2.2; 3.3]	2.77 [0.96; 3.8]
Dibenzo(*a,h*)pyrene	189-64-0	2B	nd	nd	3.9 [2.3; 7.5]	6.21 [2.9; 6.2]	22.7 [7.2; 31]
Dibenzo(*a,i*)pyrene	189-55-9	2B	nd	nd	0.35 [0.057; 2.8]	4.05 [1.1; 5]	11.6 [2.6; 18]
Dibenzo(*a,l*)pyrene	191-30-0	2A	nd	nd	23 [5.5; 73]	30.3 [22; 69]	139 [36; 200]
Fluoranthene	206-44-0	3	0.001	nd	0	0	0
Fluorene	86-73-7	3	0.001	nd	0	0	0
Indeno(*1,2,3-c,d*)pyrene	193-39-5	2B	0.1	nd	0.23 [0.11; 0.47]	0	0.36 [0.070; 0.55]
Naphtalene	91-20-3	2B	0.001	nd	0	0	0
Phenanthrene	85-01-8	3	0.001	nd	0	0	0
Pyrene	129-00-0	3	0.001	nd	0	0	0

nd: Not determined

[a] from (Nisbet and LaGoy, 1992)

[b] from (US-EPA, 1988)

[c] from this study

Figure Legends

Figure 1. Structures of the different PAHs tested in this study.

Figure

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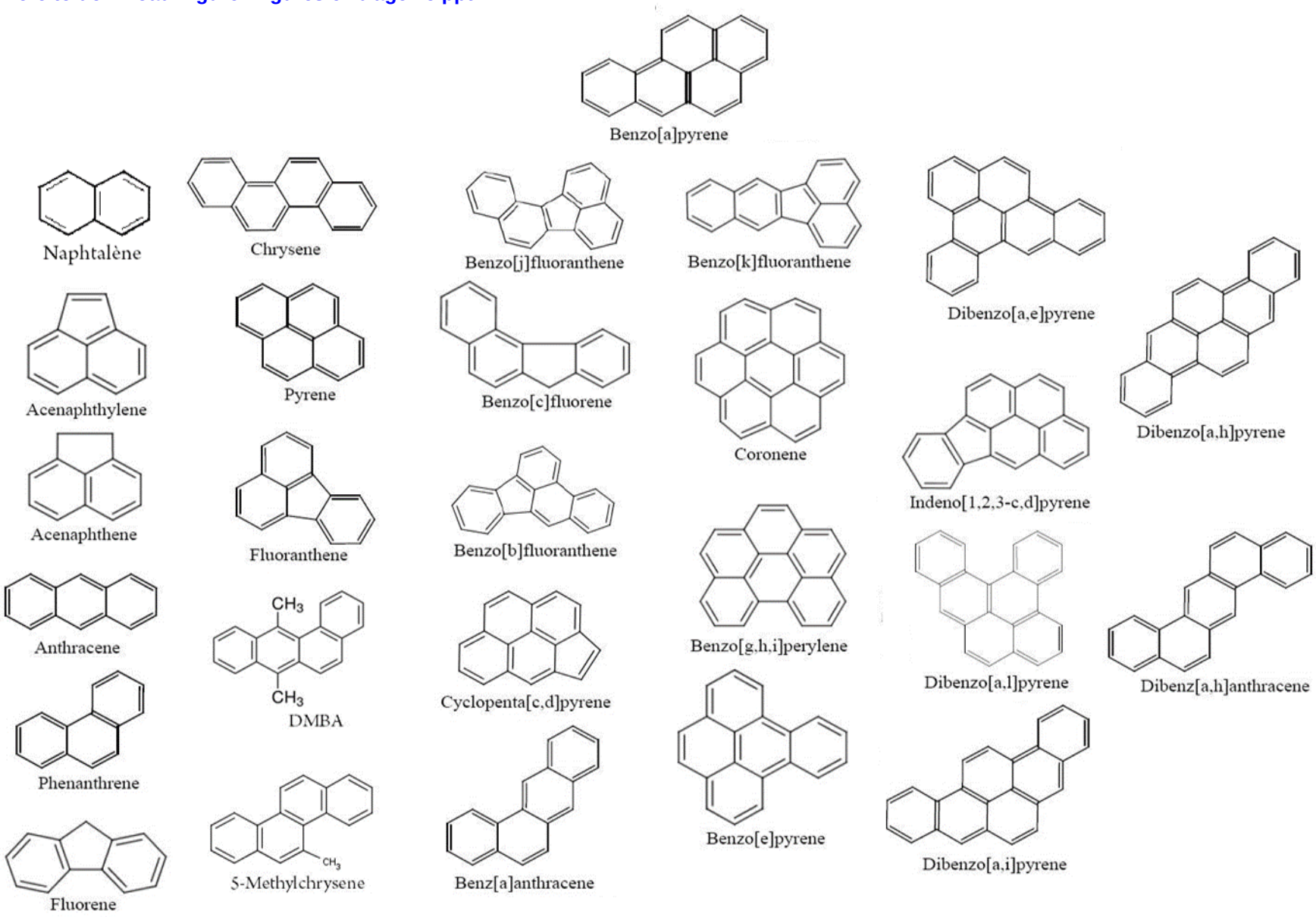

Fig 1.Tomasetig et Al.