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Quantitative comparison between *in vivo* DNA adduct formation from exposure to selected DNA-reactive carcinogens, natural background levels of DNA adduct formation and tumour incidence in rodent bioassays

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This study aimed at quantitatively comparing the occurrence/formation of DNA adducts with the carcinogenicity induced by a selection of DNA-reactive genotoxic carcinogens. Contrary to previous efforts, we used a very uniform set of data, limited to *in vivo* rat liver studies in order to investigate whether a correlation can be obtained, using a benchmark dose (BMD) approach. Dose–response data on both carcinogenicity and *in vivo* DNA adduct formation were available for six compounds, i.e. 2-acetylaminofluorene, aflatoxin B1, methyleugenol, safrole, 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline and tamoxifen. BMD_{10} values for liver carcinogenicity were calculated using the US Environmental Protection Agency BMD software. DNA adduct levels at this dose were extrapolated assuming linearity of the DNA adduct dose response. In addition, the levels of DNA adducts at the BMD_{10} were compared to available data on endogenous background DNA damage in the target organ. Although for an individual carcinogen the tumour response increases when adduct levels increase, our results demonstrate that when comparing different carcinogens, no quantitative correlation exists between the level of DNA adduct formation and carcinogenicity. These data confirm that the quantity of DNA adducts formed by a DNA-reactive compound is not a carcinogenicity predictor but that other factors such as type of adduct and mutagenic potential may be equally relevant. Moreover, comparison to background DNA damage supports the notion that the mere occurrence of DNA adducts above or below the level of endogenous DNA damage is neither correlated to development of cancer. These data strongly emphasise the need to apply the mode of action framework to understand the contribution of other biological effect markers playing a role in carcinogenicity.

Introduction

Cancer induced by exogenous as well as endogenous sources of chemicals acting through genotoxic mechanisms is a multistep process, which needs a number of critical events before the adverse effect is developed (1). Briefly, it involves an initiation step damaging the DNA and/or forming DNA adducts, followed by at least one round of DNA synthesis to fix the genetic damage. The promotion stage which is a reversible step is characterised by genetic instability and clonal expansion (2) stimulating the induction of cell proliferation, resulting in the formation of an identifiable focal lesion. Progression, considered an irreversible process, is the final step characterised by accumulation of additional genetic damage and cell proliferation. Formation of endogenously damaged DNA bases has been measured and quantified in various experimental studies. This damage has been reported to result from damage induced by oxidative stress, with reactive oxygen species being a major source for formation of damaged DNA bases (3). Furthermore, DNA damage may result from adduct formation by endogenous alkylating electrophiles, such as by-products resulting from lipid peroxidation (3). In addition to these endogenous factors, an important factor contributing to the occurrence of DNA damage can be found in exposure to exogenous DNA-reactive carcinogens. DNA adduct formation, although involved in the process of cancer formation, is generally considered a biomarker of exposure (4–6) rather than a biomarker of effect although it is also well recognised that increased levels of DNA adduct formation reflect a risk factor for cancer development. Progress has been made in understanding the mode of action (MOA) of DNA-reactive carcinogens but still not all pathways are clearly defined. The formation of DNA adducts is considered a prerequisite of cancer induction by DNA-reactive genotoxic carcinogens, which, with or without metabolic conversion to a reactive metabolite can react with DNA to form adducts resulting in mutation and eventually carcinogenic transformation (7). Several attempts have been made to correlate the occurrence of DNA adducts with the carcinogenic outcome, but the significance of their formation in the risk assessment, especially with respect to the discussion and justification of possible thresholds is a matter of ongoing debate (8,9).

As reported by Boobis *et al*. (10), there are several critical key events in the MOA of DNA-reactive carcinogens: (i) exposure of the target cells to the ultimate reactive and mutagenic species, (ii) reaction with DNA in the target cells, (iii) misreplication or misrepair of the lesion and (iv) mutations in critical genes in replicating target cells, etc. (clonal expansion, further mutations, uncontrolled growth, progression, malignancy). DNA-reactive carcinogens, directly or upon metabolic activation, can covalently bind to cellular DNA. DNA adducts occur at nucleophilic sites in the DNA strands (5), and this process can result in the formation of different types of adducts (5). DNA adduct formation can be a reversible process since living organisms contain DNA repair mechanisms that are able to repair DNA damage induced by endogenous and exogenous electrophiles. These DNA repair mechanisms are supported by ~130 genes involved in different distinct repair processes (11), including for example, direct

repair, mismatch repair, base excision repair, nucleotide excision repair and homologous recombination repair (11–13). If not repaired, DNA lesions can be misread by DNA polymerases, leading to heritable mutations (5). While DNA adducts are a biomarker of exposure (4–6), mutations are considered biomarkers of effect (6). In an attempt to fill the gap between DNA adduct formation as a biomarker of exposure and tumour incidences as a biomarker of effect, the aim of the present paper was to make a quantitative comparison between data on *in vivo* DNA adduct formation and on tumour incidences taking into account reported endogenous background levels of DNA adduct formation.

Methodology

Compound selection

DNA-reactive carcinogens included in the present study were selected based on the following criteria: (i) available data on *in vivo* DNA adduct formation and (ii) available dose–response data for tumour induction, both in the same target tissue, the liver, for the same species, the rat, and for the same gender, after oral administration (by gavage, feed or water). Data were searched using the ISI Web of Science (14), PubMed (15), the Carcinogenic Potency Database (CPDB) (16–18) and the database of the US National Toxicology Program (19).

Benchmark dose software

The tumour incidence data obtained were modelled with the benchmark dose (BMD) software version 2.1.1 developed by the US Environmental Protection Agency (EPA) (20). The BMD methodology was developed by Crump (21) and is based on statistically fitting a dose–response model to experimental data. The advantage of applying the BMD software in analysing tumour incidence data is that all information of the dose–response curve is taken into account (20–22). The BMD level thus defined is not limited to an experimental dose level and is defined as the dose producing a defined change in response, for example 10% response, above background level defined as the Benchmark Response (BMR) 10%. The parameters for the computer modelling, using dichotomous models, were set as default predefined in the EPA software: 95% confidence limit, extra risk and the BMR value set at 0.1. The BMD_{10} values were expressed as milligrams per kilogram body weight per day for each mathematical model. The following dichotomous models were selected: gamma, logistic, log-logistic, log-probit, multistage, probit, quantal-linear and Weibull. These models are each represented by a specific mathematical equation (20–22). Models were accepted or rejected based on the log-likelihood. The log-likelihood ratio test is based on the log-likelihood value associated with a fitted model compared with, and tested against, the log-likelihood value associated with the so-called 'full model' (20–22). The full model simply consists of the observed (mean) responses at each applied dose (19,20). Hence, the number of parameters equals the number of dose groups. If a model fit is not significantly worse than that of the full model, then the model may be accepted (20–22). Furthermore, an extra criteria for accepting or rejecting the models was the *P*-value. When the *P*-value was >0.1, the models were accepted (20–22), and only those BMD_{10} values were further used to estimate DNA adduct levels. In the case that several models provide an adequate fit to a given data set, the goodness-of-fit was further evaluated on the basis of the Akaike Information Criterion (AIC) value. The AIC is a deviant of the log-likelihood and penalises a model for having too many parameters (i.e. degrees of freedom), thus allowing to compare the goodness-of-fit between different models. Lower AIC values indicate a better fit within a data set (20–22).

Aspects related to ^{32}P-postlabelling

In order to use data set as uniform as possible, experimental studies using similar analytical methods for detection and quantification were applied. For four carcinogens, data on total DNA adduct levels were available, generated using ^{32}P-postlabelling followed by thin layer chromatography (TLC) separation; for methyleugenol (ME), the separation step was not specified. The types of adduct(s) formed were in most cases not characterised. ^{32}P-postlabelling is a highly sensitive method which needs only small amounts of DNA (23,24) and it has been the most widely used method for the detection of DNA adducts. However, a limitation of the ^{32}P-postlabelling method is the potential for underestimation of the adduct levels due to incomplete DNA digestion, submaximal efficiency of adduct labelling by polynucleotide kinase and/or loss of adducts during enrichment and chromatography stages (25). For aflatoxin B1 (AfB1), available data were generated using ^{3}H-HPLC, the *N*-7 guanine is the predominant adduct formed by AfB1, which is poorly labelled by ^{32}P-postlabelling technique (25).

Dose adjustment for cancer bioassays

In order to correct the doses and the duration of treatment to the standard lifespan (2 years or 104 weeks by convention), we followed the recommendations of Peto *et al*. (26) and Gold *et al*. (27). Two principal situations of dose adjustments exist: in the first situation, administration of the test compound is discontinued before the terminal sacrifice after completion of the full duration of 2 years. In this case, the dose is adjusted by multiplying with $f =$ (period of active treatment)/(actual time on test, i.e. 104 weeks). This was the case for the highest dose group in the ME study, which was stopped after 53 weeks due to toxic side effects and continued to 104 weeks without treatment (26). This dose was, therefore, adjusted by $f =$ (53/104). In the second principal situation, both the treatment and the study are terminated before completion of the standard lifespan, e.g. after 52 weeks. In this case, the f^2 correction factor is used where $f =$ (experiment time)/(standard lifespan). This was the case for the AfB1 study, where all except the lowest dose groups were terminated 'prematurely' between 54 and 93 weeks due to mortality (26,28). For the 'mixed' situation, where the study is terminated before completion of the 104 weeks, and treatment is even shorter, the adjustment was done by correcting for [(period of active treatment)/(standard lifespan)] $\times$ [(experiment time)/(standard lifespan)]. Thus, in cases where treatment time equals study duration this corresponds exactly to the f^2 approach.

When dosing was done during only 5 days of the week, as was the case for ME, the dose was corrected by $f = 5/7$.

Results

Compound selection

DNA-reactive carcinogens in the present study were selected based on (i) available data on *in vivo* DNA adduct formation and (ii) available dose–response data for tumour induction, both in the same target tissue, the liver, for the same species,

the rat, and for the same gender. *In vivo* DNA adduct formation data were available for a large number of carcinogens. However, the number of *in vivo* studies on tumour formation in the same species, sex and target tissue for which data also on *in vivo* DNA adduct formation were available, were limited. Only six DNA-reactive carcinogens were identified for which data on both tumour incidence and *in vivo* DNA adduct formation in rat liver were available. The compounds selected were: 2-acetylaminofluorene (2-AAF) (CAS # 53-96-3); AfB1 (CAS # 1162-65-8); ME (CAS # 93-15-2); safrole (SA) (CAS # 94-59-7); 2-amino-3,8-methylimidazo[4,5-f]quinoxaline (MeIQx) (CAS # 77500-04-0) and tamoxifen (TAM) (CAS # 54965-24-1). Even though the compounds were selected as to obtain relatively uniform data set regarding target tissue, species and gender, important variations were observed in the available information with respect to study design, such as duration (5 days up to 18 months), dosing (water, diet, gavage), group size ($\geq$2) and analytical methods employed. This is especially relevant concerning the DNA adduct data, for which (in contrast to, e.g. a 2 years cancer bioassay), no guideline study protocols exist.

Data on in vivo DNA adduct formation

Table I recapitulates the data collected for the dose dependent *in vivo* DNA adduct formation of the selected DNA-reactive carcinogens (2-AAF, AfB1, ME, SA, MeIQx and TAM) focussing on liver as the target organ. All data sets collected for the individual carcinogens were from rats, with the data for five of the six carcinogens being obtained in male rat liver, whereas those for TAM were obtained in female rat liver. Table I presents information on the data collected including information on dose in milligrams per kilogram body weight per day, exposure route and duration, amount of DNA adducts formed, type of adduct detected, method of analysis, species and gender. Exposure duration, dose ranges applied and mode of administration appear to vary between the studies available for the different model compounds. For instance, study duration varies from as short as 5 days (SA) up to 18 months of exposure (TAM). Due to the paucity of available data on formation kinetics, DNA adduct levels were assumed to have reached near steady state and were used as such without any normalisation or adjustments. Therefore, data from the longest available treatment duration were used. For SA, the longest available treatment duration was 5 days and this may be borderline to our selection criteria. The steady-state level of DNA adducts are a function of dose and adduct produced in a cell, modulated by processes linked to drug-metabolising enzymes that activate or detoxify the genotoxic compound. Furthermore, steady-state levels are influenced by the type of DNA adduct and the DNA adduct half-life (36). However, using a physiologically based kinetic (PBK) model for SA (37), which is based on a published PBK model for the related alkenylbenzene estragole (38,39), it can be estimated that at the dose levels used for DNA adduct analysis by Daimon *et al.* (32) of 62.5, 125 and 250 mg/kg bw/day, full conversion of SA will be obtained in 60, 62 and 70 h, respectively. Furthermore, Miller *et al.* (40) reported that the metabolite 1′-sulfooxysafrole has a half-life of 1 min and quickly binds to DNA. Therefore, we conclude that the biological responses towards SA may be rapid and may result in a steady state in DNA adduct levels in a relatively short period of time.

To allow comparison, Table I presents all data on adduct formation expressed as number of adducts in 10^8 nucleotides (nt). A graphical representation of the dose–response curves is shown in Figure 1 that appears to fit linear dose responses in accordance with the current scientific view on low dose linearity of DNA formation (6,9) (Figure 1). Therefore, and due to the lack of data points to perform a statistical curve fit with sufficient confidence, no non-linear curve fit was applied to the DNA adduct dose–response data. Instead, linear regression through the origin was applied to all data sets without any transformation. The linear regression gave satisfactory results for all six compounds ($P < 0.005$) based on the residual plot and the P-value from the run test ($P > 0.5$). The data obtained allow comparison of the relative potency of the six DNA-reactive carcinogens to form DNA adducts. To allow such a comparison, a normalisation for dose was applied by calculating for all six compounds the expected levels of DNA adduct formation at a dose of 1 mg/kg bw/day using the dose–response data plotted in Figure 1. This results in the following decreasing order for DNA adduct formation at a dose of 1 mg/kg bw/day: AfB1 (40480 adducts/10^8 nt) > 2-AAF (24002 adducts/10^8 nt) > MeIQx (159 adducts/10^8 nt) > TAM (59 and 15 adducts/10^8 nt,

Table I. Published DNA adduct data, including dose, exposure route, exposure duration, number of adducts detected in the liver, adduct type, method of detection, species and gender

Compound	Daily dose (mg/kg/day)	Exposure route	Exposure duration	Number of adducts/10^8 nt	Adduct type	Method	Species/ gender	Reference
2-AAF	0-0.0011-0.011-0.333[a]	Gavage	12 w*16 w	4-9-40-8000	Total adducts	^{32}P-postlab-TLC	Rat/M	(29)
AfB1	$2.2*10^{-6}$–$73*10^{-6}$–$2110*10^{-6}$	Water	8 w	0.091-3.2-85	Aflatoxin-DNA adducts	^{3}H/HPLC	Rat/M	(30)
ME	1-5-50	Diet	28 d	0(ND)-1-50	Total adduct	^{32}P-postlab	Rat/M	(31)
SA	0-1-10-100-250-500	Gavage	1 d	7-9-36-163-303-720	Total adduct	^{32}P-postlab-TLC	Rat/M	(32)
	0-62.5-125-250	Gavage	5 d	27-430-673-898	Total adduct	^{32}P-postlab-TLC	Rat/M	(32)
MeIQx	0.0013-0.013-0.134-1.26[b]	Diet	1 w	0.399-2.81-33.6-390	Total adduct	^{32}P-postlab-TLC	Rat/M	(33)
	0.0023-0.023-0.235-2.2[c]	Diet	12 w	1-10-100-1000[d]	Total adduct	^{32}P-postlab-TLC	Rat/M	(33)
TAM1	0-20-40[e]	Diet	18 m	0-300-610	Total adduct	^{32}P-postlab-TLC	Rat/F	(34)
TAM2	4-20-40[e]	Diet	8 w	225-1050-2000[d]	Total adduct	^{32}P-postlab-TLC	Rat/F	(35)

ND: not detected (<1/10^8 nt); M, male; F, female, d, days; w, weeks; m, months.
[a]Converted from cumulative doses.
[b]Converted from total daily doses assuming a body weight of 200 g.
[c]Converted from total daily doses assuming a body weight of 350 g.
[d]Estimated from a graph.
[e]Converted from 50–250 to 500 p.p.m. TAM in feed using 0.02 kg daily intake and 0.25 kg bw.

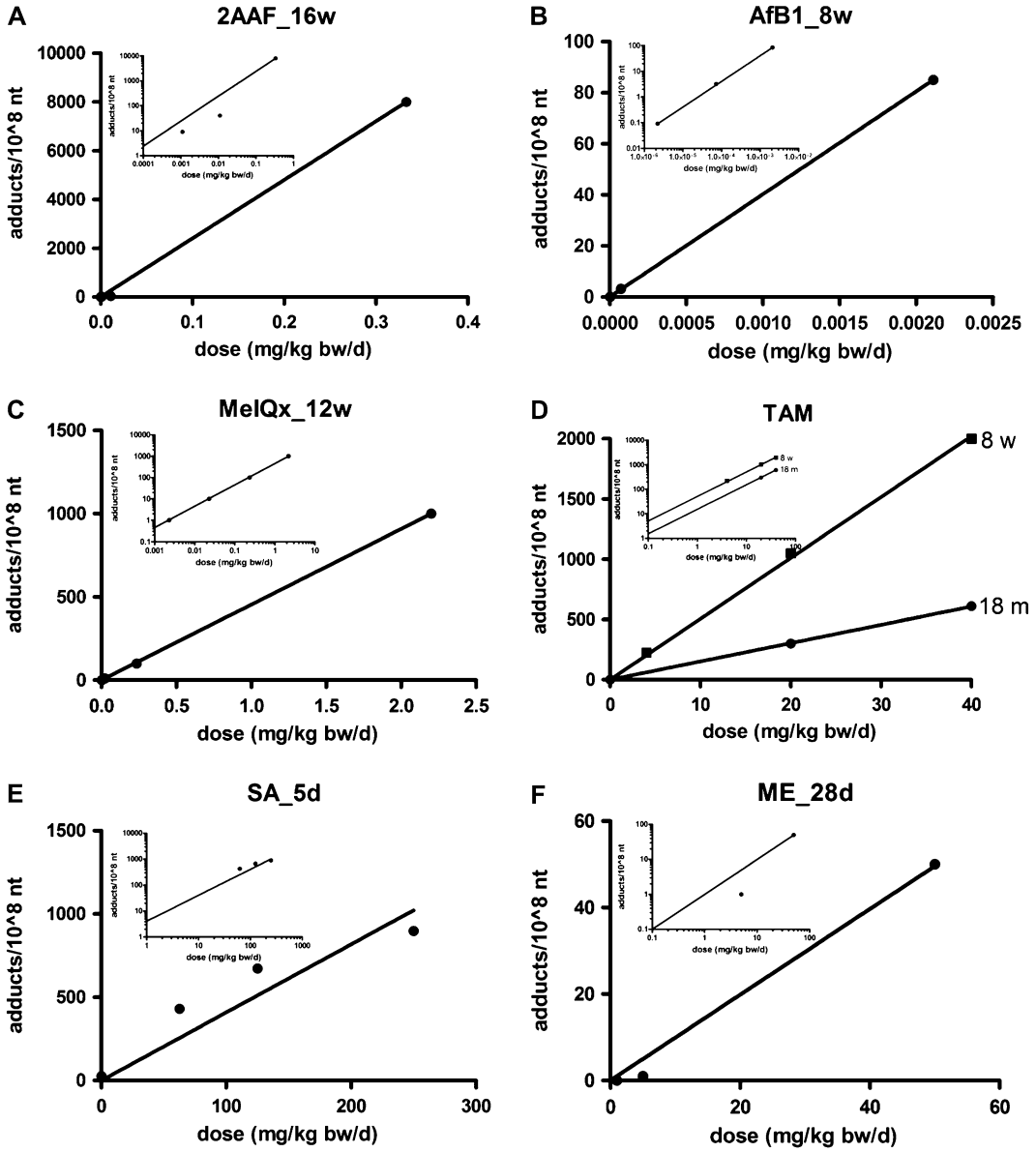

Fig. 1. Dose-dependent DNA adduct formation (number of adducts in 10^8 nt), in the liver as obtained from *in vivo* studies in male rats treated with: (**A**) 2-AAF, (**B**) AfB1, (**C**) MeIQx, (**E**) SA and (**F**) ME and in female rats treated with (**D**) TAM (34,35). For all six DNA-reactive carcinogens, linearity was assumed resulting in the following slope [(#adduct 10^8 nt)/(mg/kg bw/day)] factors, (m): 2-AAF, $m = 24002$; AfB1, $m = 40480$; ME, $m = 0.9917$; SA, $m = 4.0899$; MeIQx, $m = 454.22$; TAM, $m = 18.24$ and TAM, $m = 54.45$. Inserts are log-log plots of doses and DNA adduct levels.

respectively) (34,35) >SA (4 adducts/10^8 nt) > ME (1 adducts/10^8 nt). Assuming that the level of DNA adduct formation will increase over time in a linear way, with the number of adducts formed each day being equal to the number formed minus the number that are lost or repaired (6), one could further normalise the data to a 1-day exposure, in order to be able to compare the relative potency between compounds. The values obtained above for the total number of adducts/10^8 nt at a dose of 1 mg/kg bw/day were thus converted to numbers of adducts/10^8 nt/day by dividing the total numbers by the exposure duration; resulting in the following decreasing potency order: AfB1 (723 adducts/10^8 nt/day) > 2-AAF (214 adducts/10^8 nt/day) > MeIQx (1.89 adducts/10^8 nt/day) > TAM (1 adducts/10^8 nt/day) (35) > SA (0.82 adducts/10^8 nt/day) > ME (0.035 adducts/10^8 nt/day) > TAM (0.03 adducts/10^8 nt/day) (34). Together these data point at a different potency for each individual compound to result in DNA adduct formation, with AfB1 and 2-AAF being at least 2–3 orders of magnitude more effective in forming DNA adducts than MeIQx and SA and even 4 orders of magnitude more effective than ME and TAM.

It should be noted that rats treated with TAM in the study by Li *et al.* (34) had undergone partial hepatectomy before treatment, which, by inducing liver proliferation, may have influenced the levels of DNA adduct formation.

Tumour incidence data and BMD_{10} calculation

To allow quantitative comparison of dose levels causing DNA adduct formation and dose levels causing a significant increase above background levels in tumour incidence, data from long-term studies on tumour incidence induced by the selected set of model DNA-reactive carcinogens in rodent species were collected from the literature. Table II presents an overview of

Table II. Published tumour incidence data, including dose, exposure route, exposure duration (w = weeks, m = months), the dose adjusted to 2 years of duration and exposure, tumour incidences in the liver, type of lesion, species and gender

Compound	Dose (mg/kg bw/day)	Exposure route	Exposure duration (weeks)	Time adjusted dose (mg/kg bw/day)[a]	Liver tumor incidence	Type of lesion in the liver	Species/ gender	Reference
2-AAF	0-0.32-1.6-8	Diet	104	0-0.32-1.6-8	0/30-3/29-26/28-23/23	hpc	Rat/M	(41)
AfB1	0-0.001-0.005-0.015-0.05-0.1	Diet	104-104-93-96-82-54	0-0.00004-0.00016-0.00051-0.00124-0.00108[b]	0/18-2/22-1/22-4/21-20/25-28/28	hpc	Rat/M	(42)
ME	0-37-75-150-300	Gavage	104-104-104-53	0-26.4-53.6-107.1-101.85[b]	2/50-3/50-14/50-25/50-36/50	hpc	Rat/M	(43)
SA	0-4-20-40-200	Diet	104	0-4-20-40-200	3/25-1/25-2/25-2/25-7/25	tum	Rat/M	(44)
MeIQx	0-4-8-16	Diet	56	0-1.16-2.32-4.64	0/15-0/30-13/29-15/16	hpc	Rat/M	(45)
TAM	0-5-20-35	Gavage	104-104-87-71	0-5-14-16.31	0/104-6/52-37/52-37/52	hpc	Rat/F	(46)
TAM	0-11.3-22.6	Gavage	52	0-2.83-5.65	0/18-16/36-24/24	hpc	Rat/F	(47)

F, female; M, male; hpa, hepatocellular adenoma; hpc, hepatocellular carcinoma; tum, different liver tumour; w, weeks; m, months.
[a]For each individual data set, the dose was adjusted to the time of treatment (28), to the duration of the *in vivo* study and to the lifespan of rats of 104 w (for further explanations, see text).
[b]Highest dose was not used when performing the BMD analysis.

in vivo hepatic tumour incidence data obtained in rats as reported in the literature. Available tumour incidence data from carcinogenicity studies of the six DNA-reactive compounds (2-AAF, AfB1, ME, SA, MeIQx and TAM) are presented including information on dose, exposure route and duration, liver tumour incidence, type of tumour lesion and species/gender (Table II). Exposure to the compounds was via the diet with the exception of ME and TAM, which were dosed by gavage. In order to use data sets as uniform as possible, exclusively the development of hepatocellular carcinoma was taken into consideration since this end point was separately available for all compounds. Time of treatment was 1 year for MeIQx and >1 year for the remaining carcinogens. All data sets collected were for male rats with the exception of TAM for which only female rat data were available. In a next step, all data were modelled using the BMD software. The BMD_{10} values thus obtained for the six selected DNA-reactive carcinogens, expressed in milligrams per kilogram body weight per day, are reported in Table III. Each compound resulted in up to nine valid BMD_{10} values because of the number of models (9) used to calculate the BMD_{10} (Table III). The $BMDL_{10}$ values were also calculated but only the BMD_{10} were used for further comparison; *P*-value, log-likelihood and AIC are reported in Table III. Results obtained showed a large variation in the BMD_{10} values for the six model DNA-reactive carcinogens. For instance, the upper value of the BMD_{10} for AfB1 for male rats amounted to 0.00033 mg/kg bw/day. On the other hand, the highest BMD_{10} was found for male rats treated with SA and amounted to 169.1 mg/kg bw/day. The results from the BMD_{10} analysis show an increasing trend in the BMD_{10} value in the order AfB1 < 2-AAF < MeIQx < TAM < ME < SA. This order is identical to the order obtained on the basis of the carcinogenic potency estimates expressed as the TD_{50} that can be found in the CPDB (16). The TD_{50} is defined as the chronic dose rate (in milligrams per kilogram body weight per day), which would have 50% of tumour-free animals at the end of a standard lifespan experiment time for the species (26), or, simplified, represents the daily dose inducing tumours in half of the test animals (above background incidence). Based on the TD_{50} values, the order in the carcinogenic potency of the six model carcinogens of the present study was AfB1 (0.0032 mg/kg bw/day) > 2-AAF (1.22 mg/kg bw/day) > MeIQx (1.66 mg/kg bw/day) > TAM (3.96 mg/kg bw/day) > ME (19.7 mg/kg bw/day) > SA (441 mg/kg bw/day) and thus similar to the order defined on the basis of the BMD_{10} approach in the present study, which is not surprising since dose–response modelling and adjustments for exposure and experimental duration are also employed to derive the TD_{50} values in the CPDB (26,27). It can also be noted that this order of potencies is roughly similar to the order derived for their potency in inducing DNA adduct formation.

Comparison of the BMD_{10} data for cancer incidence to the data on in vivo DNA adduct formation

The quantitative BMD_{10} values defined from the tumour incidence data were compared to the dose–response curves for DNA adduct formation. To illustrate this comparison, Figure 2 presents the dose–response curves for DNA adduct formation already presented in Figure 1, but now including the range of accepted BMD_{10} values obtained for each carcinogen focussing on the same gender for which the DNA adduct levels were reported. The results obtained reveal that generally the BMD_{10} values (Figure 2, dashed lines) fall within the *in vivo* experimental dose ranges enabling detection of DNA adduct formation. This also allowed the calculation of the number of DNA adducts expected to occur at each BMD_{10}, using the linear extrapolation curves for the *in vivo* data for DNA adduct formation and these values are reported in Table III. The different data sets resulted in different models exhibiting the best fit (Table III). Therefore, none of the models was appropriate to model all data sets. In order to avoid averaging across the different BMD_{10} values obtained with the different models [as suggested in (28), based on (48)], accepted BMD_{10} values were computed and used individually in order to extrapolate DNA adduct levels and for visualisation in Figure 3. This approach also provided a good visual indication of values obtained using the BMD software. It can be concluded that at BMD_{10} levels for tumour induction, significant levels of DNA adduct formation can be detected for all six DNA-reactive carcinogens. The highest amount of DNA adducts formed at the BMD_{10} was found for male rats exposed to 2-AAF followed by male rats exposed to MeIQx > male rats exposed to SA > male rats exposed to AfB1 > male rats exposed to ME. The amount of DNA adducts formed at the BMD_{10} for female rats exposed to TAM was comparable to the level found for ME in male rats. The results also show that the number of adducts covers a range of 20–11 000 adducts in 10^8 nt at the dose levels causing 10% increase in tumour incidence above background levels, i.e. <3 orders of magnitude, while the carcinogenic potencies (based on TD_{50}) covers >5 orders

Table III. Name of compound, sex, species, reference, *P*-values, log-likelihood, AIC, degrees of freedom (d.f.) and BMD_{10} obtained with the different models using the EPA BMD software version 2.1.1 for the DNA-reactive compounds selected and the extrapolated amount of DNA adducts formed for each individual BMD_{10} based on the regressions in Figure 1

Model	*P*-value	Log likelihood	AIC	d.f.	BMD_{10}[a]	Number adducts in 10^8 nt
2-AAF, male rat (29,41)						
Full		−16.85				
Reduced		−76.08		3		
Gamma	1	−16.85	37.70	2	0.32	7561
Logistic	0.60	−17.67	39.34	2	0.45	10729
Log-logistic	0.99	−16.86	37.73	2	0.32	7585
Log-probit	0.999	−16.85	37.70	2	0.32	7585
Multistage	1	−16.85	37.70	2	0.31	7537
Multistage cancer	1	−16.85	37.70	2	0.31	7537
Weibull	1	−16.85	37.70	2	0.31	7537
Probit	0.68	−17.45	38.90	2	0.41	9937
Quantal linear	0.067	−21.22	44.44	3		
AfB1, male rat (30,42)						
Full		−33.51				
Reduced		−63.98	4			
Gamma	0.25	−35.28	76.57	2	0.00043	17.56
Logistic	0.41	−35.15	74.31	3	0.00039	15.75
Log-logistic	0.25	−35.28	76.56	3	0.00043	17.67
Log-probit	0.41	−34.75	75.49	2	0.00045	18.37
Multistage	0.30	−35.49	74.98	3	0.00033	13.49
Multistage cancer	0.30	−35.49	74.98	3	0.00033	13.49
Weibull	0.24	−35.27	76.55	2	0.00042	16.84
Probit	0.35	−35.19	74.37	3	0.00035	14.17
Quantal linear	0.085	−37.76	77.51	4		
MeIQx, male rat (33,45)						
Full		−23.69				
Reduced		−55.79		3		
Gamma	0.42	−24.79	53.59	2	1.59	721.6
Logistic	0.06	−26.44	56.87	2		
Log-logistic	0.57	−24.44	52.87	2	1.65	748.7
Log-probit	0.65	−24.27	52.54	2	1.62	736.2
Multistage	0.17	−28.04	58.07	3	1.04	473.7
Multistage cancer	0.17	−28.04	58.07	3	1.04	473.7
Weibull	0.19	−26.09	56.17	2	1.44	651.9
Probit	0.09	−26.45	56.91	2		
Quantal linear	0.0019	−35.40	72.81	3		
ME, male rat (31,43)						
Full		−84.01				
Reduced		−106.63		3		
Gamma	0.24	−84.72	175.43	1	35.39	35.10
Logistic	0.21	−85.58	175.14	2	40.10	39.76
Log-logistic	0.27	−84.65	175.30	1	35.16	34.87
Log-probit	0.36	−84.44	174.87	1	35.84	35.54
Multistage	0.16	−84.99	175.98	1	35.70	35.41
Multistage cancer	0.16	−84.99	175.98	1	35.70	35.41
Weibull	0.18	−84.97	175.94	1	34.20	33.92
Probit	0.27	−85.31	174.62	2	37.19	36.88
Quantal linear	0.09	−86.80	177.60	2		
SA, male rat (32,44)						
Full		−42.13				
Reduced		−45.86		4		
Gamma	0.58	−42.70	91.40	2	158.80	649.5
Logistic	0.71	−42.79	89.58	3	117.31	479.8
Log-logistic	0.58	−44.70	95.40	2	167.94	686.8
Log-probit	0.58	−42.70	91.40	2	153.51	627.8
Multistage	0.77	−42.71	89.42	3	130.69	534.5
Multistage cancer	0.77	−42.71	89.42	3	130.69	534.5
Weibull	0.58	−42.70	91.40	2	169.11	691.6
Probit	0.70	−42.81	89.62	3	112.66	460.8
Quantal linear	0.62	−42.97	89.94	3	91.20	372.9
TAM, female rat (34,46)						
Full		−81.07				
Reduced		−160.48		3		
Gamma	0.55	−81.67	167.35	2	4.56	69.31
Logistic	0.055	−84.77	173.45	2		
Log-logistic	0.65	−81.57	167.02	2	4.60	69.92
Log-probit	0.65	−81.50	167.01	2	4.66	70.83
Multistage	0.67	−81.86	165.72	2	4.44	67.49
Multistage cancer	0.67	−81.86	165.72	2	4.44	67.49
Weibull	0.46	−81.86	167.72	2	4.39	66.73

Table III. *Continued*

Model	*P*-value	Log likelihood	AIC	d.f.	BMD_{10}[a]	Number adducts in 10^8 nt
Probit	0.10	−83.83	171.66	2	4.56	69.31
Quantal linear	0.018	−86.87	175.74	3		
TAM, female rat (34'47)						
Full		−24.73				
Reduced		−54.04		2		
Gamma	0.99	−24.76	51.51	2	2.12	32.22
Logistic	0.99	−2473	53.46	2	2.54	38.61
Log-logistic	0.99	−2473	51.46	2	2.54	38.61
Log-probit	0.99	−35.70	53.46	1	2.47	37.54
Multistage	0.33	−26.47	54.93	2	1.07	16.26
Multistage cancer	0.33	−26.47	54.93	2	1.07	16.26
Weibull	0.99	−24.73	53.46	1	1.98	30.10
Probit	0.99	−24.73	53.46	1	2.27	34.50
Quantal linear	0.002	−54.04	63.39	2		
TAM, female rat (35'46)						
Full		−81.07				
Reduced		−160.48		3		
Gamma	0.55	−81.6	167.35	2	4.56	230.5
Logistic	0.055	−84.77	173.45	2		
Log-logistic	0.65	−81.57	167.02	2	4.60	
Log-probit	0.65	−81.50	167.01	2	4.66	232.5
Multistage	0.67	−81.86	165.72	2	4.44	235.5
Multistage cancer	0.67	−81.86	165.72	2	4.44	224.4
Weibull	0.46	−81.86	167.72	2	4.39	224.4
Probit	0.10	−83.83	171.66	2	4.56	221.5
Quantal linear	0.018	−86.87	175.74	3		
TAM, female rat (35'47)						
Full		−24.73				
Reduced		−54.04		2		
Gamma	0.99	−24.76	51.51	2	2.12	107.1
Logistic	0.99	−24.73	53.46	2	2.54	128.4
Log-logistic	0.99	−24.73	51.46	2	2.54	125.4
Log-probit	0.99	−35.70	53.46	1	2.47	124.8
Multistage	0.33	−26.47	54.93	2	1.07	54.08
Multistage cancer	0.33	−26.47	54.93	2	1.07	54.08
Weibull	0.99	−24.73	53.46	1	1.98	100.1
Probit	0.99	−24.73	53.46	1	2.27	114.7
Quantal linear	0.002	−54.04	63.39	2		

[a]The BMD_{10} are expressed as milligrams per kilogram body weight per day (mg/kg bw/day).

of magnitude. The data also lead to the conclusion that for all six DNA-reactive carcinogens, the actual level of DNA adducts formed at the BMD_{10} do not vary by more than ~3 orders of magnitude. This may be related to the fact that genotoxic electrophiles can react at different sites within the DNA, resulting in the formation of different types of adducts. Together, these results quantitatively support the conclusion that all individual DNA-reactive selected compounds 2-AAF, AfB1, MeIQx, TAM, SA and ME give rise to DNA adducts with a 10- to 100-fold different potential to result in mutations and ultimately cancer.

Species differences in 2-AAF DNA adduct formation

Figure 4 presents the dose-dependent DNA adduct formation measured in liver of mice chronically exposed to 2-AAF (49). In addition, literature data on tumour formation in the liver of mice dosed with 2AAF (50) were used to determine the BMD_{10} value (Figure 4). For both studies, the feed concentrations in p.p.m. were converted to the following dose range: 0, 0.65, 1.3, 1.95, 3.9, 5.85, 7.8, 9.75, 13 and 19.5 mg/kg bw/day. DNA adduct levels at 1 mg/kg bw/day, the average BMD_{10} and the average DNA adduct formation at the BMD_{10} are displayed in Table IV. When comparing the average DNA adduct levels at the average BMD_{10}, it was found that at the BMD_{10}, DNA adduct formation in rat was 8-fold greater than in mouse. Previous data derived for DNA adduct levels in rats and mice at the TD_{50} value were in line with this result (51).

Endogenous DNA adduct levels

Endogenous or background DNA damage occurs constantly in all human and animal tissues, induced, for instance, through oxidative stress, the formation of radicals or reactive, alkylating metabolites. The targets of these 'stressors' are numerous and can be the deoxyribose-phosphodiester backbone, the glycosidic linkages connecting the nucleobases to the DNA backbone or the nucleobases itself, leading to a plethora of different possible primary and secondary modifications (52) that can have profound biological consequences. For the purpose of this manuscript, we use the term 'background' or 'endogenous DNA adduct' for measurable specific covalent modifications of the nucleobases (including oxidatively damaged nucleobases) in order to define a background level for comparison with exogenous carcinogen-specific adduct formation that principally attack similar atoms of the nucleobases. Reports in the literature on endogenous levels of DNA adducts are varied and address both animal and human tissues. Historically, difficulties were encountered concerning the performance of analytical methods. For instance, the determination of oxidatively damaged guanine

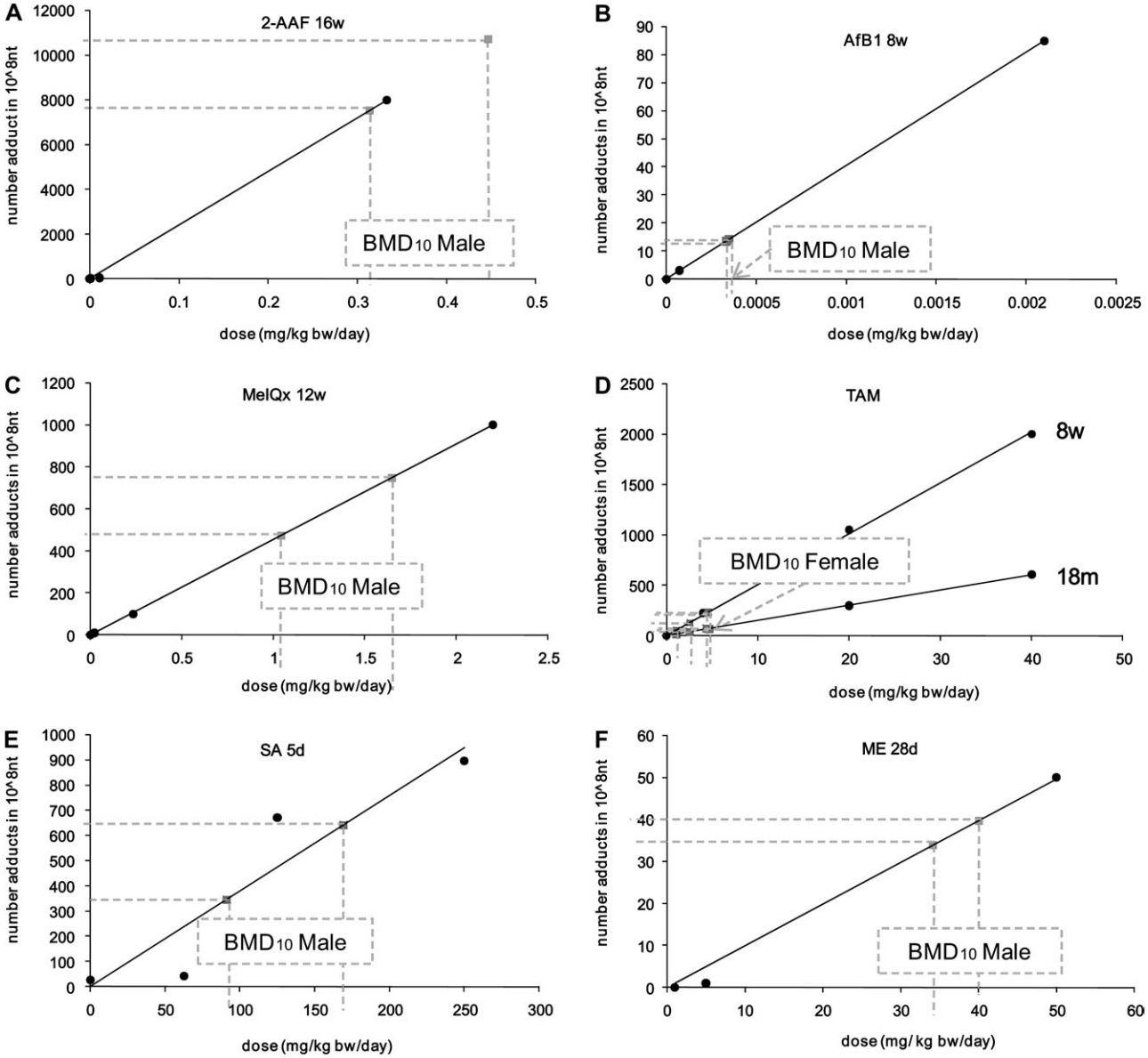

Fig. 2. Illustration of the dose–response curves for DNA adduct formation with the range of accepted BMD_{10} values (dashed lines) derived from *in vivo* data on liver tumour incidence, representing the data for male rats treated with: (**A**) 2-AAF, (**B**) AfB1, (**C**) MeIQx, (**E**) SA, (**F**) ME and female rats treated with (**D**) TAM (34,35).

(8-oxo-7,8-dihydroguanine, 8-oxo-dG) led to important overestimations due to artifactual DNA oxidation during the analytical sample workup (53–56). Due to the heterogeneity of adducts and available methods and the fact that most recent comprehensive reviews focus on human endogenous DNA adducts (3,57), detailed knowledge on endogenous DNA adduct formation in rat tissues is actually limited. Representative data on levels of the most studied endogenous adducts, in human and rat liver, were extracted from the literature and are summarised in Table V. Though not being exhaustive, the list provides a reasonable indication of the overall endogenous adduct levels. It is obvious that, even within the same species and organ, levels of a specific adduct can easily vary by >1 order of magnitude (e.g. in rats: 8-oxo-dG, 23-480/10^8 nt and M1G, 1-52/10^8 nt). The results presented in Table V revealed that oxidative damage usually exceeds other endogenous DNA adducts induced by alkylation or lipid oxidation which is in agreement with previous reviews (3,57,70). In addition, endogenous DNA adduct levels tend to be somewhat higher in humans than in rats, probably due to non-controllable and lifestyle factors such as unknown dietary and environmental sources of exposure to alkylating agents, smoking or oxidative/metabolic stress as compared to the controlled housing conditions of laboratory animals. In general, it has been reported that endogenous DNA adduct levels are present in 100 adducts in 10^8 nt (6), and additionally, the level was reported by Farmer (71,72) to be generally 10–100 adducts in 10^8 nt for low molecular weight alkylating electrophiles. Based on these data, in the present manuscript the background of the alkylating endogenous DNA adduct level was defined at 10–100 adducts in 10^8 nt and used as the relevant background level for endogenous DNA adduct formation. This level of endogenous DNA adduct formation was also previously used to compare estragole DNA adduct formation predicted by our physiologically based biodynamic model for DNA adduct formation in male rat liver after exposure to estragole (73).

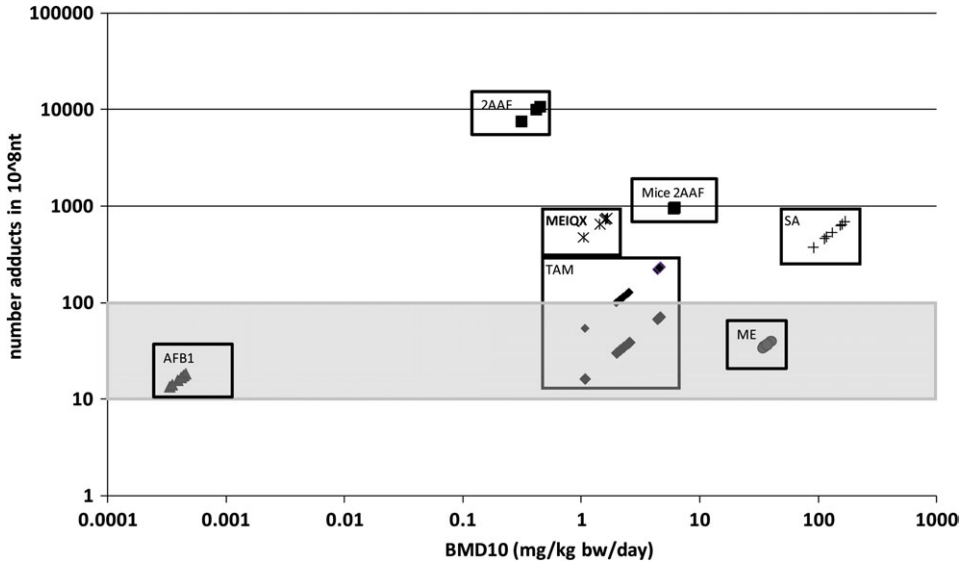

Fig. 3. Levels of DNA adduct formation at the BMD_{10} for liver tumour formation by the six model compounds as related to endogenous DNA adduct levels (10–100 adducts in 10^8 nt, grey shadow) in liver. The individual data points represent the outcome of each individual mathematical model for male rats treated with: 2-AAF, AfB1, MeIQx, SA, ME and female rats treated with TAM and male mice treated with 2-AAF.

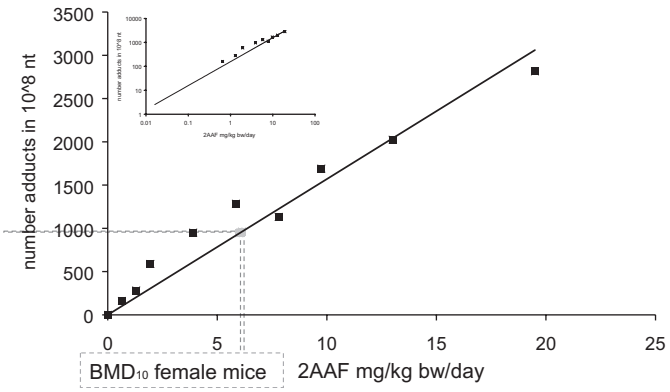

Fig. 4. Dose-dependent DNA adduct formation (number of adducts in 10^8 nt) in the liver as obtained from *in vivo* studies in male mice treated with 2-AAF, giving the following slope [(#adduct 10^8 nt)/(mg/kg bw/day)]: $m = 156.96$, with the range of accepted BMD_{10} values derived from mice *in vivo* data from liver tumour incidence. Inserts are log-log plots of doses and DNA adduct levels.

Table IV. Species differences in response to 2-AAF: female mice versus male rats (29,41)

Model	Number adducts in 10^8 nt for 1 mg/kg/bw/day	Average BMD_{10}	Average number adducts in 10^8 nt at the BMD_{10}
Mice	157.0	6.07	953.0
Rat	24002	0.343	8250

In a next step, the DNA adduct levels calculated at the BMD_{10} (Table III) were compared to reported endogenous levels of DNA adduct formation (Table V). The value of 10–100 adducts in 10^8 nt was included resulting in Figure 3, which allows comparison of the DNA adduct levels at the BMD_{10} to the endogenous DNA adduct levels in rat liver. When comparing the levels between genotoxic compounds, it appears that the number of DNA adducts in 10^8 nt formed at the highest BMD_{10}, for 2-AAF, MeIQx, SA, TAM were 100-, 7.5-, 6.9-, 2.3-fold higher than the highest value set for the endogenous DNA adduct levels (100 adducts in 10^8 nt). For AfB1 and ME, the predicted levels of DNA adduct formation at the BMD_{10} was within the background level of 10–100 adducts 10^8 nt. In the specific case of TAM, the amount of DNA adducts at the BMD_{10} resulted in four different sets of values (Figure 3) since two studies on DNA adduct formation and two tumour incidence studies were available for this compound. Two data sets falling within the endogenous background level, while one data set was above the endogenous background and the fourth data set was partially above the background level. When the TAM adducts calculated at the BMD_{10} were based on data generated using different analytical methods (35), the values obtained did not vary by more than a factor of 2 (not shown), which is much less than the differences observed between different animal studies.

Discussion

In the present manuscript, a comparison was made between *in vivo* DNA adduct formation, cancer induction and background DNA adduct formation using a series of selected DNA-reactive carcinogens: 2-AAF, AfB1, ME, SA, MeIQx and TAM, as model compounds. The aim of this study was to use a uniform data set on DNA-reactive genotoxic carcinogens that induce, with or without metabolic activation, DNA adducts which can lead to hepatocellular carcinogenic transformation, in order to analyse if a correlation between the formation of DNA adducts and cancer incidences in the target organ can be obtained.

A decade ago, Otteneder and Lutz (74), making a similar effort, indicated that there was a need of more data on DNA adduct levels in target tissues of rodents and human. After more than 10 years, little progress has been made since a thorough literature search revealed only six DNA-reactive carcinogens for which in addition to tumour data also data on *in vivo* DNA adduct formation in the same target tissue, species

Table V. Quantitative information on endogenous DNA adduct levels limited to liver of humans and rats

Adduct	Affected nucleo-base position	Quantity/10^6 nt[a]	Method	Source (h, human; r, rat) (no. of samples)	Reference
Oxidation					
8-oxo-dG	C8	0.51	HPLC/EC[b]	r (?)	(58)
8-oxo-dG	C8	0.84–1.14	HPLC[b]	r (32)	(55)
8-oxo-dG	C8	1.8–4.8[c]	LC-MS/MS	r (3)	(59)
8-oxo-dA	C8	0.23–0.34[c]	LC-MS/MS	r (3)	(59)
Lipid peroxidation					
M1G	N1	0.5–1.1	GC–MS	h (6)	(60)
M1G	N1	0.14	P^{32}/HPLC	h (?)	(3)
M1G	N1	0.9	GC/EC	h (?)	(3)
M1G	N1	0.01	NCI/MS	r (4)	(61)
M1G	N1	0.52	LC-MS/MS	r (6)	(61)
7-HEG	N7	3.0	GC/ECNCI MS	h (9)	(60)
7-HEG	N7	0.06–0.09	GC-HRMS	r (?)	(62)
7-HEG	N7	0.29	^{32}P-pl	h (?)	(63)
7-HEG	N7	0.013	^{32}P-pl	r (3)	(64·65)
7-HEG	N7	0.025	LC-MS/MS	r(12)	(64·65)
7-CEG	N7	0.05	LC-MS/MS	h (24)	(66)
7-EtG	N7	0.0084	LC-MS	h (25)	(63)
Cyclic adducts					
ϵG	N^2,C3	0.02	LC-MS	h (1)	(60)
ϵG	N^2,C3	0.012	IA-GC/HRMS	r (8)	(67)
ϵG	N^2,C3	0.007	IA-GC/HRMS	r (7)	(67)
ϵdA	C1,N^6	0.001–0.002	IA ^{32}P-pl	r (3)	(68)
ϵdA	C1,N^6	0.006	IA ^{32}P-pl	h (10)	(69)
ϵdC	C3,N^4	0.006–0.008	IA ^{32}P-pl	r (3)	(68)
ϵdC	C3,N^4	0.007	IA ^{32}P-pl	h (10)	(69)
HNE-dG	C1,N^2	0.0006–0.0018	HPLC/P^{32}-pl	h (?)	(3)
Acr-dG	C1,N^2	0.06–0.4	HPLC/P^{32}-pl	h (?)	(3)
Alkylation					
7-MG	N7	0.21–0.25	^{32}P-pl	r (?)	(62)
7-MG	N7	0.27	^{32}P-pl	r (?)	(62)
O^6-MeG	O^6	0.022–0.13	PREPI	h (?)	(3)
O^4-MeT	O^4	0.003–0.42	PREPI	h (?)	(3)
O^4-EtT	O^4	0.015–4.24	PREPI	h (?)	(3)
Abasic sites	–	8–9	ASB	h (?)	(3)

IA, immunoaffinity purification; PREPI, ^{32}P-postlabelling (32P-pl) followed by high-performance liquid chromatography and immunoprecipitation; ASB, combination of aldehyde-reactive probe and slot blot techniques; HRMS, high-resolution mass spectrometry; HNE, 4-hydroxynonenal; Acr, acrolein; ECNCI, electron capture negative chemical ionisation;?, number of samples not specified.

[a]In part corrected from the number of adducts per normal parent base by using a content of 22% G or C and 28% of A or T in mammalian DNA.

[b]Improved workup to minimise artifactual formation.

[c]Depending on extraction procedure (chaotropic extraction or anion exchange, respectively).

and gender were available. Otteneder and Lutz (74) used carcinogen-DNA adduct levels associated with a 50% tumour incidence, using data from both mice and rats, males and females. In the present manuscript, we used data on DNA adducts and cancer incidences exclusively for rat, within the same gender, and dose responses for cancer incidences were modelled using a BMD approach. In addition, data were compared to endogenous DNA adduct levels. Although for an individual carcinogen, the tumour response increases when adduct levels increase, our results demonstrate that when comparing different carcinogens, no quantitative correlation exists between the level of DNA adduct formation and carcinogenicity. Assuming that DNA adducts have a role in cancer development, the data corroborate that the different DNA adducts have different mutagenic and carcinogenic potential. The evaluation of DNA adduct data represents a particular difficulty, especially in risk assessment (8). Jarabek *et al.* (8) defined mutagenic efficiency to be: 'the probability that a DNA adduct or an adduct load converts a normal cell to a viable cell containing a heritable alteration in the DNA (i.e. a mutation)'; the efficiency of conversion of a promutagenic DNA adduct to a mutation depends on a wide range of factors including the type and chirality of the adduct; the cell-, tissue- or organ-specific DNA repair and metabolism; the local DNA sequence context of the adduct; the lability/stability of the adduct; the extent of disruption induced in the helix structure; the capacity of the affected cell to replicate to form a clone and other cellular processes such as the frequency of cell replication. All these parameters must be considered when dealing with DNA adducts in risk assessment. Furthermore, it is important to underline that unlike the rodent 2-year cancer bioassay, that is conducted according to guideline protocols since considerable time, with agreed procedures as to adjust doses for exposure and treatment duration in order to allow comparability between studies and compounds, analyses of *in vivo* DNA adduct formation have variable study designs, are usually done for very specific purposes, with particular dosing regimens and duration depending on the question asked. Therefore, we corrected for these diverting factors as much as possible.

Thus, various DNA-reactive carcinogens for which data on *in vivo* DNA adduct formation were available, such as for example dimethylnitrosamine, 2-amino-3-methylimidazo[4,5-f]quinolone (IQ) and 2,4-diaminotoluene, which were used in

Otteneder and Lutz (74) did not have tumour incidence data meeting the criteria set in this manuscript. And for many other DNA-reactive compounds for which long-term carcinogenicity data are available, data on *in vivo* DNA adduct formation are lacking. The selection of the compounds in the present study was done following the following criteria: (i) available data on *in vivo* DNA adduct formation and (ii) available dose–response data for tumour induction, both in the same target tissue, the liver, and for the same species, the rat and for the same gender. In further analysis of the results obtained, it was important to take into account other critical steps underlined by Otteneder and Lutz (74) such as: (i) DNA adduct measurement is a balance between formation of the adduct, repair, cell death and dilution of the adduct formed by DNA replication (ii) the period of treatment should be long enough to achieve a steady state and (iii) the relationship between DNA adducts detected and tumour incidence may be influenced by the time of sacrifice of the animal after administration of the compound. DNA adduct data obtained from literature revealed that all the experimental data sets, although limited, appear compatible with linearity to the origin, a finding that is in line with the current understanding of DNA-adduct dose–response behaviour (75). DNA adduct levels detected can be expected to reflect a balance between DNA adduct formation and DNA repair, cell death and dilution of the adduct formed by DNA replication (76).

The data obtained point at a different potency for each individual compound to result in DNA adduct formation, with AfB1 and 2-AAF being at least 2–3 orders of magnitude more effective in forming DNA adducts than MeIQx and SA and even 4 orders of magnitude more effective than ME and TAM. Such differences can be expected to result for example from differences in the reactivity of the DNA-reactive intermediate formed, in the relative percentage of the dose that is actually converted to the DNA-reactive metabolite and/or in the stability of the DNA adduct formed. DNA adducts are produced when electrophilic compounds covalently bind to a base of the DNA structure, forming different types of DNA adducts (6). DNA adducts vary greatly in their half-lives, which depends on their chemical stability, their repair by DNA repair processes and/or their elimination through apoptosis and/or cell death. The site of formation of the electrophilic metabolite, combined with its chemical stability, influences the cellular and tissue distribution of DNA adducts. For instance, highly unstable electrophiles do not persist long enough to form adducts in tissues distant to the site of metabolic activation (6). On the other hand, relatively stable metabolites can circulate in the blood and form DNA adducts in many tissues (6). Furthermore, tissue-specific possibilities for DNA repair can greatly affect the amount of DNA adducts present in a tissue. In a repair-proficient tissue, the amount of DNA adduct rapidly decreases but if the tissue is deficient in repair mechanisms, then the amount of DNA adducts remains unaffected (6).

Furthermore, as outlined by Otteneder and Lutz (74), it is important to note that, when comparing the relative potency of the different model compounds to form DNA adducts, the period of treatment underlying the data for which the comparison is made should be long enough to achieve a steady state. Steady-state levels are reached when the number of DNA adducts formed are equal to the number that are destroyed and repaired and this may be achieved either after a single exposure or only after repeated exposure to a DNA-reactive compound, the latter leading to an increase in DNA adduct formation over time. For highly unstable or rapidly repaired DNA adducts (such as *N*-3 methyladenine or *N*-7 oestrogen adducts on adenine), steady state may be reached as quickly as following the first daily dose (6). However, in other cases (i.e. *N*7-alkylguanine adducts), a steady state may only be achieved in 7–10 days, whereas more persistent adducts (such as *O*4-ethyl thymidine) can accumulate over a period of 4 weeks (6).

In the present paper, we have normalised the data either only with respect to the dose or with respect to both the dose and duration of exposure. Some data are available that could provide at least some insight into the actual influence of dose and duration of exposure on the normalised levels of DNA adduct formed. For example, for SA, DNA adduct levels were measured after one injection and after five repeated daily injections. The DNA adduct levels at the same dose (1 mg/kg bw/day) for the single exposure, amounted to 1.40 10^8 nt/day, whereas it amounted to a normalised value of 0.8 10^8 nt/day after five daily injections, indicating that the repeated daily exposure gave a somewhat lower amount of DNA adducts formed when normalised to 1 day. Similarly, for MeIQx, DNA adduct measurements obtained after 1- and 12-week administration of the same dose level revealed that after normalisation, the longer treatment period (12 weeks) resulted in a lower number of DNA adducts amounting to 1.89 10^8 nt/day than the 1-week exposure period, which resulted in 8.8 10^8 nt/day. Clearly, however, these differences due to varying duration of the dosing remain within an order of magnitude and are thus smaller than the differences in the levels of DNA adduct formation at a dose of 1 mg/kg bw/day obtained for the model compounds of the present study. Furthermore, the amount of DNA adducts detected may be influenced by the time of sacrifice of the animal after administration of the compound (77). Therefore, it could be important to take into account the time period between the last treatment and sacrifice when analysing DNA adduct levels *in vivo*. In the studies discussed in the present manuscript, two studies reported that the period between dosing and sampling was 24 h (29,32), whereas for the remaining studies, this time was not specified, hampering further evaluation of this possible factor of influence. In the special case of 2AAF, complete data sets (DNA adducts and tumour incidence in liver) were available for both rats and mice. Analysis of these data revealed that at the BMD_{10}, the levels of DNA adduct formation in rat appeared to be 8-fold higher than the levels in mouse. This suggests that, for this compound, there is a species-dependent difference in sensitivity with rats requiring higher levels of DNA adduct formation for a similar level of tumour formation, and the difference between species (rat versus mice) indicating that mice are more sensitive to this compound than rats.

The present manuscript also focuses on the possible role of DNA adduct formation in the MOA of the different DNA-reactive carcinogens and the endogenous DNA adduct levels. With respect to these MOAs, it has been reported for example that DNA adduct formation by AfB1 produces 8,9-dihydro-8-(N7-guanyl)-9-hydroxyaflatoxin B1 (AfB1-N7-Gua), which is converted naturally to two secondary lesions, an apurinic site and an AFB1-formamidopyrimidine (AfB1-FAPY) adduct. AfB1-FAPY is detected at near maximal levels in rat DNA days to weeks after AfB1 exposure, underscoring its high persistence *in vivo* (78). Furthermore, the DNA adduct formation by AfB1 does not occur randomly within the DNA but rather at so-called mutational hot spots causing mutations

with high frequency in the *p53* gene (79). It has been shown in humans that AfB1 acts preferably on Codon 249 and 250 of the *p53* gene, resulting in AGG to AGT and CCC to ACC transversions with a relative mutation frequency of 15×10^{-6} (79). The putative hot spot in humans, Codon 249, corresponds to Codon 243 in the rat gene, which is located in Exon 6. Results suggested that mutation at the site corresponding to the hot spot Codon 243 in the rat gene is rare (80). Given, however, that the occurrence of these mutational hot spots for AfB1 may not occur in rats (80), the first explanation pointing at high persistence of the AfB1 DNA adducts may provide an alternative explanation for their high mutagenic and carcinogenic potential compared to that of endogenous DNA modifications. Reduction of aflatoxin-DNA adduct levels by chemopreventive agents could reduce the number of foci present and could be used as predictive of cancer preventive efficacy (81,82). For TAM, mutations are found in the liver, including predominantly GC→TA transversions (83) and Liapis *et al*. (84) predicted, using an in silico model, GC→TA mutational hot spots for TAM at Codons 244 and 273 of *p53*. Among the 393 codons of the human *p53* gene, 222 are the targets of 698 different mutational events (85). By reacting at different spots, the ability of different genotoxic carcinogens to result in tumour formation can be expected to differ. Given this situation, it is of interest to note that the actual levels of DNA adduct formation observed for the different DNA-reactive carcinogens at their BMD_{10} differ by only 1–2 orders of magnitude.

The general background level of DNA adducts was defined at 10–100 adducts in 10^8 nt (5,70,71,76). Based on the data obtained, a comparison of the level of DNA adducts calculated to be observed at a dose level amounting to the BMD_{10} for tumour formation by the model carcinogens selected for the present study, to this endogenous DNA adduct levels in rat liver tissue could be made (Figure 3). For 2-AAF, 2-MeIQx and SA, DNA adduct formation at the BMD_{10} was above background levels for DNA adduct formation and may thus contribute to their MOA. The levels of DNA adduct formation expected at the BMD_{10} were actually below or close to the background DNA adduct level set a 100 adduct in 10^8 nt for (i) the study with female rats treated with TAM, (ii) the study with ME in male rats and (iii) the study with male rats treated with AfB1. Theoretically, this observation of DNA adduct formation at the BMD_{10} at levels that are below the background levels of DNA adduct formation might reflect that either the DNA adducts formed are far more mutagenic and carcinogenic than the type of lesions included in the background levels or it might imply that the mechanism underlying the tumour induction is not related to the DNA adduct formation but rather due to another mechanism of action such as for example cytotoxicity. Given the present proposals for the MOA of ME, TAM and AfB1, one might conclude that the first explanation holds for AfB1 pointing at formation of DNA adducts that are more mutagenic and carcinogenic than background type DNA lesions. The AfB1 mutation at high frequency in a hot spot in the *p53* tumour suppressor gene (79) and/or leading to DNA adducts with relatively high persistence (86) supports this hypothesis. An efficacy of at least 1 order of magnitude higher than the efficacy of background DNA lesions to result in tumour induction would already explain that the level of adduct formation by AfB1 can result in tumour incidences above background level. Poirier and Beland (87) reported that the high carcinogenic potency of AfB1 may result in tumour formation at concentrations that do not saturate metabolic pathways, stimulate cell proliferation or induce extensive toxicity. For ME, literature data point at a possible role for liver toxicity in the mechanism underlying tumour formation (88) and the results of the present analysis may corroborate involvement of another mechanism than merely DNA adduct formation in the MOA of this DNA-reactive carcinogen. Finally, for the specific case of TAM, there are still several options for the MOA of this genotoxic carcinogen. Formation of DNA adducts at mutational hot spots in the *p53* tumour suppressor gene as suggested by Liapis *et al*. (84) may support formation of DNA adducts with higher potency for tumour induction than background DNA adducts, whereas on the other hand, involvement of TAM or its metabolite 4-hydroxytamoxifen as a partial oestrogen receptor alpha agonist (84) may point at a MOA including increased cell proliferation and tumour formation induced by oestrogen receptor alpha agonist activity, representing a MOA other than the association of hepatic tumors with accumulation of DNA adducts in the liver (47). Oestrogen receptor alpha activation may indeed play a role in hepatic tumour formation in rodents (46).

Overall, the results of the present paper reveal that for some DNA-reactive carcinogens, the level of DNA adduct formation at the BMD_{10} for tumour formation does not significantly exceed endogenous DNA adduct levels, pointing at either the formation of DNA adducts that are more effective in inducing carcinogenesis than background DNA adducts or corroborating the involvement of other MOAs than DNA adduct formation underlying their carcinogenicity.

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