Mechanisms of chemical carcinogenesis and their impact on dose-response relationships — the examples of dioxin and benzo[*a*]pyrene

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Executive summary

This review summarises the current knowledge and future research needs on the mechanisms of chemical carcinogenesis with regards to dose response relations and potential thresholds. Emphasis is given to the best studied model carcinogens benzo[a]pyrene and dioxin (TCDD) as representatives for DNA damaging and non-genotoxic carcinogens, respectively, but where required other carcinogens are also included. The goal of this review was to better define the nature of dose responses in chemical carcinogenesis as either linear, non-thresholded non-linear or even having practical or absolute thresholds. Furthermore, the potential mechanisms relevant for the shape of the dose response curves are discussed.

For risk assessment data on chemical carcinogens are often derived from high-level exposure experiments in rodents. Whether extrapolations from high doses and different species are meaningful to predict the risk of low-level exposed humans can only be answered by considering the underlying mechanisms of carcinogenesis. As the development of a tumour is a multi-step process for each and every step different dose response curves may apply. In the chapters of the review data on dose responses and potential thresholds for the following steps in carcinogenesis were collected and critically discussed.

Dose response and potential thresholds in a) activation and inactivation of procarcinogens, b) in DNA adduct formation, c) in induction of mutagenesis and the influence of DNA repair and cell cycle progression, d) in gene expression, e) proliferation and cell survival and death, and f) in tumour development.

In conclusion, there is no evidence for a practical threshold in DNA adduct formation. However, there are current limitations of sensitivities of experimental methods to detect very low levels of adducts relevant for human exposure. Similarly, firm conclusions on dose response relations in mutagenesis and gene expression are hampered by the lack of sensitive enough methods. On the other hand, there is data to support the existence of thresholds for effects on cell toxicity and proliferation by non-genotoxic compounds. Most often toxicity and altered proliferation in conjunction with DNA adduct formation seems to be required for chemically induced tumours. Hence, dose response relations are determined by independent dose response curves of the individual processes leading to cancer. More reliable markers and methods are needed in the future to ultimately solve the problem of dose response and potential thresholds in chemical carcinogenesis.

Introduction

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The risk assessment models of chemical carcinogenesis often rely on data derived from high-level exposure experiments in rodents. Consequently, hazard identification and risk assessment in the low-dose range require extrapolation across species, as well as extrapolation of high dose-response curves over many orders of magnitude down to the low levels relevant for human exposures. Even though dose-response extrapolation based on the mode of action suggests the use of two different models, threshold and non-threshold, the former to be used for non-genotoxic carcinogens, the latter for DNA damaging carcinogens, this approach may be simplistic, in so far as recent findings suggest the existence of at least practical thresholds even for some DNA damaging carcinogens, a decisive new situation which may profoundly affect health risk assessment. Thus, mechanistic studies are of the utmost importance to better understand the cellular response at low level exposure of carcinogens for improving the basis for risk assessment methodology and for guiding the choice of risk assessment strategy.

Carcinogenesis by DNA damaging agents is a multistep process, involving in most cases procarcinogen activation to an ultimate genotoxin immediately responsible for the DNA damage, induction of mutations in critical genes and stimulation of processes that favour the proliferation of initiated cells which, after clonal expansion, eventually give rise to a tumour, which after further mutations progresses to a malignant cancer. Cellular defence mechanisms counteract carcinogenesis at multiple levels e.g. carcinogen metabolism, DNA repair, cell cycle arrest and/or apoptosis. The sum of several processes finally determines whether a compound acts via a linear response mechanism, a non-linear but not thresholded dose response, a practical threshold or even an absolute threshold.

The aim of this review is to summarise the current knowledge and future research needs on the mechanisms of chemical carcinogenesis with regards to dose response relations and potential thresholds with emphasis on the model carcinogens benzo[*a*]pyrene and dioxin (TCDD) as representatives for DNA damaging and non-genotoxic carcinogens, respectively, without excluding the additional treatment of other compounds where this is felt useful for the purpose of this review. The topic of this review can be viewed from divers angles, many, but of course not all, of which are addressed here. Thus, besides the immediate target tissues where the tumour will develop, this review also covers the importance of non-target tissues, such as metabolically especially active organs (e.g. the liver) or organs of entry/first contact (such as intestine, lung and skin), but some further aspects such as immune competent cells

are not. Also genotypes related to differences in interindividual susceptibility to PAHs and dioxin as well as tissue and cell type specificities are not within the remit of this review and will only be touched upon where it is felt advantageous for putting the aspect in question into proper perspective.

The following fundamental topics will be specifically addressed in the present review.

Dose response and potential thresholds in:

— activation and inactivation of procarcinogens,
— DNA adduct formation,
— induction of mutagenesis and the influence of DNA repair and cell cycle progression,
— gene expression,
— proliferation and cell survival and death,
— tumour development.

1. Dose response and potential thresholds in activation and inactivation of procarcinogens

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Numerous genes have evolved to protect cells from the deleterious effects of environmental chemicals, which are pivotal in determining individual responses to the activation or inactivation of procarcinogens. Over the past few years the use of transgenic technologies has contributed to the *in vivo* analysis of gene function. Many genes associated with xenobiotic metabolism have been deleted in mice. Further details can be found in a recent review by Henderson et al. [1,2], and a list of transgenic animals is provided in Table 1.1. Research in Dundee has focused on several transgenic mouse lines relevant to describing chemically induced mechanisms of carcinogenesis and determining threshold levels of exposure. These include: Hepatic P450 Reductase Null (HRN) [3]; GstP null [4]; CYP1A1/bGAL reporter [5]. Furthermore, we have developed recombinant cells to decipher the role of polymorphic human P450s for the activation of carcinogens.

Here we examine the use of these powerful tools for understanding the molecular mechanisms of carcinogenesis, and the development of new paradigms for risk assessment.

Table 1.1. Deletions of drug metabolising enzymes and related proteins in the mouse

Gene	Citation*	Constitutive Phenotype	Genetic background	Comments
Cytochrome P450s				
Cyp1a1	Dalton et al. (2000)	None	129xC57BL/6	Protection against BaP toxicity
Cyp1a2	Pineau et al. (1995)	Respiratory distress	129xC57BL/6	Disruption of exon 2 Incomplete penetrance
	Liang et al. (1996)	None	129xCF-1	Disruption of exon 2, removal of exons 3–5 Deficient zoxazolamine metabolism

Table 1.1. Deletions of drug metabolising enzymes and related proteins in the mouse — cont.

Gene	Citation*	Constitutive Phenotype	Genetic background	Comments
Cyp1b1	Buters et al. (1999)	None	129xC57BL/6	Protection against DMBA-induced bone marrow cytotoxicity and ovarian cancer
Cyp2e1	Lee et al. (1996)	None	129xC57BL/6	Resistant to acetaminophen, chloroform and carbon tetrachloride hepatotoxicity
CPR (liver-specific) [HRN™]	Henderson, Otto et al. (2003)	Altered hepatic lipid homeostasis	129xC57BL/6 C57BL/6	No hepatic P450 function Altered drug pharmacokinetics Reduced acetaminophen toxicity
Cyp7a1	Ishibashi et al. (1996) Schwarz et al. (1996)	Lethal in postnatal period (< 18d)	129xC57BL/6	Rescued by dietary supplementation Alternative bile acid biosynthetic pathway induced
Cyp19	Toda et al. (2001)	Females infertile Precocious follicular depletion	129xC57BL/6	Disruption of exon 9 Partial rescue with oestradiol treatment
	Fisher et al. (1998)	Females infertile Corporea lutea absent	129xC57BL/6	Disruption of exon 9 Enlargement of prostate gland Increased adiposity
Cyp26a1	Abu-Abed et al. (2001)	Embryonic lethal, mid-late gestation	129xC57BL/6	Deletion of exons 2–6 Spina bifida, sirenomelia, caudal truncation
	Skai et al. (2001)	Lethal in perinatal period (< 24 h)	129xC57BL/6	Deletion of entire gene Caudal truncation, vertebra transformation, hindbrain mispatterning
Other DMEs				
GstP	Henderson et al. (1998, 2000)	None	129xMF1 or C57BL/6	Knock outs protected against DMBA toxicity and acetaminophen toxicity

Table 1.1. Deletions of drug metabolising enzymes and related proteins in the mouse — cont.

Gene	Citation*	Constitutive Phenotype	Genetic background	Comments
NQO-1	Radjendirane et al. (1998)	None	129xC57BL/6	Increased menadione toxicity Myelogenous hyperplasia
mEH	Miyata et al. (1999)	None	129xC57BL/6	Protection against DMBA toxicity
sEH	Sinal et al. (2000)	Lowered systolic blood pressure (males)	129xC57BL/6	Altered renal arachidonic acid metabolism (males and females)
GPx	Ho et al. (1997)	None	129xC57BL/6	Increased sensitivity to Diquat
GCLM	Yang et al. (2002)	None	129xC57BL/6	Depleted GSH levels in liver, lung, pancreas and blood plasma
Associated transcription factors and nuclear receptors				
AhR	Fernandez-Salguero et al. (1995)	Hepatic fibrosis and impaired immune system	129xC57BL/6	Disruption of exon 1 50% mortality in perinatal period Resistant to TCDD toxicity Reduced retinoic acid metabolism
	Schmidt et al. (1996)	Viable, fertile but with hepatic defects	129xC57BL/6	Disruption of exon 2
	Mimura et al. (1997)	Viable, fertile	129xC57BL/6	Disruption of exon 2; lacZ reporter Resistant to BaP carcinogenicity
ARNT	Kozak et al (1997)	Embryonic lethal	129xC57BL/6	Disruption of bHLH domain Defects in neural tube closure, embryo rotation
	Maltepe et al. (1997)	Embryonic lethal	Not stated	Disruption of bHLH domain Defective angiogenesis of yolk sac & branchial arches, stunted development
	Tomita et al. (2000)	Loss of induction via AhR in liver	129xC57BL/6	Disruption of exon 6 Conditional deletion (Mx-1-Cre)

Table 1.1. Deletions of drug metabolising enzymes and related proteins in the mouse — cont.

Gene	Citation*	Constitutive Phenotype	Genetic background	Comments
Nrf2	Chan et al. (1996)	None	129xC57BL/6	Disruption of exons 4,5; lacZ reporter Increased sensitivity to acetaminophen
	Itoh et al. (1997)	Develop autoimmune nephritis > 60 weeks old	129xICR	Disruption of exon 5; lacZ reporter Increased sensitivity to acetaminophen
AhR/Nrf2	Noda et al. (2003)	None* (see comments)	129xC57BL/6xICR	Response to 3MC and BHA abolished * 50% perinatal mortality, survivors apparently viable, fertile and normal
PPARα	Lee et al. (1995)	None	129xC57BL/6	Disruption of exon 8 Lack of response to peroxisome proliferators
PPARδ	Peters et al. (2000)	Reduced growth; Smaller gonadal adipose stores; Reduced brain myelination; Increased epidermal hyperplastic response to TPA;	129xC57BL/6#	Disruption of last exon # Genetic background enriched to at least 75% C57BL/6 to increase postnatal survival of PPARδ null pups to Mendelian ratios
PPARγ	Barak et al. (1999)	Embryonic lethal	Not stated	Disruption of zinc finger coding region
	Akiyama et al. (2002)	Altered cholesterol homeostasis	129xC57BL/6	Conditional deletion (macrophages)

* Individual gene deletion references can be found in Henderson et al. [1].

In vitro Model Systems for investigating carcinogen metabolism

P450s are key enzymes associated with catalyzing the toxication and detoxication of drugs and environmental pollutants. Mammalian genomes contain 50–100 different genes, thus the development of functional *in vitro* expression systems in *E. coli* and mammalian cells has been key to identifying those P450s affecting chemical transfor-

mations of hazardous potential. In addition, these models enable researchers to evaluate the influence of allelic changes in carcinogen metabolising enzymes on the toxication and detoxication of xenobiotics. While bacterial systems can be established within a short time, they lack several endpoints indicative of the biological actions of carcinogens. Employing bacterial expression of five polymorphic CYP1B1 variants, we were able to demonstrate that they did not differ in the K_m and the V_{max} for the epoxidation of the penultimate carcinogenic metabolite of benzo[*a*]pyrene, namely benzo[*a*]pyrene 7,8-dihydrodiol, which leads to the formation of the ultimate carcinogenic metabolite 9,10-epoxy 7,8-dihydrodiol benzo[*a*]pyrene [6]. However one allelic protein, namely CYP1B1*4, when compared to other variants, displayed a higher K_m for the 4-hydroxylation of 17β-estradiol, yielding a carcinogenic catechol estrogen [6]. Here it seems that a single polymorphic change at residue 432 altered the catalytic properties of CYP1B1 towards estradiol. This is at some variance with other investigations, which found that only combinations of certain allelic changes in CYP1B1 resulted in alterations, albeit modest, of enzyme activity towards steroids [7]. Irrespective of some differences between both studies, the enzymatic data indicate that polymorphisms of CYP1B1 should be associated with the incidence of hormone dependent cancers. Surprisingly, however, for breast cancer this does not seem to be the case, as shown by several epidemiological investigations, including a study with 3000 case controls of a mainly Caucasian population [8]. A recent study found, unlike in our study [6], that polymorphisms in CYP1B1 impact on the metabolic activation of PAH [9]. Thus it may be reasonable to assume that these allelic changes should have some influence on the incidence of smoking associated tumors. Indeed, the allelic change Leu432Val was found to have an influence on the incidence of lung cancer [10]. Interestingly, the same allelic change had a pronounced effect on the incidence of head and neck squamous carcinoma, which is also related to exposure to tobacco products [11].

Similar to CYP1B1, polymorphisms have been discovered in CYP1A1. Four allelic changes have been identified in CYP1A1 and were termed m1, m2, m3, and m4. M1 is a nucleotide change in the 3'-untranslated region and can be identified by MspI restriction. This allelic variation seems to have an effect on the inducibility of this P450 isoform, even though not all studies agree (reviewed in [12]). M2 encodes the change ^{462}Ile to Val and it was reported that this affected the catalytic properties of the enzyme [ref]. However, in an other study this variation did not modulate the catalytic parameters of the O-dealkylation of ethoxyresorufin and the 3-hydroxylation of benzo[a]pyrene [ref]. Interestingly, numerous epidemiological studies indicated that the M2 polymorphism in CYP1A1 affected the incidence of lung cancer [refs]. However, a recent meta-analysis of these studies concluded that this polymorphism was by itself not significantly associated with lung cancer risk [13]. Nevertheless, in conjunction with polymorphisms in GSTP1-1 and GSTM1, CYP1A1 polymorphisms show a correlation with the incidence of lung cancer and with PAH DNA adducts [14,15].

While these data illustrate that recombinant polymorphic P450s expressed in micro-organisms are suitable for predicting toxico-kinetics in humans, including the

extrapolation to low concentrations, they are not suitable for predicting more subtle effects that polymorphisms might have on post-transcriptional processes or on cellular homeostasis. To address this question, we have expressed polymorphic CYP1B1 forms in mammalian cells. We were able to demonstrate that CYP1B1*3 had a much lower expression level than the other CYP1B1 variants [6]. This resulted from a shorter half-life of the P450 protein. Thus, carriers of the CYP1B1*3 variant will have a lower rate of CYP1B1-mediated activation of exogenous and endogenous carcinogens compared to other CYP1B1 genotypes. This observation provides mechanistic support for epidemiologic investigations that show that carriers of the CYP1B1*3 genotypes display a lower incidence of endometrial cancer compared to carriers of other genotypes [16].

These studies illustrate that bacterial and mammalian expression systems for P450s complement each other to assess the role of P450s and their polymorphisms in the toxication of endogenous and exogenous carcinogens. Results coming from such studies are extremely valuable to support the findings of epidemiological studies.

In vivo Model Systems for investigating mechanisms of carcinogenesis

Phase I metabolism — the HRN mouse

The multigene CYP family is central to the Phase I metabolism of xenobiotics. To date, most research on the involvement of hepatic CYPs in drug metabolism has been carried out *in vitro* using liver microsomes, fresh and cryopreserved hepatocytes, or recombinant enzymes. Although these *in vitro* tools have provided valuable insights into the pathways of disposition mediated by the cytochrome P450 system, a definitive method for correlating *in vitro* effects from these *in vitro* approaches remains elusive. Despite the fact that rats have been used extensively for toxicity and carcinogenicity testing by the pharmaceutical industry over many years, a variety of technical issues has meant that mice are almost exclusively employed in transgenic work. Although a number of mouse lines have been generated in which individual cytochrome P450s have been deleted, these have either no overt phenotype, with differences only becoming apparent following xenobiotic challenge (drug metabolising P450s from Families 1–4), or exhibit problems ranging from embryonic lethality to varying degrees of post-natal health issues (P450s involved in 'house-keeping' or essential chemical reactions) [1,17–19]. For some P450s, mouse lines have been crossed to generate models in which more than one enzyme is deleted, i.e. Cyp1a1/Cyp1a2 and Cyp1a1/Cyp1b1 [20]. Carcinogenicity studies with benzo[*a*]pyrene in such mice has demonstrated the importance of the balance between Cyp-mediated detoxification versus metabolic activation in specific tissues, and shown that consideration must also be given to the route of carcinogen exposure [21,22].

A mouse model has been developed where the key electron transfer protein in all CYP-mediated reactions, cytochrome P450 reductase (POR), has been conditionally deleted in the liver [Hepatic cytochrome P450 Reductase Null (HRN)]. These mice have a profoundly reduced capacity for hepatic CYP-mediated drug metabolism [3,23,24],

providing a powerful *in vitro* model for unravelling the role of CYPs in relation to xenobiotic metabolism and toxicokinetic studies.

In relation to dose-response, HRN mice have proved successful for understanding cyclophosphamide metabolism, in which quantitative measurements and comparisons of the *in vitro* metabolism and intrinsic clearance of the anticancer drug in wild-type and hepatic POR-null animals have confirmed that hepatic metabolism is the major route of CPA elimination and disposition [25]. By altering the pharmacokinetics of CPA in the HRN mice, we have been able to study the toxicokinetics of this drug, i.e. the relationship between therapeutic efficacy and myelotoxicity, the major side-effect associated with CPA. This has allowed us to determine that by increasing the time over which CPA is administered, from bolus to infusion, the overall exposure to 4-OH CPA (a measure of therapeutic efficacy) is unchanged, whilst the C_{max} of this metabolite (proportional to myelotoxicity) is significantly reduced [25].

Similarly, the HRN mouse may be used to demonstrate efficacy of a compound using doses far below those which would have to be employed in a wild-type animal. For example, when HRN mice are treated with a dose of pentobarbital which is non-narcotic to wild-type mice, those mice lacking hepatic POR are anaesthetised for extended periods [3]. Thus, pharmacological effects of pentobarbital were discovered which might not have otherwise been observed, and a much lower drug dose was required. The removal of hepatic P450 metabolism enables dose-response relationships to be investigated in a much more meaningful manner.

With regard to exposure to environmental chemicals, the potential cancer risk of the environmental pollutants nitro–polycyclic aromatic hydrocarbons (nitro-PAH) has most recently been examined. Following different exposures of 3-NBA (3-nitrobenzanthrone [3-nitro-7H-benz[*de*]anthracen-7-one]) (0.2 or 2 mg/kg body weight), no differences in DNA adduct formation in the various tissues examined were found between hepatic POR-null mice and wild-type littermates, indicating that cytosolic nitroreductases activate 3-NBA rather than microsomal POR [26]. In separate examination of the main metabolite of 3-NBA, 3-aminobenzanthrone (3-ABA) by ^{32}P-postlabelling, DNA binding by 3-ABA in the livers of the null mice are undetectable at a low dose (0.2 mg/kg) and substantially reduced (by up to 80%), relative to wild-type mice, at a higher dose (2 mg/kg). Thus POR-mediated CYP enzyme activities, most likely Cyp1a1 and Cyp1a2, are important for the oxidative activation of 3-ABA in livers [27].

Although rodents have great value as a model system in which to investigate drug toxicity or mechanisms of carcinogenesis, it is recognised that the differences between species could potentially interfere with the extrapolation of data from mice (or rats) to humans. To this end, a number of groups have begun to 'humanise' mouse lines in order to address this problem [28,29].

Phase II — The GstP null mouse as a model system

Glutathione S-transferase P (GstP) is a member of a multigene family of Phase II drug metabolising enzymes, the expression of which has been found to be significantly elevated in chemically-induced tumours in rodents, and in many human tumours also,

and in cell lines made resistant to anti-cancer drugs. Several years ago we generated a mouse line in which GstP is inactivated, and showed that such mice are far more susceptible to the formation of skin papillomas than wild-type mice in response to a two-stage chemical bioassay [4], although there was no change in the level of DNA adducts in the skin (David Phillips, unpublished). Further, in a collaboration with Professor Peter Farmer, we have recently shown a similar difference in chemically-induced lung tumourigenesis, with GstP null mice demonstrating a higher level of pulmonary adenomas after treatment with polycyclic aromatic hydrocarbons, including benzo[*a*]pyrene, and interestingly, that this increase in adenomas is not necessarily associated with an increase in DNA adducts in the lung (Ritchie, Henderson et al., unpublished data). However, others have demonstrated that the absence of GstP is protective in terms of colon carcinogenesis [30], indicating that much remains to be learnt about the role(s) of GstP in tumourigenesis and indeed that this enzyme may function in more than one mechanism with regard to carcinogenesis.

Future work with this model will involve defining the role which GstP plays in the tumourigenic process. To this end, we will be crossing the GstP null mice with a number of transgenic lines, including TgAC, which is genetically initiated by virtue of carrying the Ha Ras gene under the control of a globin promoter, and the APC^{min} mouse. These experiments will allow us to determine the relationship between GstP and carcinogen dose, and define a threshold exposure above which adduct formation, and carcinogenesis, occurs.

Reporter Systems — the CYP1A1 gene promoter

Reporter systems provide exquisite levels of sensitivity and definition in studying protein expression, and are unrivalled in providing real-time information on cellular changes within living animals. They are therefore extremely useful for assessing concentration thresholds. Many *in vitro* reporter systems have been devised around the inducible *CYP1A1* gene promoter, due to the fact that this system exists in essentially only two states, either on or off, depending on the presence or absence of an inducing agent, usually a polycyclic aromatic hydrocarbon. For instance, linked to a β-galactosidase reporter, the rat *CYP1A1* promoter has been shown to be a faithful reproduction of the endogenous gene and to be highly inducible (> 10,000-fold in liver) by 3-methylcholanthrene [5], and has been used to investigate the expression of CYP1A1 during murine embryonic development [31]. We and other groups have used variations of this system to provide fine regulation of, for example, blood pressure via the renin/angiotensin system [32] and the expression of Cre recombinase for conditional gene deletions ([33] and Scott, Finn et al., unpublished data).

The CYP1A1/βGAL mouse will allow us to 'titrate' the induction of the CYP1A1 gene following treatment by dioxin and by benzo[*a*]pyrene, and relate induction to DNA adduct formation, thus establishing thresholds for carcinogen exposure.

Conclusion and perspectives

The relationship between carcinogen dose and tumour response is a complex one, dependent on an unknown number of variables and involving mechanism(s) which are not yet fully elucidated [34,35]. For humans, defining this relationship is particularly important at low levels of carcinogen exposure, since most experimental animal models employ significantly higher doses of carcinogens than those to which we are routinely exposed on a daily basis, in order to elicit a significant tumour response. Carcinogenicity studies are also complicated by the fact that very few give consideration to the cumulative carcinogen dose, and this is of particular importance to short-term carcinogenicity studies. The relationship between DNA adducts and tumourigenesis is also controversial, with DNA adducts often being measurable without tumours being present; directly linking DNA adducts to subsequent tumour formation is experimentally difficult, since in *in vivo* models it is usually necessary to kill the animal to measure adduct levels.

A further confounding factor for risk assessment is hormesis by non-genotoxic carcinogens, whereby some chemicals apparently induce a protective response at low levels [36] (explanation: a threshold is also part of a non-monotonic dose response relationship: it is a change from an increasing (or decreasing) to a flat curve). Rationalising the public health consequences of this phenomenon is difficult, since the mechanisms behind hormesis are unknown. Possible explanations include alterations in expression of genes involved with DNA repair, receptor mediated signalling pathways, and detoxification. The phenomenon of hormesis underlines the importance of carrying out *in vivo* carcinogenicity studies at lower carcinogen doses, in order to determine whether there are truly thresholds below which such compounds no longer exhibit a carcinogenic effect.

Investigating the role of Phase I and Phase II metabolism in the activation or inactivation of chemical carcinogens and their relationship to genotoxic or nongenotoxic effects, is important for understanding initiation mechanisms of carcinogenesis. A major step forward has been the development of *in vitro* and *in vivo* models to assess the contribution of individual or groups of xenobiotic metabolising enzymes. The HRN mouse, in particular, allows the contribution of extrahepatic CYPs to be assessed. This provides a starting point for tackling some of the numerous complicating factors in understanding the toxicokinetics of chemical exposure, such as the route of exposure, absorption, and involvement of different genes. In collaboration with Professor David Phillips at the Institute of Cancer Research, we are using the HRN mouse model to define the role of P450s in the generation of DNA adducts following carcinogen exposure. For example, we have recently found a significant increase in DNA adducts in the liver of HRN mice after treatment with benzo[*a*]pyrene, (Henderson & Arlt, unpublished data) reinforcing the recent concept that the role of CYP1A1 may be concerned more with carcinogen detoxification than activation [20]. Furthermore we use, in collaboration with Peter Farmer, recombinant cell lines to investigate the activation to DNA damaging metabolites of endogenous compounds by cytochrome P450s.

With the available transgenic tools at hand it will be feasible to build quantitative biological models to measure the influence of Phase I and II xenobiotic enzymes on the dose-effect curve. With respect to the initiation of cancer from chemical exposure research in future should aim to:

— Titrate dose vs. carcinogenic effects and to relate biomarker data with thresholds.
— Distinguish between acute and chronic effects on susceptibility, with emphasis on cumulative exposure.
— Investigate inter-organ effects on carcinogens i.e. hepatic metabolism versus target organ activation.

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2. Dose response and potential thresholds in DNA adduct formation

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Many chemical mutagens and carcinogens are electrophiles or are metabolised to such. These electrophilic species can bind covalently to DNA, forming DNA adducts. The initial level of a particular adduct is a function of the dose of the reactive chemical (intermediate) in the vicinity of the DNA and its reactivity towards the particular nucleophilic atom at its present stage of structural conformation [1]. Besides the exposure levels, the dose will depend on rates of formation and elimination of species that react with macromolecules, and on the uptake and transport of parental chemicals and/or reactive intermediates. Adducts are eliminated from DNA as a consequence of chemical instability, enzymatic repair and turn-over of cells. At chronic exposures, a steady-state level of adducts will accordingly be reached.

In vitro reactions between nucleophilic sites in DNA and electrophilic chemicals could be considered to be pseudo first-order reactions, as long as the nucleophilic sites in the DNA have not been substantially used up in the reaction. Consequently there will be a linear relationship between adduct formation and dose. A linear dose response relationship for adduct formation is also expected after *in vivo* exposures, as long as no passive uptake, transport mechanisms or enzymatic processes involved in metabolism and DNA repair are saturated, inhibited or induced [2–5]. Deviation from linearity could also occur if the exposure is causing cell killing or proliferation. Furthermore, if the chemical is poorly absorbed from the site of application, such as after i.p. injection of a strong lipophilic compound, this effect could be more pronounced at high doses and thus influence the slope of the dose response curve. Therefore, linearity is expected primarily at low dose exposures. The subject has been addressed previously in several reviews [2,3,5–9], but the entire available literature data have never been put together.

This review includes data from studies where DNA adducts were analysed after single, as well as after intermittent or chronic dosing. Studies where less than 3 doses were used were excluded and data have only been collected from studies in rodents (mice and rats). About 30 chemicals have been tested for DNA adduct formation in rodents after single dosing [10–43] and about 20 following multiple or chronic dosing [15,35,44–78] Most chemicals used in these tests need metabolism to become reactive and the most common types are alkylating agents and polycyclic aromatic hydrocarbons. For

the majority of compounds there was just one study reported in the literature, but for some, like benzo[*a*]pyrene, tamoxifen, butadiene, the tobacco specific nitrosamine NNK, dimethylnitrosamine, butadiene and aflatoxin B_1, data were available from several studies, i.e. giving more reliable assessments of the true shapes of the curves. Aflatoxin B1 is the most well studied chemical for dose response relationship of DNA adduct formation in experimental animals and an extensive review has been published [76]. The range of doses tested in different studies span from a factor of 4 up to 10^6 (largest spans for aflatoxin, benzene, trichloroethylene, diethylnitrosamine and the fried food mutagens Trp-P-1 and MeIQx) and the number of doses range from 3 to more than 10. The routes of exposures were most often oral or i.p. injection and liver was the most common tissue analysed. I.p. injection is unnatural route of exposure, but in spite of that it has been frequently used, since it is relatively easy to apply and it will give a direct passage to the liver (the tissue which normally has the highest metabolic capacity). A majority of the tests covering a large dose interval have been carried out with accelerator mass spectrometry, which is an extremely sensitive method, i.e. very low doses can be used. The disadvantage with this assay is that specific adducts are normally not measured, therefore, one has to consider false positive responses as a consequence of unspecific binding, radioactive contamination and metabolic incorporation. Studies in which total radioactivity bound to DNA (and not a specific adduct) has been measured by liquid scintillation counting suffer from the same limitations. Other commonly used methods are ^{32}P-postlabelling and analysis of specific adducts by HPLC using radiolabelled test compounds. This latter way of analysis would normally give very reliable measurements since a radioactive trace of the used chemical can be followed.

For all the studies examined where single dosing had been used, the dose response curve was clearly linear at low doses or a linear component could at least not be excluded. For several of the compounds tested over a very large dose interval DNA adduct formation was linear over the entire dose interval, even at relatively high doses, e.g. for aflatoxin, benzene, MeIQx and trichloroethylene [14–17,35]. This would indicate that the metabolism of the chemical (if metabolism is needed) and/or its reactive intermediate is not induced by the treatment itself and is not saturated. If there is enough time between termination of exposure and analysis of adducts, in relation to the rate of repair of the adducts, these findings could also indicate that the repair of the adducts in question was also not saturated. Effects of inducible DNA repair on slopes of dose response curves for DNA adduct formation have not yet been unequivocally shown in rodents *in vivo*. For other chemicals the adduct level did not increase proportionally at high doses. For some studies, an increased slope above a certain dose level could be linked to saturation of metabolism [6,7,21,76] or DNA repair, such as for O6-methylguanine following exposure to dimethylnitrosamine [22]. Almost all of the used test chemicals are also genotoxic and DNA adducts have been identified and shown to be formed *in vivo*, but for a few compounds (methyl-t-butyl ether and trichloroethylene [17,23] the reported study is the first attempt to find adducts, i.e. data have to be considered as preliminary as long as specific adducts have not been identified.

Following intermittent or chronic dosing a similar dose response curve is expected as after single dosing, i.e. linear at low doses, as long as no processes involved in metabolism or DNA repair have been saturated or induced. The data show that this is indeed the case, but changed slopes of the adduct formation curves at high doses are common. Many of the chemicals used during long term exposures exist in gaseous state at normal condition and inhalation was, therefore, the normal route of exposure. The dose response curve for most of these compounds showed a Michaelis-Menten type of slope which is not strictly linear at low doses, but with the few doses normally used the exact slope of the curve could not be analysed [44,52–54,75].

Conclusion and perspectives

DNA adduct formation in rodents is a linear function of the dose at low doses, but deviations from such often occur at high doses, due to the limited capacities of the metabolic systems and/or saturation of DNA repair. The general observation is that the slope of the dose response curve for adduct formation of chemicals that are direct reacting increases at high dose (most often due to saturation of detoxification) and for chemicals that need metabolism to become reactive it will decrease at high doses (saturation of activation). Thus, if data from high dose experiments are used for extrapolation the adduct formation per unit dose could be overestimated for the first type of chemicals and underestimated for the second type.

A limitation with many of the studies is that rather few and often high doses have been used. Therefore, the true shape of the curve at low doses might not have been established. Another drawback is that there are for most compounds data available from just one study, i.e. no confirmation is available. More data are therefore needed, using several reliable methods, to study the shape of dose response curves for DNA adduct formation. In these future studies doses that humans are exposed to, and lower if possible, should be included, i.e. the methods applied must also be the most sensitive.

Dose response relationships for DNA adduct formation have been studied in humans [43,79–83]. However, individual differences due to genetic polymorphisms in genes involved in transport, metabolism and DNA repair makes it difficult to establish exact slopes of dose response curves for adduct formation in humans. In most studies one can at best observe a correlation between exposure and adduct levels [80]. However, also in these cases non-linearity at high exposure levels have been indicated [82,83]. To really study the kinetic of a dose response curve in humans one would have to expose one and the same individuals to different doses of an adduct forming chemical, an experiment which would be difficult to carry out for ethical and other reasons.

The observed linearity for DNA adduct formation in rodents, and most likely also in humans, does not necessarily mean that there will be a linearity between exposure and biological effect (ultimately cancer disease). Tumour formation is a multistep process where genotoxic chemicals will contribute primarily to the initiation step, but downstream factors will be very important. The slope of the dose response for tumour

formation will therefore depend upon the interactions between all contributors to this process and will in most cases be impossible to predict. However, for genotoxic (tumour initiating) effects of DNA adduct forming chemicals linear dose response relationships can in most cases not be excluded and one should probably use a conservative approach when making risk assessment for such chemicals.

Chemicals causing DNA adducts which are present as background adducts from other sources might be a special case where one can talk about "practical thresholds". A number of DNA adducts, particularly those of low molecular weight, have multiple sources, out of which some might be endogenous. If a risk assessment is carried out for a specific chemical which is causing such adducts and the levels formed are considerably lower than the "normal" background one can talk about a threshold, i.e. whatever risk the newly formed adducts are adding could be considered negligible compared to the risk from the same adducts originating from other sources.

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3. Dose-response relationships and potential thresholds in the induction of mutagenesis and the influence of DNA repair and cell cycle progression

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3.1. Introduction

Initiation of carcinogenesis is believed to involve induction of mutations in critical sequences of DNA that can lead to the activation of oncogenes and/or the inactivation of tumour suppressor genes. Such mutations can be induced by carcinogen-derived DNA damage and/or by errors associated with DNA repair or DNA replication. The dose-dependence of mutagenesis reflects a balance between, on one hand, the formation of DNA damage and, on the other hand, the operation of processes such as error-free or error-prone DNA repair, DNA replication occurring spontaneously or under the influence of mutagen-induced mitotic signals, and loss of genetically damaged cells as a result of necrosis or apoptosis [1]. For example, following treatment of lacZ transgenic mice with benzo[*a*]pyrene (B[*a*]P), higher levels of formation and initial rate of repair of DNA adducts were observed in tissues with significant cell proliferation (lung, spleen, liver) than in non-proliferating tissues [2]. On the other hand, the tissue-specific rate of accumulation of mutations in different tissues exhibited a complex dependence on adduct formation, DNA repair and cell proliferation. Furthermore, the decrease of the mutant frequencies to background levels observed some weeks after B[*a*]P treatment suggested that cell loss, probably due to apoptotic processes, also plays an important role in determining the efficiency of the accumulation of mutations.

3.2. Point mutations and DNA repair

DNA repair plays an important role in the protection of cells and tissues against genotoxins. Evidence demonstrating that absence or substantial reduction of the level of DNA repair results in increased cellular sensitivity to genotoxic effects comes not only from studies of experimental carcinogenesis but also from human syndromes combining deficiency in DNA repair with increased susceptibility to cancer [3,4]. This leads to the

anticipation that any variation in the efficiency of DNA repair at different levels of exposure, e.g. changes reflecting saturation or, conversely, induction of repair, may affect the shape of the dose-response curve for carcinogenesis or for intermediate stages such as accumulation of DNA damage or mutations. It has been hypothesised that the initiation of chemically induced transformation is a mutational event and the formation of adducts on DNA, including those of PAH carcinogens, have mutagenic consequences [5]. While it is well known that adduct formation per se does not determine carcinogenicity [5], the rate of repair of lesions prior to cell division can be a determining factor [6].

Relatively few studies have been conducted to assess in detail the shape of the dose-response curve linking mutagenesis with preceding processes such as DNA repair. Perhaps the most extensive database on such quantitative relationships exists for simple alkylating agents. In an early study, Pegg and Hui [7] administered to rats single doses of the methylating carcinogen N-nitrosodimethylamine (NDMA), in the range 1 μg–20 mg/kg, and measured the levels of N7-methylguanine (N7-meG) and O^6-methylguanine (O^6-meG) in liver DNA. They observed a linear increase in the amount of N7-meG formed, but a sublinear (higher slope at higher doses) increase in O^6-meG above a dose (approx. 0.5–1 mg/kg), which caused saturation of the DNA repair protein O^6-methylguanine-DNA methyltransferase (MGMT). Given that O^6-meG is believed to be responsible for most of the mutagenicity and carcinogenicity of NDMA, saturation of its repair would be expected to lead to a corresponding non-linear dose-response in mutage-nesis and carcinogenesis.

The dose-response relationship of NDMA mutagenesis was investigated by Souliotis et al. [8] who measured DNA adducts and mutant frequencies in the liver of lambda-lacZ transgenic mice treated with NDMA at single doses of 1, 5 and 10 mg/kg. The levels of O^6- and N7-meG were approximately linearly related to the dose, although the proportionality constant for O^6-meG was somewhat reduced at 1 mg/kg, reflecting the lower level of saturation of MGMT at this dose. On the other hand, a significantly increased efficiency of mutagenesis in the lacZ gene, relative to the administrated dose or the corresponding DNA adduct levels, was observed in going from 5 mg/kg to 10 mg/kg, probably reflecting mutagenesis-enhancing influence of cell proliferation caused by the toxicity of high doses of NDMA.

Treatment of rats with single doses of diethylnitrosamine (DEN) results in the formation of O^6-etG at a rate approximately eight times greater than O^4-ethylthymine (O^4-etT) [9]. However, following 4 weeks exposure to 40 ppm DEN, O^4-etT accumulates at levels fifty times higher than O^6-etG, because of efficient repair of the latter lesion as a result of the induction of MGMT by the chronic treatment. On the other hand, repair of a third lesion induced by DEN, O^2-etT, appears to become saturated as the dose of DEN in the drinking water is increased from 0.4 to 100 ppm (4 weeks' treatment), resulting in an additional increase in this adduct, relative to O^4-etT. Thus, during chronic treatment with DEN, the dose-response relationship for the accumulation of different DNA adducts exhibit linearity for O^4-etT and deviation from linearity for O^6-etG (as a result of induction of its repair) and for O^2-etT (as a result of saturation of its repair).

The relationship between dose, DNA damage and DNA repair in colon and liver mutagenesis induced by 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) was investigated in lacI transgenic rats fed diets with 20–200 mg IQ/kg for 3 weeks [10]. Non-linear dose-response relationships of DNA adducts in both colon and liver were observed, with an abrupt increase in adduct levels being observed above 20–70 mg/kg (depending on the tissue). On the other hand, a more or less linear induction of mutations was observed. Furthermore, the highest levels of DNA damage were observed in the colon while 2-fold more mutations were found, for the same dose, in the liver compared with the colon. This observation, which is in line with analogous observations of Ochiai et al. [11], is surprising in view of the higher rate of cell turnover known to exist in the latter tissue. These results demonstrate the complexity of the parameters, which determine the quantitative relationship between dose, DNA damage and mutagenesis.

The mutation frequency and the levels of DNA adducts were measured in the Chinese hamster V79 cells (*HPRT* mutants) exposed to various doses of BPDE (0, 50, 100, 200, 400, 700 nM) [12]. The BPDE-induced DNA adducts and the mutation frequencies were proportional to the given dose. Moreover, the profile of mutations induced by BPDE in the *hprt* gene of V79 cells, was found to be dependent on the dose employed [13]. When the BPDE dose was decreased, the proportion of base substitutions targeted at GC base pairs decreased and the proportion of base substitutions, targeted at AT base pairs increased, suggesting dose-dependent differences in DNA repair acivities for the removal of diol epoxide-induced guanine and adenine adducts. Furthermore, Schiltz et al. [14] examined the effect of the concentration of BPDE on the mutational profile in the *hprt* gene in transcription-coupled repair (TCR)-deficient V-H1 cells (a derivative of V79 cells) to explore the role of DNA repair in the dose-dependent mutational profile of BPDE. Thus, V-H1 cells were exposed to low (4–6 nM) or high (40–48 nM) doses of BPDE, while V79 cells were treated with low (10–20 nM), intermediate (40–100 nM) or high (300–480 nM) doses of BPDE. They found that V-H1 cells were 9-fold more sensitive to the cytotoxic effects of BPDE than V79 cells (proficient in both subpathways of nucleotide excision repair — NER, e.g. TCR and global genome repair -GGR), while the mutation frequency in V-H1 cells was similar to that observed in V79 cells. BPDE-induced mutations at guanine on the transcribed strand of the *hprt* gene were common in V-H1 cells but were rare in V79 cells; however, mutations at adenine on the transcribed strand of the *hprt* gene were common in both V-H1 and V79 cells. Finally, although exposure of V79 cells to different doses of BPDE resulted in a dose-dependent mutational profile at the *hprt* gene, this was not observed in V-H1 cells, indicating that the defect in TCR is essential for the dose-dependent mutational profile observed with BDDE in V79 cells.

3.3. Point mutations, cell cycle progression and cell proliferation

Unrepaired DNA damage may activate cell-cycle checkpoints to allow more time to repair damage and thereby avoid irreversible events [15]. Alternatively, if damage is too great or persists too long, a signal might be transduced that initiates an inactive programme, leading to a permanent growth arrest (in proliferative cell types) or apoptosis. Depending on the cell type, its proliferative history and current position in the cell cycle, in addition to the type and amount of damage, a cell might need to choose between DNA repair and apoptotic death. The overall response to DNA damage must be tightly regulated. One major element in this regulation is the tumour suppressor protein p53, which is thought to have evolved in multicellular organisms to protect against cancer. Exposure of cell systems to genotoxic agents often results in the stabilization of the p53 protein as a common response to DNA damage [16]. Consequently, p53 levels increase and this transcription factor turns on the expression of many genes, including $p21^{waf1}$, a known inhibitor of cyclin-dependent protein kinases. Since these kinases phosphorylate the Rb protein in order to allow the cells to enter the S-phase of the cell cycle, their inhibition causes cells to arrest in the G1 phase. Such p53-mediated G1 arrest permits a choice between three paths: escape into S-phase and cell proliferation, protracted G1 arrest pending DNA repair or cell deletion via apoptosis. This G1 decision point is crucial to the expression of genotoxicity and represents a defence mechanism for the cell that can help to prevent mutation and maintain genomic integrity [17]. It has been proposed that p53-deficient cells have a mutator phenotype, due to their abnormal apoptotic response and/or abnormal cell cycle control [18].

Dipple and coworkers, based on their studies in human breast carcinoma MCF-7 cells, proposed the concept of "stealth carcinogens", i.e. carcinogens with the ability to damage DNA without inducing a G1 arrest, thus allowing cells to replicate on a damaged template [19–22]. The non-induction of the G1/S arrest adds an additional modulating parameter, the likelihood of the particular DNA damage leading to cell cycle arrest, so that repair may be affected or apoptosis initiated before DNA replication on a damaged template can give rise to mutation. This effect may be cell-type specific and dose-dependent. Thus, when human diploid fibroblast cells were exposed to various doses of dibenzo[*a,l*]pyrene (DB[*a,l*]P; 0.014, 0.028 and 0.07 μM) [23], while the lowest dose used did not arrest a significant proportion of cells in G1 phase compared with the control (stealth effect), at the higher doses a dose-dependent G1 arrest was observed which correlated well with the levels of DNA binding determined. This discrepancy with the reports of Dipple et al. [22] could simply result from using different cell types that may respond in a different manner to PAH-induced DNA damage. Alternatively, PAHs may present a dose-dependent effect on the induction of the G1/S arrest, indicating that exceedence of a critical threshold level of PAH-DNA adducts is required before a cellular response occurs. This would imply that low doses of a carcinogen may induce a low level of mutations that allows cells to escape cellular arrest and thus lack sufficient time for DNA repair processes to remove the damage prior to DNA replication. Consequently, even low levels of carcinogen-DNA adducts could generate mutations in the

genome and, possibly, also within critical genes that may lead to the generation of cancer cells [19–21,24].

The significant impact of cell proliferation on mutagenesis following genotoxic treatment is demonstrated by studies on the mutagenicity of the polycyclic aromatic hydrocarbon 5,9-dimethyldibenzo[*c,g*]carbazole (DMDBC) in lacZ transgenic mice [25]. Single, s.c. doses of DMDBC (3 to 180 mg/kg) resulted in liver mutagenesis which increased abruptly at 30 mg/kg or higher. Examination of the accumulation of DNA adducts and hepatocyte proliferation suggested strongly that increased cell proliferation caused by hepatotoxicity at the higher doses, rather than a change in the rate of accumulation of DNA damage, was responsible for the increase in mutagenic efficiency.

The BigBlue mouse system was also used to investigate the role of cell proliferation in mutation fixation in the mouse back skin model of carcinogenesis [26]. Cell proliferation was stimulated with phorbol 12-myristate-13 acetate (TPA) and B[*a*]P-induced mutations were measured in the skin. Parameters of cell proliferation (mitotic index, epidermal thickness and cell cycle stages) were measured. As the dose of B[*a*]P increased (4 to 64 μg), DNA adduct levels and mutant frequency also increased in a dose-dependent manner. Co-treatment with TPA enhanced both mutant frequency and DNA adducts but not always mitotic index, which measures only those cells that have successfully completed the cell cycle. Mutant frequencies were positively correlated with the levels of DNA adducts caused by B[*a*]P, but tended to be inversely associated with mitotic index. Tumour promoters, such as TPA, and carcinogens affect cell proliferation in different ways. TPA increases the number of cells in mitosis, with a peak at above 48 h after application. B[*a*]P on the other hand, can increase mitotic index and labelling index almost immediately when applied in a low dose such as 4 μg B[*a*]P, but higher doses produce an initial inhibition of mitotic index, while simultaneously increasing labelling index through a lengthening of the S phase [27]. These changes are reversed many hours to days later by a rebound increase in mitotic index and return to a lower labelling index (shorter S phase). With TPA and B[*a*]P together the interplay of these different influences on cell proliferation will determine the overall mitotic index.

Of relevance to the above observations are studies on the dose-response relationships governing the induction of carcinogenesis, DNA adducts and cell proliferation in the liver of Wistar rats treated chronically with NDMA. Lifetime administration of NDMA in the drinking water resulted in the induction mainly of hepatocellular carcinomas, with a dose-dependency that was linear at low doses (and showed no evidence of a threshold) but increased abruptly above a dose-rate of approx. 200 μg/kg/day [28]. On the other hand, under comparable conditions of treatment, accumulation of the premutagenic and precarcinogenic lesion O^6-meG in liver DNA showed no significant deviation from dose-linearity (and no evidence of MGMT saturation) [29]. However, the labelling index of hepatocytes increased significantly above 200 μg/kg/day, suggesting that a change in cell proliferation and not in DNA damage accumulation caused the abrupt increase in carcinogenic efficiency at high doses [30].

A role for cell proliferation, arising as a result of NDMA toxicity, in enhancing mutagenesis is also suggested by the results of Mirsalis et al. [31]. Daily treatment,

for 5–21 days, of lambda-lacZ transgenic mice with 2 mg/kg/day did not cause any increased mutagenesis in the liver of 6-week old animals, whereas 10- to 20-fold elevations were observed in the case of younger (3-week old) animals.

3.4. Micronuclei — chromosomal aberrations

In recent years, increasing attention has been paid towards the use of micronuclei (MN) as an index of cytogenetic damage in fish and clams exposed environmentally to a variety of toxic and genotoxic pollutants. Different recognised mechanisms may give rise to MN and MN-like structures in cells exposed to genotoxic agents and, excluding apoptosis, cell division is essential for MN which are indicative of cytogenetic damage. Nowadays, MN detection represents a widely used parameter, easily performed, which also allows molecular approaches in studying the effects of many clastogenic or aneugenic agents. The human bronchial epithelial lung cell line BEAS-2B, immortalised with a virus construct, was treated for 24 h with five different concentrations of B[a]P (4, 20, 100, 500 and 1000 nM) to assess the relationship between DNA adduct levels, cell cycle distribution and micronuclei formation [32]. There appeared to be a strong linear correlation between B[*a*]P concentration and DNA adduct formation ($r = 0.99$, $p = 0.001$), while no difference in cell cycle distribution was observed after incubation with these concentrations of B[*a*]P. On the other hand, MN increased with increasing B[*a*]P concentration and DNA adduct levels, while no substantial increase in apoptosis after 24 h was found.

Nitro-aza-benzo[*a*]pyrenes (1- or 3-N-6-ABP) and their N-oxides (1- or 3-N-6-ABPO) are potent mutagens for *Salmonella* strains [33]. To further investigate the mutagenic properties of these derivatives, micronucleus induction in mice and chromosomal aberrations in Chinese hamster lung (CHL) fibroblasts were studied [34]. The induction of MN was dependent on the dose response of 10–40 mg of 3-N-6-ABP, and of 10–40 mg for 1-N-6-ABP and in addition 1- and 3-N-6-ABPOs markedly increased MN in a dose range of 10–400 mg and from 1 to 80 mg, respectively. The results of chromosomal aberrations of the four compounds, also showed a dose-dependent induction of aberrations of the chromatid type, chromatid breaks and exchanges for 1- and 3-N-6-ABP and mainly chromatid exchanges for 1- and 3-N-6-ABPO.

Two non-transformed epithelial-like cell lines (C6 and C2.8) derived from fetal mouse liver were treated with B[*a*]P (8, 20 and 40 μM) and a dose-dependent induction of DNA adducts was found [35]. In additional experiments a significant and reproducible increase of MN was evident in C6 and C2.8 cells treated with increasing doses of B[*a*]P (0, 0.2, 2 and 20 μM) for one replication time. The percentage of mitotic cells was increased following exposure of cells to the lowest dose (0.2 μM), while a significant reduction was found in cells treated with the two higher doses (2 and 20 μM). This decrease of C6 and C2.8 mitotic cells observed at the highest B[*a*]P doses indicated a broad effect of the compound on the progression of cell replication and, consequently, on the

expression of chromosomal damage (slower appearance of micronuclei) indicating that the MN frequencies observed at the doses of B[*a*]P inhibiting cell replication were probably underestimated. No significant changes in the MN frequency were observed in the CHO cell line, tested for comparison. Notably, the proliferation of CHO cells was not affected by these doses of B[*a*]P.

Increasing attention has been paid towards the use of MN as an index of cytogenetic damage in fish and clams exposed environmentally to a variety of toxic and genotoxic pollutants [36]. In B[*a*]P-exposed mussels (0, 5, 50, 100, 500, 1000 μg/l), a dose-dependent increase in micronuclei was obtained for doses > 50 μg/l, in both the large gill cells and the agranular haemocytes ($p < 0.05$) [37]. It is worth noting that the MN assay and ^{32}P-postlabelling assay (DNA adducts analysis) detected the same lowest effective dose in mussels treated independently, that is 50 μg/l B[*a*]P [38]. In this study, mussels were exposed to various B[*a*]P doses (0, 0.5, 5, 50 and 100 μg/l) for 48 h or 72 h. Statistically significant dose-response and time-dependent increase in DNA adducts in gills were observed at both time-points examined (48 h and 72 h), while a threshold for adduct induction was found: DNA adducts were measured only at doses greater than 50 μg/l.

Other investigators exposed groups of mussels exposed for 2 days to 1, 5 and 10 μg/l of B[*a*]P. A dose-dependent induction of CA in gills was found [39]. The correlation coefficient was $p = 0.392$, yielding significance to the existing correlation between the concentration of B[*a*]P added and the percentage of aberrant metaphases at $p < 0.025$. In another study, mussels were exposed to higher concentrations of B[*a*]P (25, 75, 225, 675 μg/l) [40]. A dose-dependent increase in the induction of MN was also found in gills of mussels up to the concentration of 75 μg/l. Maximum MN frequencies were obtained at a concentration of 75 μg/l while at the two highest B[*a*]P doses (225 and 675 μg/l) a reduction in the mean level of micronuclei was detected. This decrease may be due to the strong clastogenic effect of the mutagen that would delay or stop the division of most damaged cells.

The micronucleus test was also performed in adults and spats of the oyster Crassostrea gigas following exposure to various doses of B[*a*]P (0, 0.5, 5, 500 and 1000 μg/l) to evaluate the genotoxic effect of the marine environment [41]. The MN frequency observed for adult oysters showed a slight increase in MN for the 0.5 μg/l and then a rapid increase for 5 μg/l to 500 μg/l concentrations. MN measurement was impracticable for the 1000 μg/l concentration since the cells were very fragilised by the high B[*a*]P concentration and unstained or considerably damaged. MN frequency for spat haemocytes (obtained from gill tissue), following exposure to B[*a*]P were much lower than that for adult haemocytes (obtained from pericardial cavity) indicating that the greater cell division activity responsible for micronucleus multiplication did not seem to increase MN assay response in spats. A possible explanation for this discrepancy could be the greater capacity of adult oysters for repair of DNA damage. Alternatively, the weaker response of treated spat may have been due to the greater vulnerability of young organisms, whose cell division could be rapidly inhibited by B[*a*]P effects.

3.5. Discussion

The formation of DNA damage and the subsequent disappearance results from an interplay of molecular events involving absorption of the agent, distribution to different tissues, metabolic activation to form reactive intermediates, detoxification processes, DNA repair, signalling to the cell cycle or apoptosis [1]. These two events, DNA repair and signalling to the cell cycle or apoptosis machineries, comprise what is known as the "DNA damage response". Damage signalling might behave as a binary switch, producing one of two outcomes — DNA repair or cell death — depending on a preset DNA damage threshold. Lowering this damage threshold might shift the balance in favour of apoptosis independently of DNA damage repair systems. However, the damage threshold, as well as repair capacity, is both cell type- and species-specific [42]. This is likely to explain the observed tissue specificity of cancer with particular defects in the DNA damage response.

DNA repair is a factor playing an important role in the deviation of the dose-response linearity in the mutagenesis. DNA repair can be induced and it can be saturated. Rusyn et al. [43] demonstrated increased gene expression for oxidative DNA repair enzymes in liver RNA from rats and mice treated with peroxisome proliferators. In addition, it must be recognised that most DNA repair pathways utilise multiple enzymes. In the case of base excision repair, glycosylases, AP-endonuclease, polymerase B and ligase are required for complete repair. It is conceivable that imbalances in such a pathway could result from either induction or saturation of repair that would result in removal of the adduct, but also accumulation of a subsequent lesion such as strand breaks.

Most of the studies described here show a linear dose-dependent induction of mutagenesis; however, a few studies showed a deviation from linearity which, as explained above, could be attributed to changes in DNA repair [7,9]. Moreover, some studies showed a deviation from linearity of the dose-response in mutagenesis resulting in increased mutagenesis at higher doses due to chemical-dependent induction of cell proliferation [8,25], and a similar effect probably lied behind the abrupt increase above a critical dose in NDMA-induced hepatocellular carcinomas in rats [28,29].

Although the formation of DNA damage may not always have a threshold, it is conceivable that the conversion of such damage into mutations may exhibit a threshold effect. For example, at high dose of a genotoxic chemical, cells may engage the apoptotic pathway. At intermediate doses, the DNA damage may be below the level required for apoptosis, but might favour, instead, a G1/S arrest pending repair, coupled with some G1/S leakage giving rise to mutations. At low doses, the G1/S arrest may be absolute until repair is complete. Even an acceptance of the existence of such threshold does not permit an accurate prediction of the impact of changing doses: with decreasing dose, cells with reduced levels of damage may survive rather than be deleted, enhancing the resulting rate of mutation. Conversely, a decreased dose could fall below the threshold of cytotoxicity, removing stress cytokines and thus the stimulus for replication in the remaining viable, but DNA damaged cells. This would be associated with a decreased rate of mutation.

The frequency of mutants per mutagen-induced DNA adduct in the genome or the coding sequence of the gene is an important parameter reflecting the probability that a primary lesion leads to mutation following repair, apoptosis and other processes that modify the manifestation of a mutation event. Thus, the frequency of mutants per DNA adduct for the coding region of the HPRT gene was estimated to be 0.04 for BPDE [12]. In agreement with this value, earlier studies in V79 cells for various PAHs [syn- and anti diol-epoxide of B[*a*]P, benzo[*c*]chrysene, benzo[*g*]chrysene and benzo[*c*]phenanthrene] found values in the range of 0.01–0.04 per DNA adduct in the *HPRT* exons [44]. Moreover, in *in vivo* studies, the number of mutants per DNA adduct was estimated to lie in the range of 0.01–0.05 in mouse liver, lung and spleen, i.e. a range overlapping with that found for *HPRT* mutations *in vitro* [2,45]. Finally, for a few simple alkylating agents, similar frequencies of mutants per DNA adduct were obtained: ethylene oxide gives 0.03 and ethylating agents 0.08 [46], while for MNNG figures in the range 0.1–0.2 mutants per O^6-meG residue have been reported [47,48].

The biological significance of the relationship between adduct formation and mutation induction has not yet been established due to the fact that methods for measuring mutations are not as sensitive as those measuring DNA adducts [49]. DNA adducts can be detected at frequencies of 10^{-10} [50], whereas mutations are only generally detectable at frequencies of about 10^{-6} [51], this limit being primarily determined by the frequency of background mutations which reflect spontaneous events occurring during DNA replication. What happens below these detection limits? It is possible that there are non-linear effects below the detection limit that we are currently missing, or conversely, that detected thresholds are a consequence of the insensitivity of the methods used at low doses. Further developments are required to produce more sensitive methods for detecting rare DNA mutations. One way of approaching this question using currently available methodologies might be to employ chronic exposure using low doses of mutagens. Surprisingly there is an almost complete lack of studies, while the few that have been reported do not permit an evaluation of the dose-response relationships [52–54]. On the other hand, on a more theoretical ground it should be recognised that, while in principle even a single adduct per cell, if present in a gene under observation, may be sufficient to induce a mutation (which could lead to an observable biological event such as cancer), assuming a near random distribution of adducts in the genome would imply the need for a minimum number of such adducts being present in the genome overall before a mutation in a specific gene can be induced.

In conclusion, while the studies reported so far focusing on the shape of the dose-response relationships for mutagenesis provide some evidence of deviation from linearity as a consequence of variation in DNA repair, no direct evidence of a resulting real or practical threshold has been produced. On the other hand, clear evidence is available regarding the modulation of mutagenesis by cell proliferation or apoptotic processes, usually induced by high-dose toxicity. Assessment of the modulation of mutagenesis by similar processes and in the absence of such toxicity constitutes an important question and a current research challenge.

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4. Dose response and potential thresholds in gene expression

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When cells are exposed to xenobiotic compounds, including carcinogens, the physiology of the cell may change. This can vary from subtle alterations, such as on metabolic capacity, cell-cell interaction, proliferation, to the most severe effect: cell death. Many of these changes will be accompanied by alterations in the expression of genes, either as a direct/initial or an indirect/secondary response. Ligand-activated nuclear receptors, for instance the Aryl hydrocarbon receptor, will, upon activation directly act as transcription factors and enhance the expression of specific genes. Many non-genotoxic carcinogens, but also some genotoxic carcinogens, appear to act according to this mechanism. The DNA damage response induced by many genotoxic carcinogens, may be an example of an indirect/secondary response, since first several succeeding events are required (e.g. activation of the carcinogen by metabolic enzymes, formation of sufficient DNA damages to trigger a signalling pathway, the successive steps in that pathway [many by protein kinases]), ultimately leading to the activation of transcription factors.

Here, the aim is to discuss the dose response relations and potential thresholds on modulation of gene expression due to exposure of cells to carcinogens, with a focus on the dioxin TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) as model for non-genotoxic carcinogens, and polycyclic aromatic hydrocarbons (especially benzo[*a*]pyrene, B[*a*]P) for genotoxic carcinogens. Furthermore, where feasible, the overview is restricted to studies on effects in animal models, with a special attention for those using DNA micro-arrays as the method for measuring gene expression, and with investigation of at least 3 different doses.

The most comprehensive *in vivo* study on TCDD-induced gene expression changes, was recently published by Boverhof et al. [1]. Dose-response analyses were performed on hepatic tissue from immature ovariectomized C57BL/6 mice treated with TCDD (0, 0.001, 0.01, 0.1, 1, 10, 100, or 300 μg/kg) and sacrificed after 24 h. Gene expression profiles were monitored using cDNA microarrays containing ca. 13,000 genes. The data indicate that TCDD elicits gene specific dose-dependent responses as the ED50 values may vary drastically. This may be due to gene-specific upper thresholds (saturation of gene expression), differential temporal regulation, or differing basal expression levels, which would affect the dose at which transcriptional regulation may be initiated or detected. Whether thresholds exist below which no expression changes occur, is not described. However, taking into account the large dose-steps and the variation in observations, it appears not feasible to establish the existence of such thresholds for most genes.

In general, the gene expression responses preceded or paralleled the observed histopathology for the various functional categories. This suggests that apparent thresholds for histopathological parameters, may not be confirmed at gene expression level.

Another study addressing the effects of TCDD on gene expression *in vivo*, was undertaken during murine cardiovascular development in C57Bl/6N pregnant mice dosed with 1.5, 3.0, or 6.0 μg TCDD/kg [2]. Although a few genes showed typical dose-related effects on expression, many more of the genes showed significant alterations in expression only at the highest dose or only at the lower doses.

Numerous studies have been conducted in many mouse and rat organs, on TCDD-induced effects on the expression *CYP450* genes by RT-PCR methods. In a dose–response study on *CYP1B1, CYP1A1,* and *CYP1A2* in the liver of female rats following chronic exposure to TCDD, results showed that while the liver concentration of TCDD required for half-maximal induction was similar, the shaping parameter of the dose–response curve for *CYP1B1* was significantly higher than that for *CYP1A1* or *CYP1A2* [3]. Even at the lowest dose (3.5 ng/kg/day), however, all genes were clearly induced. Another study in the liver of female mice 24 h following a single exposure, however, showed that for *CYP1A1* a steeper dose-response was observed than for *CYP1B1* [4]. Furthermore, it appears that a threshold for induction of gene expression may exist, although differences between mouse strains are evident. In organs from the immune system of the rat, this thresholded effect on CYP1A1 induction is not that clear following acute [5] or chronic treatments [6].

For the genotoxic class of carcinogens of polycyclic aromatic hydrocarbons, including B[*a*]P, much less data are available. Despite that several gene expression studies on B[*a*]P have been described, few of the microarray or RT-PCR based gene expression studies apply at least 3 dose levels or are conducted in animals. To our knowledge, the only *in vivo* study fulfilling our selection criteria was from Bartosiewicz et al. [7]. In this study, DNA arrays containing 148 genes for relevant toxic processes were used to examine gene expression patterns in the liver of mice in dose-related response to B[*a*]P. Administration of B[*a*]P up-regulated only *CYP1A1* and *CYP1A2* and produced no significant increases in any of the stress response genes or any of the DNA repair genes present on the array. Whether or not a threshold occurs at low doses is not apparent from the current data.

Due to the lack of *in vivo* data, *in vitro* studies using cell lines or primary cells have also been reviewed. Surprisingly few studies on multiple doses, genotoxicants, and gene expression by microarray or RT-PCR methods (focussing on *CYPs, p21/Cip1/Waf1* and *GADD45*) were retrieved. In one study, the human TK6 cell line was used as an in vitro model system, and reactive metabolites of human carcinogens, N-hydroxy-4-acetylaminobiphenyl (N-OH-AABP) and benzo[*a*]pyrene diol epoxide (BPDE), were used as model compounds [8]. Each compound was tested at low, medium, and high toxicities (5, 15, and 40%) and analysed for mutagenic activity and gene expression modulation using microarrays comprising approximately 18,000 genes. The microarray data from the treatment groups were compared using self-organising map clustering algorithms, as well as a statistical regression modelling approach. Gene clusters were identified that showed differential

dose-dependent responses to BPDE and N-OH-AABP, such as a continuous dose-dependent increase or decrease, saturation at the two high doses, or possible thresholded effects at low doses. Whether these differential dose-dependent responses are linked to specific functional processes or pathways is unclear. Another study in TK6 cells using BPDE showed that DNA adduct formation was the most sensitive indicator of DNA damage [9]. DNA adduct formation was clearly evident at low doses, where the number of genes with significantly altered expression was minimal. Alterations in gene expression were more robust at doses associated with cellular toxicity and induction of mutations. For both of these studies it is not evident whether thresholded effects on gene expression occur. For RT-PCR based studies, no examples were found that fulfilled all criteria.

Summarising, both for the non-genotoxic and genotoxic carcinogens, no firm conclusion can be given whether their effects on gene expression in target tissue *in vivo* or in cells in vitro show a threshold or not at low doses. Besides some conflicting data, most studies simply do not have the power for conclusive statements.

Conclusion and perspectives

As indicated above, most dose-response studies on modulation of gene expression by non-genotoxic and genotoxic carcinogens in target tissue *in vivo* or in cells *in vitro*, simply do not have the power for firm conclusions whether or not thresholds for induced effects exist at low dosages. The main problem is to discriminate true thresholds from apparent thresholds due to technical limitations, mostly the observed variations for the investigated effect parameters. Obviously, the only answer lies in large-scale studies, with many dose levels with small intercepts, lots of biological repeats, combined with appropriate statistics that can significantly discriminate thresholded dose-response curves from linear dose-response curves.

For microarray studies on gene expression changes where thousands of genes are investigated, an additional problem appears: namely the chance of false positive conclusions. With such a huge number of genes, it is likely that statistical tests will always — by chance — come up with some significant thresholded responses. This specification of the multiple testing problem, raises the question of how to discriminate true thresholded responses from false positive responses. Possibly, new statistical approaches are required to solve this problem for dose-response analyses. Also in this case, the answer lies in large-scale studies, with many dose levels with small intercepts, and lots of biological repeats. The application of smaller arrays rather then whole-genome arrays (e.g. after selection of the affected genes by limited preliminary studies), may reduce the otherwise huge costs for microarray analyses. Furthermore, the verification of array data with independent methodologies, such as quantitative RT-PCR, will aid to discriminate true thresholded responses from false positive responses.

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5. Dose response and potential thresholds in proliferation and cell survival and death

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Tumourigenicity is the result of the balance between mutations, epigenetic changes, cell proliferation and cell death. Cell proliferation can be a primary effect of the carcinogen or a secondary effect consequent to cell toxicity [1]. For cancer risk assessment, the role of cell proliferation and cell death (apoptosis and necrosis) is particularly critical for non-genotoxic agents because a threshold effect is likely (Fig. 5.1). Whether induction or inhibition of apoptosis (or necrosis) is carcinogenic may be dependent on the type and concentration of the carcinogen. Apoptosis is considered to be anti-carcinogenic when eliminating mutated cells after exposure to genotoxic carcinogens or "epigenetically" modified cells after exposure to non-genotoxic carcinogens. On the other hand, excessive elimination of cells can induce compensatory cell proliferation to restore homeostasis. This process will contribute to expansion of mutated or modified cells. Moreover, rapid proliferation may in itself lead to genomic instability.

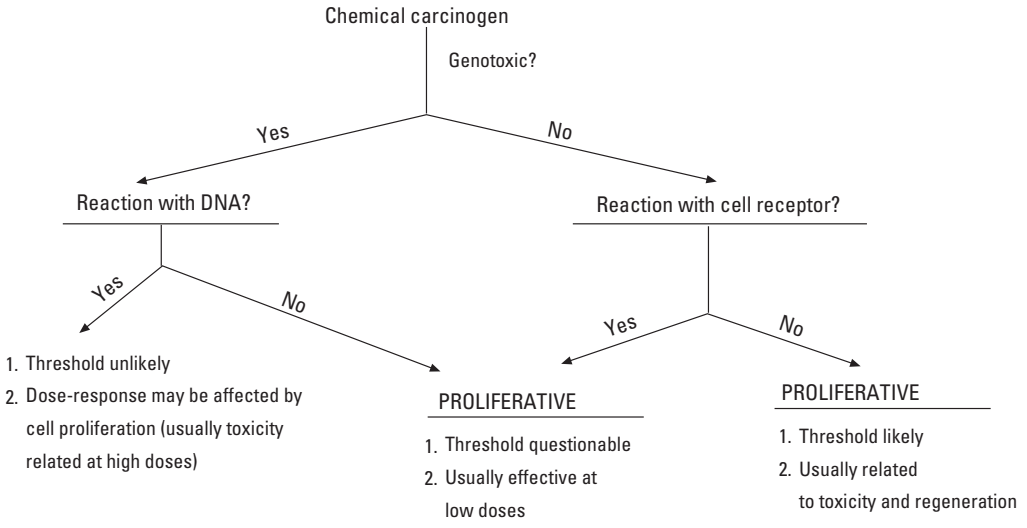

Fig. 5.1. Classification scheme for carcinogens (Adapted from [1]).

5.1. Do polycyclic aromatic hydrocarbons (PAHs) induce apoptosis and if yes does it support a threshold dose-effect for mutations and/or cancer? Benzo[*a*]pyrene (B[*a*]P) as an example

Introduction

A number of PAHs are known or suspected carcinogens and have been extensively studied as genotoxic, initiating agents [2]. It has been proposed that PAHs contribute to cancer development by modulating various signalling pathways and transcription factor activities linked to cell proliferation and apoptosis [3].

The most studied member of the PAH family is the unsubstituted five-ring compound benzo[*a*]pyrene (B[*a*]P), which was recently evaluated by the International Agency for Research on Cancer as carcinogenic to humans (Group 1) [4]. Most of the biological effects of B[*a*]P and other PAH are considered to be mediated via the aryl hydrocarbon receptor (AhR)-dependent gene expression (for review see [5]). AhR is a ligand-activated transcription factor which binds carcinogens, including aryl hydrocarbons (AH) such as B[*a*]P [6]. Once activated, AhR translocates from the cytosol to the nucleus, binds with the aryl hydrocarbon nuclear translocator (ARNT) and induces the transcription of *CYP1A1* gene, a cytochrome P450 family member. CYP1A1 metabolises B[*a*]P and other PAHs to reactive electrophilic metabolites that form DNA adducts and induce oxidative DNA damage, thereby causing mutations and cancer initiation [7].

Apoptotic effects of B[*a*]P

Besides its carcinogenic and mutagenic effects, B[*a*]P has been shown to activate apoptotic pathways in a number of studies [7–14].

Lei et al. [8] reported that upon 24 h treatment of murine Hepa1c1c7 hepatoma cells with 0.01–30 μM B[*a*]P, a decreased cell viability was observed in the 1 to 10 μM concentration range (Table 5.1). Based on this result of cell killing concentrations, apoptosis induction was assessed upon treatment of Hepa1c1c7 cells with 1 or 5 μM B[*a*]P for 8–36 h. An increased DNA fragmentation was noticed after 24 and 36 h of exposure, indicating that both B[*a*]P concentrations induced Hepa1c1c7 cells to undergo apoptosis. A dose-dependent activation of caspase-3 and caspase-1 activities by B[*a*]P was also investigated. 10 μM of B[*a*]P stimulated a substantial induction of caspase-3 which preceded B[*a*]P-induced apoptosis, while no caspase-1 activity was observed at any of the studied concentrations (0.01–10 μM). Kinetic studies showed that substantial caspase-3 activation was observed between 12 and 24 h of B[*a*]P (10 μM) treatment, while no caspase-1 activation was seen at any time point. Furthermore, 90 minutes treatment with 0.001–1 μM B[*a*]P caused a dose-dependent activation of JNK1 activity, which was first observed at 0.005 μM B[*a*]P and peaked at 0.1 μM B[*a*]P concentration. At this peak concentration, JNK1 activity rapidly increased at 15 minutes, peaked at 60 minutes, and was sustained through 150 minutes after exposure to B[*a*]P. This activation pattern indicated that B[*a*]P

stimulated rapid activation of JNK1 activity at a much lower concentration than required for activating caspase-3 or inducing apoptosis, implicating differential activation of JNK1, caspase-3 activities and apoptosis by B[*a*]P. The authors concluded that B[*a*]P induces apoptosis in Hepa1c1c7 cell line via a caspase-dependent pathway, which may be independent of JNK1 activation.

Ko et al. [9] demonstrated that treatment of Hepa1c1c7 cells with 1–5 μM B[*a*]P concentrations for 24, 36 and 48 h resulted in a nearly linear time-and dose dependent decrease in cell viability (Table 5.1). Moreover, 24 h exposure of Hepa1c1c7 cells to 1–5 μM B[*a*]P resulted in a concentration-dependent increase in ARNT and CYP1A1 expression. Incubation of Hepa1c1c7 cells with various concentrations of B[*a*]P (1–5 μM) for 36 h, or 5 μM B[*a*]P for 24/36 h markedly increased the ladder-pattern fragmentation of genomic DNA in a time and dose dependent manner, showing that the observed decrease in cell viability was due to apoptosis. A significant time-dependent activation of caspase-3 and caspase-9 was also observed upon treatment of Hepa1c1c7 cells with 5 μM B[*a*]P, with a peak in enzymatic activity at 30 h and 24 h, respectively. At the same B[*a*]P concentration, a time-dependent increase in the total and phosphorylated p53(Ser15) was clearly apparent after 12 h of treatment, and was sustained for 24 h. A dose-dependent experiment of cytochrome c expression indicated that cytosolic cytochrome c was predominantly increased by the addition of B[*a*]P (1–5 μM), while cytochrome c in the mitochondrial fraction was consistently decreased. Moreover, the translational expression level of pro-apoptotic Bid and Bax proteins was dose-dependently increased by the addition of B[*a*]P up to 2.5 μM, while the anti-apoptotic Bcl-2 and Bcl-XL proteins were decreased in expression according to the increase in B[*a*]P doses up to 2.5 μM. Taken together, the results of Ko et al. [9] suggest that B[*a*]P–induced apoptosis of Hepa1c1c7 cells occurs via intrinsic caspase cascade activation, mitochondrial dysfunction and p53 activation.

Solhaug et al. [7] examined the possible apoptotic effects of B[*a*]P (and other PAHs) in Hepa1c1c7 cells and primary rat lung cells at B[*a*]P concentrations of 0.03–30 μM (Table 5.1). 20 h treatment of Hepa1c1c7 cells with 0.03–30 μM B[*a*]P induced a concentration-dependent apoptosis as assessed by flow cytometry, Hoechst 33342 and propidium iodide (PI) staining, with a nearly linear increase in the number of apoptotic cells. 25 h incubation of Hepa1c1c7 cells with 0.03–10 μM B[*a*]P was shown to elicit a concentration-dependent expression of CYP1A1. Upon treatment of Hepa1c1c7 cells with 30 μM B[*a*]P, a time-dependent increase in apoptotic cells as assessed by flow cytometry, an activation of caspase-3, the cleavage of its intracellular substrate [poly(ADP-ribose)polymerase (PARP)] and DNA fragmentation were observed. Moreover, Hepa1c1c7 exposure to 30 μM and 20 μM B[*a*]P also resulted in the accumulation of the tumour suppressor protein p53 and its increased translocation into the nucleus, respectively. No changes in the level of Bax and Bcl-2 proteins were observed in response to 30 μM B[*a*]P, whereas the anti-apoptotic Bcl-xl protein was down-regulated, as determined by Western blot analysis. Furthermore, reduction in pro-caspase-8 and cleavage of Bid were also observed following exposure to 30 μM B[*a*]P.

However, the levels of anti-apoptotic phospho-Bad (Ser155 and Ser112) had a biphasic increase after 20 μM B[*a*]P treatment. In contrast, neither accumulation of p53 nor apoptosis could be seen in primary cultures of rat lung cells (Clara cells, type 2 cells and lung alveolar macrophages) after exposure to B[*a*]P, possibly due to a lack of CYP1A1 induction. The authors concluded that B[*a*]P induces both apoptotic and anti-apoptotic effects in Hepa1c1c7 hepatoma cells; the latter effect may result in an increased probability for the cells to survive with DNA damage, which could explain the carcinogenic effects of B[*a*]P.

B[*a*]P has also been shown to induce apoptosis in Daudi human B cells [10] (Table 5.1). Terminal deoxynucleotidal transferase-mediated dUTP-biotin nick end labeling (TUNEL) analysis revealed a 75% increase in the number of apoptotic cells after 18 h of B[*a*]P (10 μM) treatment over DMSO vehicle control cultures. By 36 h, the trend toward increasing numbers of apoptotic cells continued with B[*a*]P (10 μM) producing a 125% increase over control values. Distinct DNA fragmentation and cleavage of the PARP protein were also observed after 18 and 36 h of B[*a*]P (10 μM) treatment. Moreover, Daudi cells undergoing apoptosis at 36 h in response to B[*a*]P (10 μM) treatment expressed moderately reduced amounts (78%) of Bcl-2 compared to vehicle controls, whereas the Bax levels were increased by 130%. The apoptotic response to B[*a*]P in Daudi cells was sensitive to 4 h pretreatment with 0.3 μM α-naphtoflavone (ANF), a known inhibitor of cytochrome P450. After 18 h of B[*a*]P (10 μM) exposure, cultures pre-treated with ANF showed a 33% reduction in the number of apoptotic cells compared to cultures receiving no ANF. Although this study does not provide a dose-response evaluation of B[*a*]P-induced apoptosis, it points, however, to the importance of P450-mediated metabolic activation of B[*a*]P in triggering apoptosis in Daudi human B cells.

B[*a*]P coated on carbon black or hematite particles, used as a model of airborne particulate matter (PM), has also been shown to induce apoptosis in macrophages [11] and in Sprague-Dawley rat lungs [12] (Table 5.2). Exposure of murine cultured macrophages (RAW 264.7) to 2 μg/ml PM (B[*a*]P adsorbed on carbon black) triggered a time-dependent expression and release of TNF-α [11]. 24 and 48 h treatment of RAW 264.7 cells with 2 μg/ml PM resulted in DNA laddering at 24 h, with pronounced DNA fragmentation after 48h of exposure. No evidence of DNA fragmentation was observed after 24 h PM-treatment in the presence of a neutralizing antibody against TNF-α, suggesting that PM-induced apoptosis in RAW 264.7 cells may be mediated by TNF-α. Interestingly, neither untreated carbon black nor B[*a*]P alone induced apoptosis or caused the release of TNF-α in RAW 264.7 cells. Concurrent short-term intratracheal exposure of Sprague-Dawley rats to B[*a*]P (3 mg) and hematite (3 mg) significantly increased TNF-α and p53 expression, cytochrome c concentration and caspase-3, caspase-8 and caspase-9 activities by 2.3-, 1.53-, 1.49-, 1.15-, 1.21- and 1.27-fold, respectively [12]. However, no detectable activation of initiator caspases 8 and 9 was seen in response to either chemical alone.

Table 5.1. B[*a*]P-induced apoptosis

Endpoint	Cell type	Treatment	Concentration range of B[*a*]P	Endpoint – inducing concentration		Dose-response data	Ref.
				Highest negative concentration	Lowest positive concentration		
Apoptosis (Flow cytometry, Hoechst 33342 and PI staining) ↑	Mouse hepatoma Hepa1c1c7 cells	20 hours	0.03(?)–30 μM B[*a*]P	0.03 μM (?)	2.8 μM (?) B[*a*]P	Nearly linear dose dependent increase in the no. of apoptotic cells	[7]
Apoptosis (Flow cytometry) ↑		8–45 hours	30 μM B[*a*]P	–	30 μM B[*a*]P	Not available	
DNA fragmentation ↑		8, 20, 30 hours	30 μM B[*a*]P	–	30 μM B[*a*]P	Not available	
Capase-3 activity ↑		8, 20, 30 hours	30 μM B[*a*]P	–	30 μM B[*a*]P	Not available	
PARP cleavage ↑		8, 20, 30 hours	30 μM B[*a*]P	–	30 μM B[*a*]P	Not available	
p53 expression ↑		8, 20, 30 hours	30 μM B[*a*]P	–	30 μM B[*a*]P	Not available	
p53 translocation ↑		15 hours	20 μM B[*a*]P	–	20 μM B[*a*]P	Not available	
Bid expression ↓		8, 20, 30 hours	30 μM B[*a*]P	–	30 μM B[*a*]P	Not available	
Bax expression (no change)		8, 20, 30 hours	30 μM B[*a*]P	–	–	Not available	
Bcl-2 expression (no change)		*8, 20, 30 hours*	*30 μM* B[*a*]P	–	–	*Not available*	
Bcl- X_L expression ↓		*8, 20, 30 hours*	*30 μM* B[*a*]P	–	*30 μM* B[*a*]P	*Not available*	
Pro-caspase-8 expression ↓		8, 20, 30 hours	30 μM B[*a*]P	–	30 μM B[*a*]P	Not available	

Table 5.1. B[*a*]P-induced apoptosis — cont.

Endpoint	Cell type	Treatment	Concentration range of B[*a*]P	Endpoint – inducing concentration		Dose-response data	Ref.
				Highest negative concentration	Lowest positive concentration		
phospho-Bad expression ↑		*0.5, 2, 4, 8, 20 hours*	*20 μM* B[*a*]P	–	*20 μM* B[*a*]P	*Not available*	
CYP1A1 expression ↑		25 hours	0.03–10 μM B[*a*]P	–	0.03 μM B[*a*]P	Dose-dependent expression	
Cell viability ↓	Mouse hepatoma Hepa1c1c7 cells	24 hours	0.01–30 μM B[*a*]P	0.01 μM (?)B[*a*]P	1 μM B[*a*]P	Dose-dependent decrease in the 1–10 μM range	[8]
DNA fragmentation ↑		8, 12, 24, 36 hours	1, 5 μM B[*a*]P	–	1 μM B[*a*]P	Not available	
Caspase-3 activation ↑		24 hours	0.01–10 μM B[*a*]P	0.01 μM (?)B[*a*]P	0.67 μM (?) B[*a*]P	Dose-dependent activation	
Caspase-3 activation ↑		0–30 hours	10 μM B[*a*]P	–	10 μM B[*a*]P	Not available	
JNK1 activation ↑		90 min	0.001–1 μM B[*a*]P	0.001 μM B[*a*]P	0.005 μM B[*a*]P	Dose-dependent activation	
JNK1 activation ↑		15, 30, 60, 90, 120, 150 min	0.1 μM B[*a*]P	–	0.1 μM B[*a*]P	Not available	

Cell viability ↓	Mouse hepatoma Hepa1c1c7 cells	24, 36, 48 hours	1–5 μM B[*a*]P	–	1μM (2.5 μM[xx]) B[*a*]P	Nearly linear dose dependent decrease in cell viability for each time point	[9]
DNA fragmentation ↑		36 hours	1–5 μM B[*a*]P	–	1 μM B[*a*]P	Dose-dependent increase	
DNA fragmentation ↑		24, 36 hours	5 μM B[*a*]P	–	5 μM B[*a*]P	Not available	
Cytochrome -c expression ↑		24 hours	1–5 μM B[*a*]P	–	1 μM B[*a*]P	Dose-dependent increase in cytosolic cytochrome-c	
Capase-3 activity ↑		6, 12,18, 24, 30, 36 hours	5 μM B[*a*]P	–	5 μM B[*a*]P	Not available	
Capase-9 activity ↑		6, 12,18, 24, 30, 36 hours	5 μM B[*a*]P	–	5 μM B[*a*]P	Not available	
p53 expression ↑		6, 12, 18, 24 hours	5 μM B[*a*]P	–	5 μM B[*a*]P	Not available	
Bid expression ↑		24 hours	1–5 μM B[*a*]P	5 μM B[*a*]P	1 μM B[*a*]P	Dose-dependent increase	
Bax expression ↑		24 hours	1–5 μM B[*a*]P	5 μM B[*a*]P	2.5 μM B[*a*]P	Dose-dependent increase	
Bcl-2 expression ↓		*24 hours*	*1–5 μM* B[*a*]P	*5 μM* B[*a*]P	*1 μM* B[*a*]P	*Dose-dependent decrease*	

Table 5.1. B[*a*]P-induced apoptosis — cont.

Endpoint	Cell type	Treatment	Concentration range of B[*a*]P	Endpoint – inducing concentration		Dose-response data	Ref.
				Highest negative concentration	Lowest positive concentration		
Bcl- X_L expression ↓		*24 hours*	*1–5 μM* B[*a*]P	*5 μM* B[*a*]P	*1 μM* B[*a*]P	*Dose-dependent decrease*	
ARNT & CYP1A1 expression ↑		24 hours	1–5 μM B[*a*]P	–	1 μM B[*a*]P	Dose-dependent increase	
TUNEL ↑	Daudi human B cells	18, 36 hours	10 μM B[*a*]P	–	10 μM B[*a*]P	Not available	[10]
DNA fragmentation ↑		18, 36 hours	10 μM B[*a*]P	–	10 μM B[*a*]P	Not available	
PARP cleavage ↑		18, 36 hours	10 μM B[*a*]P	–	10 μM B[*a*]P	Not available	
Bcl-2 expression ↓		*36 hours*	*10 μM* B[*a*]P	–	*10 μM* B[*a*]P	Not available	
Bax expression ↑		36 hours	10 μM B[*a*]P	–	10 μM B[*a*]P	Not available	
TUNEL following ANF (0.3 μM) pre-treatment (4 h) ↓		18 hours	10 μM B[*a*]P	–	10 μM B[*a*]P	Not available	
Cell adhesion ↓	Human macrophages	1,4, 7 days	10 μM B[*a*]P	–	10 μM B[*a*]P	Not available	[13]

Cell adhesion ↓		7 days	0.1–10 μM B[*a*]P	0.1 μM B[*a*]P	1 μM B[*a*]P	The conc. of 1 and 10 μM were similarly active; 0.1 μM dose had no effect	
$\Delta\psi m^{\alpha}$ ↓			10 μM B[*a*]P	–	10 μM B[*a*]P	Not available	
Annexin-V/Hoechst 33342 ↑		7 days	1, 10 μM B[*a*]P	–	1 μM B[*a*]P	Slightly higher no. of apoptotic cells at 10 μM B[*a*]P dose	
		7 days	10 μM B[*a*]P	–	10 μM B[*a*]P	Not available	
Caspase-3 activity ↑		7 days	10 μM B[*a*]P	–	10 μM B[*a*]P	Not available	
			10 μM B[*a*]P	–	10 μM B[*a*]P	Not available	
p53 expression ↑		7 days	10 μM B[*a*]P	–	10 μM B[*a*]P	Not available	
c-FLIP$_L$ expression ↓		*7 days*	*10 μM* B[*a*]P	–	*10 μM* B[*a*]P	*Not available*	
Bcl-X$_L$ expression ↓		*7 days*	*10 μM* B[*a*]P	–	*10 μM* B[*a*]P	*Not available*	
Cell proliferation ↓	Human hematopoietic CD34+ progenitor cells	3, 6, 10 days	0.1–10 μM B[*a*]P	–	1 μM B[*a*]P	Not available	[14]

Table 5.1. B[*a*]P-induced apoptosis — cont.

Endpoint	Cell type	Treatment	Concentration range of B[*a*]P	Endpoint – inducing concentration		Dose-response data	Ref.
				Highest negative concentration	Lowest positive concentration		
Cell proliferation ↓		6 days	1, 10 μM B[*a*]P	0.1 μM B[*a*]P	1 μM B[*a*]PP	Similar inhibitory role of B[*a*]P at 1, 5 and 10 μM; no effect at 0.1 μM	
Δψm ¤ ↓		6 days	1, 10 μM B[*a*]P	–	1 μM B[*a*]P	Loss of Δψm after B[*a*]P treatment but no clear difference between 1 and 10 μM B[*a*]P –induced depolarization	
Annexin-V staining ↑		6 days	10 μM B[*a*]P		10 μM B[*a*]P	Higher no. of apoptotic cells at 10 μM B[*a*]P conc.	
Caspase-3 activity ↑		6 days	10 μM B[*a*]P	–	10 μM B[*a*]P	Not available	
Caspase-3/9 cleavage ↑		6 days		–	10 μM B[*a*]P	Not available	
CYP1A1/B1 expression ↑		8 hours	1, 10 μM B[*a*]P	–	1 μM B[*a*]P	Not available	

(?) – these concentrations were not clearly stated in the studies and were extrapolated from graphs.

¤ Mitochondrial membrane potential. ¤¤ Statistically significant lowest positive concentration.

Table 5.2. Particulate matter (PM)1,2-induced apoptosis

Endpoint	Cell /Animal type	Treatment	Concentration range of PM	Endpoint – inducing concentration		Dose-response data	Ref.
				Highest negative concentration	Lowest positive concentration		
TNF-α expression ↑	Murine peritoneal macrophages (RAW 264.7)	1, 2, 4, 8, 24 hours	2 μg/ml PM[1]	–	2 μg/ml PM[1]	Not available	[11]
DNA fragmentation ↑		24, 48 hours	2 μg/ml PM[1]	–	2 μg/ml PM[1]	Not available	
DNA fragmentation in the presence of Anti-TNF-α (no)		24 hours	2 μg/ml PM[1]	–	–	Not available	
TNF-α expression ↑	Sprague-Dawley rat bronchoalveolar lavage fluid (BALF)	48 hours	3 mg PM[2]	–	3 mg PM[2]	Not available	[12]
Cytochrome c conc. ↑	Sprague-Dawley rat lung tissue	48 hours	3 mg PM[2]	–	3 mg PM[2]	Not available	
Caspase-3 activity ↑	Sprague-Dawley rat lung tissue	48 hours	3 mg PM[2]	–	3 mg PM[2]	Not available	
Caspase-8 activity ↑	Sprague-Dawley rat lung tissue	48 hours	3 mg PM[2]	–	3 mg PM[2]	Not available	
Caspase-9 activity ↑	Sprague-Dawley rat lung tissue	48 hours	3 mg PM[2]	–	3 mg PM[2]	Not available	
p53 expression ↑	Sprague-Dawley rat lung tissue	48 hours	3 mg PM[2]	–	3 mg PM[2]	Not available	

[1] B[*a*]P adsorbed on carbon black (2 μg/ml); [2] (3 mg) B[*a*]P coated on (3 mg) hematite.

Table 5.3. Lack of apoptosis after *in vitro* exposure to B[*a*]P

Endpoint	Cell/Animal type	Treatment	Concentration range of B[*a*]P	Endpoint – inducing concentration		Dose-response data	Ref.
				Highest negative concentration	Lowest positive concentration		
Cell proliferation ↓	Human choriocarcinoma JEG-3 cells	5 days	1–50 μM B[*a*]P	–	1 μM B[*a*]P	Dose-dependent decrease in cell proliferation	[15]
Cell cycle distribution (G0/G1 ↓; G2/M ↑)		48 hours	10 μM B[*a*]P	–	10 μM B[*a*]P	Not available	
DNA fragmentation (no)		2, 3, 4, 5 days	10 μM B[*a*]P	–	–	Not available	
p21CIP1 expression ↑		48 hours	10 μM B[*a*]P	–	10 μM B[*a*]P	Not available	
CDK 1 expression ↓		48 hours	10 μM B[*a*]P	–	10 μM B[*a*]P	Not available	
Bax and cyclin B expression (no effect)[a]		48 hours	10 μM B[*a*]P	–	–	Not available	
Total p53 (no effect)[a]		48 hours	10 μM B[*a*]P	–	–	Not available	
Phosphorylated p53 ↑		48 hours	10 μM B[*a*]P	–	10 μM B[*a*]P	Not available	

[a] No effect of treatment on the endpoint.

Interestingly, B[*a*]P can also induce apoptosis in macrophages [13] and hematopoietic stem cells [14], in this way possibly modulating the immune response to tumour development (Table 5.3.).

Adherent cells in macrophagic cultures were exposed to 10 μM B[*a*]P for 1,4, and 7 days [13]. The number of adherent macrophages was reduced to approximately 20% after 7 days of B[*a*]P treatment; a 4-day exposure to B[a]P also resulted in a reduced cell adhesion (30%) whereas a shorter treatment (1 day) was inactive. 7 day exposure of macrophagic cultures to 0.1–10 μM B[*a*]P resulted in a dose-dependent effect on macrophage adhesion: the doses of 1 and 10 μM were similarly active whereas a lower dose of B[*a*]P (0.1 μM) had no effect. Macrophages cultured for 7 days in the presence of B[*a*]P (1 or 10 μM) showed significantly enhanced numbers of both Annexin V- and Hoechst 33342-positive cells when compared to untreated macrophage cultures. Moreover, exposure to 10 μM B[*a*]P for 7 days also led to a significant increase in caspase-3 activity, a reduced mitochondrial membrane potential, a markedly induced expression of the pro-apoptotic protein p53, the suppression of the anti-apoptotic protein c-FLIP$_L$ expression, and the reduction of Bcl-X$_L$ levels. Such deleterious effects were associated with B[*a*]P metabolite production, whose inhibition by the CYP1A1 inhibitor α-naphthoflavone fully abolished B[*a*]P toxicity.

B[*a*]P exposure was also shown to alter both proliferation and differentiation of human hematopoietic CD34+ progenitor cells [14]. Alteration of cell expansion in CD34+ cell cultures was first observed after 3-day B[*a*]P (10 μM)-treatment and it was more pronounced after a 6-day or 10-day treatment. After 6-day exposure to 0.1–10 μM B[*a*]P, the inhibitory role of B[*a*]P toward CD34+ cell expansion was similarly observed for the doses of 1, 5 and 10 μM, whereas a lower B[*a*]P dose (0.1 μM) had no effect. Moreover, cultured CD34+ cells exposed to 1 or 10 μM B[*a*]P for 6 days were found: a) to undergo apoptosis as assessed by Annexin V staining; b) to display increased caspase-3 activity and enhanced expression of the cleaved form of caspase-3; c) to exhibit a reduced mitochondrial membrane potential; d) to display an increased expression of the cleaved forms of caspase-9. Further treatment of CD34+ progenitor cells with 1 or 10 μM B[*a*]P for 8 h was found to induce the up-regulation of CYP1A1 and CYP1B1 expression, suggesting that B[*a*]P can be metabolized and form adducts in these cells.

A unique pattern of G_2/M cell cycle arrest, without any evidence of apoptosis, was reported by Drukteinis et al. [15] following 48 h treatment of human choriocarcinoma JEG-3 cells with 10 μM B[*a*]P (Table 5.3). They presented evidence that B[*a*]P related changes involved phosphorylation of p53 at serine 15 and induction of p21^{CIP1}, a pattern consistent with oxidative stress and DNA damage response. The authors explained that their findings might be concentration or cell type specific, or might also reflect possible anti- apoptotic effects of B[*a*]P.

Figure 5.2. (adapted from [16,17]) shows the apoptotic and anti-apoptotic pathways triggered by B[*a*]P.

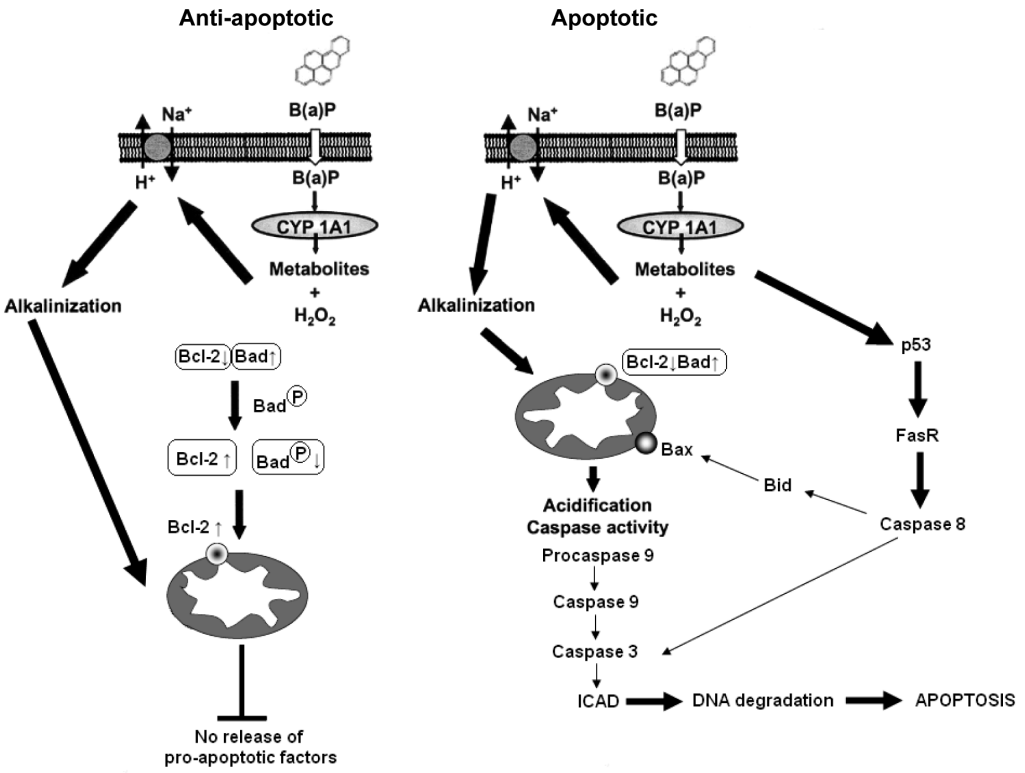

Fig. 5.2. Apoptotic and anti-apoptotic pathways triggered by B[a]P (adapted from [16,17]).

In summary, in most of the cited studies B[*a*]P was inducing an apoptotic effect which was preceded by a metabolic activation step. This and the apoptotic pathways involved, strongly suggest that DNA damage could be the crucial event for B[*a*]P induced apoptosis. When data was available, the dose-dependent increase in apoptosis showed a nearly linear trend. The lowest positive concentration for apoptosis induction was around 1 μM. However, as described by Solhaug et al. [7], B[*a*]P can trigger both apoptotic and an anti-apoptotic pathways (Fig. 5.2) in the same range of concentrations. Therefore, as long as more *in vivo* studies and studies showing frequencies of cells with known genetic damage in apoptotic and non-apoptotic fractions are not available, no conclusion on the implication of apoptosis on dose-response curves for B[*a*]P-induced mutations can be drawn. As far as thresholds are concerned, B[*a*]P is a DNA interacting mutagen. Since both apoptotic and anti-apoptotic effects are described at low concentrations, it is not proven that the induced mutations are eliminated. Therefore, the sole argument to consider a threshold might be the fact that B[*a*]P needs to be metabolised before inducing mutations. The latter, of course, is dependent on genetic polymorphisms of the different enzymes involved.

5.2. Do dioxins induce apoptosis and if yes does it support a threshold dose-effect? 2,3,7,8,-Tetrachlorodibenzo-*p*-dioxin (TCDD) as an example

Introduction

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is one of the most harmful dioxins. Recently, the International Agency for Research on Cancer evaluated TCDD as carcinogenic to humans (Group 1) [18]. Most of the biochemical and toxic effects of TCDD require an initial interaction with the aryl hydrocarbon receptor (AhR). One consequence is the subsequent transcription of *CYP1A1* gene, TCDD being the most potent known cytochrome *P4501A1* inducer [19]. TCDD was also shown to alter the binding capacity of the epidermal growth factor receptor (EGFR) for its ligand, by a mechanism that requires AhR [20]. *In vivo* and *in vitro* studies have demonstrated that TCDD also alters the estrogen receptor (ER) [20–24] and estrogens can, in turn, alter EGFR receptor binding and cellular distribution [25–27]. TCDD induction of UDP-glucuronosyltransferase (UDPGT) by a mechanism requiring AhR [28], has also been shown in several studies [29–31].

Apoptotic effects of TCDD

As TCDD does not appear to bind or damage DNA directly, several studies have addressed the effect of TCDD on cell proliferation and apoptosis (for review see [32]). Interestingly, TCDD has been reported to induce either apoptotic or anti-apoptotic effects (see Fig. 5.3) in a number of studies [32–40]. Thus, Pryputniewicz et al. [33] showed a differential induction of apoptosis in activated [popliteal lymph node (LN)] and resting (axillary LN) T cells from TCDD treated C57BL/6 mice (50 μg/kg body weight) (Table 5.4). *In vivo* T cell activation was carried out either at the same time or two days after TCDD treatment, by injection of both mice rear footpads with 25 μl of anti-CD3 mouse antibodies (mAbs). 3 days and 1 week following TCDD treatment, the axillary and popliteal LNs were harvested separately and cultured for 48 h in the presence of anti-CD3 mAbs.

For both exposure time points the resting T-cells from the axillary LNs of TCDD-treated mice showed no significant differences from the vehicle-treated controls in response to anti-CD3 mAbs. In contrast, activated T cells from the popliteal LNs of TCDD-treated mice exhibited a marked decrease in response to anti-CD3 restimulation, with a more pronounced effect observed 1 week after TCDD administration. Two weeks after TCDD treatment, the responsiveness of both activated and resting T cells to anti-CD3 stimulation was restored to control levels, suggesting that the effect of TCDD on activated T cells is both temporary and reversible. Additionally, TUNEL analysis was performed to detect apoptosis in LN cells from TCDD-treated mice following 24 h *in vitro* culture with tissue culture medium. Axillary LN cells screened 3, 5 and 7 days after

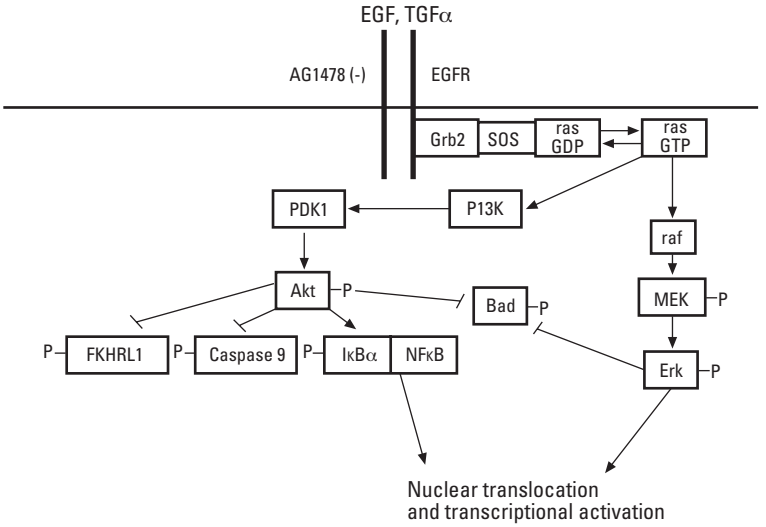

Fig. 5.3. Anti-apoptotic pathways triggered by TCDD [38].

TCDD treatment were more resistant to apoptosis than vehicle controls, while on day 14 there was no difference in apoptosis between the treated and non-treated cells. In contrast, popliteal LN cells from TCDD-treated mice showed increased levels of apoptosis compared to the controls at all time points tested.

TUNEL analysis was also carried out to detect apoptosis following 24 h *in vitro* culture of LN cells in the presence of anti-CD3 mAbs. TCDD treatment was not shown to alter the level of apoptosis in resting T cells, while the activated T cells from TCDD-treated animals exhibited ~ 32% more apoptotic cells than the corresponding controls. Further characterization of LN apoptotic cells by flow cytometry showed that after 24 h culture with medium alone, axillary LN cells from TCDD-treated animals showed a decreased percentage of CD3+ apoptotic cells when compared to cells from vehicle-treated controls (55.1 vs. 66.4%), while popliteal LN cells from TCDD treated animals showed an increased proportion of CD3+ apoptotic cells when compared to vehicle controls (22 vs. 2.4%). The authors concluded that TCDD exerts differential effects on activating and resting T cells even within the same animal, by inhibiting the proliferative responsiveness of activated but not resting T cells.

Park et al. [34] showed that upon treatment of the EL-4 murine thymoma cell line with 10 nM TCDD for 24–96 h, a significant decrease in cell viability was observed after 24 h of exposure (Table 5.5). Thereafter, the EL-4 cell viability decreased steadily, but not rapidly. EL-4 cells transfected with vectors leading to expression of insulin-like growth factor-binding protein-6 (IGFBP-6) sense or anti-sense mRNA were further incubated in the presence of TCDD (10 nM) for increasing duration of time and assayed for cytotoxicity by the MTT assay. Clones expressing IGFBP-6 sense mRNA displayed increased sensitivity to TCDD-mediated cytotoxicity, whereas clones expressing IGFBP-6

Table 5.4. TCDD-induced apoptosis after *in vivo* treatment

Endpoint	Cell/Animal type	Treatment	Tested concentration of TCDD	Response	Ref.
	C57BL/6 mice:	3 days, 1 week	50 μg/kg body weight TCDD		[33]
Cell proliferation (TCDD ≈ Control)	Axillary lymph node cells (resting T cells)	48 hours (+anti-CD3 antibodies)		–	
Cell proliferation ↓	Popliteal lymph node cells (activated T cells)	48 hours (+anti-CD3 antibodies)		+	
	C57BL/6 mice:	2 weeks	50 μg/kg body weight TCDD		
Cell proliferation (TCDD ≈ Control)	Axillary and popliteal lymph node cells	48 hours (+anti-CD3 antibodies)		–	
	C57BL/6 mice:	3, 5, 7, 14 days	50 μg/kg body weight TCDD		
TUNEL (3, 5, 7 days: ↓) (14 days: TCDD ≈ Control)	Axillary lymph node cells (resting T cells)	24 hours (+culture medium)		+	
TUNEL ↑	Popliteal lymph node cells (activated T cells)	24 hours (+culture medium)		+	
	C57BL/6 mice:	7 days	50 μg/kg body weight TCDD		
TUNEL (TCDD ≈ Control)	Axillary lymph node cells (resting T cells)	24 hours (+anti-CD3 antibodies)		–	
TUNEL ↑	Popliteal lymph node cells (activated T cells)	24 hours (+anti-CD3 antibodies)		+	
Flow cytometry (% CD3+) ↓	Axillary lymph node cells (resting T cells)	24 hours (+culture medium)		+	
Flow cytometry (% CD3) ↑	Popliteal lymph node cells (activated T cells)	24 hours (+culture medium)		+	

Table 5.5. TCDD-induced apoptosis after *in vitro* treatment

Endpoint	Cell/Animal type	Treatment	Tested concentration of TCDD	Response	Ref.
Cell viability ↓	Murine thymoma cells (EL-4)	24–96 hours	10 nM TCDD	+	[34]
Cell viability ↓	EL-4 transfected with IGFBP-6 sense*	24–96 hours	10 nM TCDD	+	
Cell viability (not affected)	EL-4 transfected with IGFBP-6 anti-sense*	24–96 hours	10 nM TCDD	–	
DNA fragmentation ↑	EL-4 transfected with IGFBP-6 sense*	24 hours	10 nM TCDD	+	
DNA fragmentation (no)	EL-4 transfected with IGFBP-6 anti-sense*	24 hours	10 nM TCDD	–	
Caspase-3 activation ↑	EL-4 transfected with IGFBP-6 sense*	6, 10, 24 hours	10 nM TCDD	+	
Caspase-3 activation (no)	EL-4 transfected with IGFBP-6 anti-sense*	6, 10, 24 hours	10 nM TCDD	–	
PARP cleavage ↑	EL-4 transfected with IGFBP-6 sense*	6, 10, 24 hours	10 nM TCDD	+	
PARP cleavage (no)	EL-4 transfected with IGFBP-6 anti-sense*	6, 10, 24 hours	10 nM TCDD	–	
AhR expression (no)	EL-4 cells	3, 24 hours	10 nM TCDD	–	
ARNT expression (no up-regulation)	EL-4 cells	3, 24 hours	10 nM TCDD	–	

* IGFBP-6 sense/anti-sense – insulin-like growth factor-binding protein-6 sense/anti-sense.

anti-sense mRNA displayed reduced sensitivity. Moreover, 24 h incubation of EL-4 clones expressing IGFBP-6 sense with TCDD (10 nM) led to an increased DNA fragmentation, while no significant increase in DNA fragmentation was observed in EL-4 cells expressing IGFBP-6 anti-sense.

Consistently, caspase-3 activation and PARP cleavage were also observed in TCDD-treated IGFBP-6 sense clones, while neither activation of caspase-3 nor PARP cleavage were observed in TCDD-treated IGFBP-6 anti-sense clones. Interestingly, the effects of TCDD were exerted without AhR. Taken together, the results of Park et al. [34] have shown that TCDD (10 nM) induces apoptosis in mouse thymoma EL-4 cells, in part by activating caspase 3 in an AhR-independent pathway. Decreased expression of insulin-like growth factor-binding protein-6 (IGFBP-6) in EL-4 cells prevents TCDD-induced caspase 3 activation, resulting in the reduction of TCDD-mediated cytotoxicity. The role of apoptosis in TCDD-induced thymic involution following perinatal exposure to TCDD was recently addressed by Camacho et al. [35] (Table 5.6). TCDD was administered as a single dose of 10 μg/kg body weight into pregnant C57BL/6 mice on gestational day (GD) 14 and thymic cellularity was determined on GDs 15, 16, 17, 18 and on post-natal day (PD) 1 in fetal and neonatal mice. A remarkable reduction in thymic cellularity was observed 3-7 days post-TCDD exposure. 24 h *in vitro* culture of thymocytes from mice exposed perinatally to TCDD showed increased apoptosis (TUNEL assay) when compared to the controls. Maximum change in apoptosis was detected on GD 17 thymocytes 3 days post TCDD treatment. TUNEL analysis showed that TCDD induced apoptosis in all four subpopulations of T cells (single positive CD8, double positive, double negative, single positive CD4), with the double-positive T cells undergoing the highest level. Moreover, a 5-fold increase in caspase-3 enzymatic activity was observed in freshly isolated TCDD-exposed GD 17 thymocytes when compared to those exposed to vehicle only. Perinatal exposure to TCDD was also shown to cause apoptosis-associated phenotypic changes in PD 1 thymocytes, characterized by an increased expression of CD3, αβTCR, IL-2R, and CD44, and a decrease in CD4, CD8, and J11d markers. Finally, thymocytes from mice exposed perinatally to TCDD and sacrificed 3 or seven days post treatment showed higher levels of Fas, TRAIL, and DR5 mRNA, while the levels of Bcl-2, Bcl-X_L, and Bax were either unaltered or changed moderately. The authors concluded that the TCDD-induced thymic atrophy following perinatal mice exposure may result from increased apoptosis mediated by the death receptor pathway involving Fas, TRAIL, and DR5.

However, several *in vivo* and *in vitro* mammalian studies have demonstrated that TCDD treatment results in inhibition of apoptosis ([36–40], for review see [32]). Thus, TCDD suppressed UV- and 2-acetylaminofluorene (2-AAF)-stimulated apoptosis but not transforming growth factor beta 1 (TGF-β1)-stimulated apoptosis in Wistar rat hepatocyte primary cultures [36] (Table 5.7). A significant reduction in the incidence of UV (90 J/m^2)-stimulated apoptosis, as assessed by Hoechst H333258 staining, was observed after 6 and 12 h incubation of primary rat hepatocytes with 1nM TCDD. The addition of the same TCDD concentration to rat hepatocytes 12 h after 2-AAF treatment (20 μM) abrogated the wave of apoptosis observable without TCDD almost completely.

Table 5.6. TCDD-induced apoptosis after *in vivo* exposure

Endpoint	Cell/Animal type	Treatment	Tested concentration of TCDD	Response	Ref.
	pregnant C57BL/6 mice: [gestational day (GD) 14]		10 μg/kg body weight TCDD		[35]
Thymic cellularity ↓	Thymi GDs 15, 16, 17, 18, PD 1[£]	3, 4, 7 days		+	
TUNEL ↑	GD 15, 16, 17, 18, PD1[£] thymocytes	1–7 days (+24 hours cultivation)		+	
TUNEL ↑	GD 17 thymocytes (spCD8, DP, DN, spCD4)[¤]	3 days (+24 hours cultivation)		+	
Caspase-3 activation ↑	GD 17 thymocytes	3 days (without cultivation step)		+	
CD3, αβTCR, IL-2R, CD44 expression ↑	PD 1 thymocytes	7 days		+	
CD4, CD8, J11d expression ↓	PD 1 thymocytes	7 days		+	
Fas, TRAIL, DR5 expression ↑	GD 17, PD 1 thymocytes	3, 7 days		+	
Bax expression (slight) ↑	GD 17 thymocytes	3 days		+	
Bax expression (unaltered)	PD 1 thymocytes	7 days		–	
Bcl-X_L expression (unaltered)	*GD 17, PD 1 thymocytes*	*3, 7 days*		–	
Bcl-2 expression (slight) ↓	*PD 1 thymocytes*	*7 days*		+	

PD1 – post-natal day 1; [¤]spCD8 – single-positive CD8 (CD4-CD8+); DP – double positive (CD4+CD8+); DN – double negative (CD4-CD8-); spCD4 – single-positive CD4 (CD4+CD8-).

Table 5.7. TCDD-induced inhibition of apoptosis after *in vitro* exposure of rat hepatocytes to UV light/2-acetylaminofluorene (2-AAF)/TGF-β1

Endpoint	Cell/Animal type	Treatment	Tested concentration of TCDD	Response	Ref.
Hoechst H33258 staining ↓	Primary Wistar rat hepatocytes	6, 12, 24 hours	90 J/m^2 UV/1 nM TCDD	+	[36]
Hoechst H33258 staining ↓	Primary Wistar rat hepatocytes	6, 12, 48, 72, 114, 192 hours	20 μM 2-AAF*/1 nM TCDD	+	
Hoechst H33258 staining no)	Primary Wistar rat hepatocytes	6, 12, 48, 96, 144, 192 hours	200 pg/ml TGF-β1¤/1 pM TCDD	-	
Hoechst H33258 staining ↓	Primary Wistar rat hepatocytes	96, 144 hours	200 pg/ml TGF-β1¤/1 nM TCDD	+	
DNA fragmentation ↓	Primary Wistar rat hepatocytes	30 min after irradiation	60, 90, 120 J/m^{2}UV/1 nM TCDD	+	
p53 immunoprecipitation ↓	Primary Wistar rat hepatocytes	30 min after irradiation	90, 120, 150 J/m^{2}UV/1 nM TCDD	+	

* 2-AAF – 2-acetylaminofluorene; ¤ TGF-β1 – transforming growth factor beta 1.

Table 5.8. TCDD-induced inhibition of apoptosis after *in vitro* exposure

Endpoint	Cell/Animal type	Treatment	Concentration range of TCDD	Endpoint – inducing concentration		Dose-response data	Ref.
				Highest negative concentration	Lowest positive concentration		
Cell number (no effect)	Human mammary epithelial cell line (MCF-10A)	2, 4, 6 days (SFIHE*)	30 nM	–	–	Not available	[37]
Cell number ↓		2, 4, 6 days (SFH*)	30 nM	–	30 nM TCDD	Not available	
Cell number ↓		2, 4 days (SFIH*)	30 nM	–	30 nM TCDD	Not available	
Cell number ↑		6 days (SFIH*)	30 nM	–	30 nM TCDD	Not available	
Cell number ↑		4, 6 days (SFHE*)	30 nM	–	30 nM TCDD	Not available	
Cell recovery (no effect)		6 days (SFIHE, SFH*)	0.3, 3, 30, 300 nM	–	–	No effect of TCDD on cell recovery at any conc.	
Cell recovery ↑		6 days (SFIH, SFHE*)	0.3, 3, 30, 300 nM	0.3 nM	3 nM TCDD	Dose-dependent increase in cell recovery	
Annexin-V staining ↓		4 days (SFH, SFIH*)	30 nM	–	30 nM TCDD	Not available	
Annexin-V staining ↓		3 days (SFH, SFIH*)	0.3, 3, 30, 300 nM	0.3 nM	3 nM TCDD	Dose-dependent inhibition of apoptosis in SFIH	
PARP cleavage ↓		18 hours (SFIH*)	30 nM	–	30 nM TCDD	Not available	
Akt phosphorylation ↑		6, 8 hours (SFIH*)	30 nM	–	30 nM TCDD	Not available	

* SFIHE – serum-free medium supplemented with insulin, hydrocortisone and EGF; SFH – serum-free medium supplemented with hydrocortisone; SFIH – serum-free medium supplemented with insulin and hydrocortisone;
* SFHE – serum-free medium supplemented with hydrocortisone and EGF.

Table 5.9. TCDD-induced inhibition of apoptosis after *in vitro* exposure

Endpoint	Cell/Animal type	Treatment	Concentration range of TCDD	Endpoint – inducing concentration		Dose-response data	Ref.
				Highest negative concentration	Lowest positive concentration		
Cell number ↑	Human mammary epithelial cell line (MCF-10A)	3 days (SFH*)	10 nM TCDD	–	10 nM TCDD	Not available	[38]
Cell number (Control ≈ Treated))		3 days (SFH*)	10 nM TCDD/1 μM AG1478¤	–	–	Not available	
Cell number (slight) ↑		3 days (SFH*)	10 nM TCDD/10 μM AG825£	–	10 nM TCDD/10 μM AG825	Not available	
Annexin-V staining ↓		3 days (SFH*)	10 nM TCDD	–	10 nM TCDD	Not available	
Annexin-V staining (slight) ↓		3 days (SFH*)	10 nM TCDD/1 μM AG1478¤	–	10 nM TCDD/1 μM AG1478	Not available	
Annexin-V staining ↓		3 days (SFH*)	10 nM TCDD/10 μM AG825£	–	10 nM TCDD/10 μM AG825	Not available	
Akt phosphorylation ↑		6, 8 hours (SFH*)	1, 3, 10 nM TCDD	–	1 nM TCDD	Transient increase in Akt phosphorylation with the greatest effect occurring at 6 h treatment	
Erk1,2 phosphorylation ↑		6, 8 hours (SFH*)	1, 3, 10 nM TCDD	–	1 nM TCDD	Dose-dependent increase in phosphorylation at 6 h treatment	
TGF-α expression ↑		6 hours (SFH*)	10 nM TCDD	–	10 nM TCDD		

* OSFH – serum-free medium supplemented with hydrocortisone.
¤ AG1478 – a specific inhibitor of EGRF; £ AG825 – erbB2 inhibitor.

Table 5.10. TCDD-induced inhibition of apoptosis after *in vitro* exposure

Endpoint	Cell/Animal type	Treatment	Concentration range of TCDD	Endpoint – inducing concentration		Dose-response data	Ref.
				Highest negative concentration	Lowest positive concentration		
AhR signaling (luciferase activity) ↑	Human mammary epithelial cell line (MCF-10A) transfected with the DRE-driven luciferase vector pGud-Luc6.1	24 hours (SFIHE*)	10 nM TCDD	–	10 nM TCDD	Not available	[39]
AhR signaling (luciferase activity) ↓		24 hours (SFIHE*)	10 nM TCDD/(0.1–1000 nM) MNF ¤	10 nM TCDD/ 1 nM MNF ¤	10 nM TCDD/10 nM MNF ¤	Co-treatment with TCDD and conc. of MNF as low as 10 nM inhibited luciferase activity	
Annexin-V staining ↑		3 days (SFIH*)	10 nM TCDD	–	10 nM TCDD	Not available	
Annexin-V staining ↑		3 days (SFIH*)	10 nM TCDD/(0.1–1000 nM) MNF ¤	10 nM TCDD/ 10 nM MNF ¤	10 nM TCDD/100 nM MNF ¤	Co-treatment with TCDD and conc. of MNF as low as 100 nM reversed the TCDD-mediated inhibition of apoptosis	
Akt and Erk1,2 phosphorylation ↓		6 hours (SFH*)	10 nM TCDD	–	10 nM TCDD	Not available	
Akt phosphorylation ↓		6 hours (SFH*)	10 nM TCDD/(0.1–1000 nM) MNF ¤	10 nM TCDD/ 0.1 nM MNF ¤	10 nM TCDD/1 nM MNF ¤	Co-treatment with TCDD and conc. of MNF as low as 1 nM decreased TCDD-mediated phosphorylation of Akt, while inhibition did not occur until 100 nM MNF	

Erk1,2 phosphorylation ↓	6 hours (SFH*)	10 nM TCDD/(0.1–1000 nM) MNF ¤	10 nM TCDD/1 nM MNF ¤	10 nM TCDD/10 nM MNF ¤	TCDD-dependent Erk1,2 phosphorylation was inhibited at concentrations of 10 nM MNF and above
TGF-α expression ↑	6 hours (SFH*)	10 nM TCDD	–	10 nM TCDD	Not available
TGF-α expression ↓	6 hours (SFH*)	10 nM TCDD/(0.1–100 nM) MNF ¤	10 nM TCDD/0.1 nM MNF ¤	10 nM TCDD/1 nM MNF ¤	Inhibition of TCDD-dependent expression of TGF-α occurred at concentrations as low as 1 nM MNF

Abbreviations as in Table 5.8. ¤ MNF – 3′Methoxy-4′nitroflavone.

Table 5.11. Lack of apoptosis after *in vitro* exposure toTCDD

Endpoint	Cell/Animal type	Treatment	Concentration range of TCDD§	Endpoint – inducing concentration		Dose-response data	Ref.
				Highest negative concentration	Lowest positive concentration		
Cell proliferation (no effect)	Human choriocarcinoma JEG-3 cells	5 days	1–100 nM TCDD	–	–	No alteration in cell proliferation after TCDD exposure	[15]
Cell cycle distribution (no effect)		48 hours	10 nM	–	–	Not available	

In contrast, no suppressing effect of TCDD (1 pM) on TGF-β1 (200 pg/ml)-induced apoptosis was observed after 6–192 h incubation time. However, at time points ≥ 96 h, when the apoptotic effect of TGF-β1 was no longer observable, 1 nM TCDD suppressed the rate of background apoptosis also obtained in untreated cultures. In addition to the morphological detection of apoptotic nuclei by Hoechst H333258 staining, internucleosomal fragmentation of DNA was also analysed after UV treatment (60, 90, 120 J/m^2). 24 h after irradiation with 90 or 120 J/m^2, a marked increase in DNA fragmentation was observed. However, when 1 nM TCDD was added 30 min after irradiation, the increase in DNA fragmentation was blocked almost completely. In agreement with the DNA fragmentation experiment, the addition of TCDD (1 nM) 30 minutes after irradiation abrogated the UV (90, 120, 150 J/m^2)-dependent increase in p53 almost completely.

The treatment of the human mammary epithelial cell line MCF-10A with 30 nM TCDD inhibited EGF-withdrawal induced apoptosis and increased the phosphorylation status of the anti-apoptotic serine/threonine kinase Akt [37] (Table 5.8). Moreover, dose-response analyses showed that TCDD was able to suppress EGF-withdrawal induced apoptosis at concentrations as low as 3 nM. Consistently, dose-response analyses of cell recovery following TCDD treatment yielded similar observations. A later study of the same authors [38] demonstrated that inhibition of apoptosis in MCF-10A cells occurs through an EGFR-dependent pathway (Table 5.9). 3 day treatment of MCF-10A cells with 10 nM TCDD in the presence of AG1478 (1 μM), a specific inhibitor of EGRF, completely abolished TCDD-induced cell growth. In contrast, the structurally similar erbB2 inhibitor AG825 had little effect on cell growth. Analysis of apoptosis by Annexin-V staining showed a 50% reduction in apoptotic cells upon 3 day treatment of MCF-10A cells with 10 nM TCDD. As expected, cotreatment with the specific EGFR inhibitor AG1478 attenuated the TCDD response, whereas AG825 plus TCDD was not different from TCDD alone. Treatment of MCF-10A cells with concentrations up to 10 nM TCDD transiently increased Akt phosphorylation, with the greatest effect occurring at 6 h. Likewise, TCDD also stimulated Erk1,2 phosphorylation at similar time points and concentrations. Moreover, TCDD (10 nM) was also shown to increase TGF-α (a ligand for EGFR) expression in MCF-10A cells after 6h treatment. Taken together, these results [38] suggest that TCDD can positively regulate the EGFR pathway by increasing the production of TGF-α, thus activating PI3K/Akt and ras/raf/MAPK, two distinct branches of the EGFR pathway (see Fig. 5.3). More recently, Davis et al. [39] demonstrated that the TCDD-mediated apoptosis in MCF-10 cells transfected with the dioxin response element (DRE)-driven luciferase vector pGud-Luc6 appears to be dependent on AhR signaling (Table 5.10). 3'methoxy-4'nitroflavone (MNF), a known AhR antagonist, was shown to suppress TCDD-dependent DRE-driven luciferase activity at concentrations as low as 10 nM. Moreover, MNF also attenuated TCDD's ability to inhibit apoptosis and to activate Akt and Erk1,2. Finally, the TCDD-dependent increase in TGFα mRNA was also suppressed by MNF. MNF's effects on TCDD action in the MCF-10A cell line occurred at concentrations ranging from 1 nM for Akt phosphorylation and TGFα expression to 100 nM

for inhibition of apoptosis. Attenuation of TCDD-dependent luciferase activity occurred at concentrations as low as 10 nM, which suggests that TCDD inhibits apoptosis in human mammary epithelial cells by multiple mechanisms. Recently, Park et al. [40] demonstrated that TCDD treatment of RAW 264.7 murine macrophages induced a transient upshift in the extracellular signal-regulated kinase (ERK) activit, followed by a decline, but a concomitant dramatic activation of p38. Their results also suggested that TCDD activates the MAPK pathway via an AhR-independent mechanism in RAW 264.7 murine macrophages.

Finally, Drukteinis et al. [15] found no evidence of apoptosis following 48 h treatment of human choriocarcinoma JEG-3 cells with TCDD (10 nM) (Table 5.11). Moreover, proliferation of JEG-3 cells was not altered after 5 days exposure to concentrations of TCDD as high as 100 nM.

Although in most of the cited studies dose-response data was not available, TCDD was shown to induce an apoptotic effect at concentrations as low as 10 nM and an anti-apoptotic effect at concentrations as low as 1 nM. These results suggest that the effect of TCDD on cell proliferation/apoptosis may be concentration-, cell- or tissue- type specific and may depend on the activated pathway. Therefore, an analysis of dose-response data should use, whenever possible, information on the quantitative relationships among ligand (i.e. TCDD) concentration, receptor occupancy and biological response, taking into account cell- or tissue-specific factors. At present, therefore, there is no clear general answer about a threshold effect in the apoptotic elimination of cells damaged by TCDD.

5.3. Conclusion and perspectives

The influence of apoptosis on dose-effect at low concentrations and its possible contribution to thresholds should be assessed *in vivo* to take into account the modulating factors. However, this is technically difficult and, therefore, in a first approach, *in vitro* studies employing highly sensitive methodologies should be advised. Once the mechanism behind the induction of a possible threshold dose-response is identified, *in vivo* studies on the appropriate tissues can be started.

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6. Dose-response and potential thresholds in tumour development

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The development of a tumour is a multi-step process including genetic and epi-genetic events. In experimental models, the process of tumourigenesis is divided into different phases which are referred to as tumour initiation and tumour promotion. Accordingly, carcinogens are classified by their mode of action in either genotoxic or non-genotoxic compounds. Depending on their application schedule, genotoxic compounds serve as tumour initiators and/or promoters, whereas non-genotoxic compounds solely act as tumour promoters.

There is a highly controversial debate over the shape of dose-response curves and the existence of thresholds for genotoxic and non-genotoxic carcinogens. Defining the shape of a dose response curve and thresholds has high impact on risk assessment concerning carcinogenic compounds and human health, especially in the low-dose zone. Assuming dose-linearity or deviation from dose-linearity for tumour induction is of great importance in the context of extrapolation from experimentally available high dose data to the low dose range of in reality occurring exposure of humans.

Considering their (presumed) fundamentally different mode of actions, dose-linearity is often proposed for the carcinogenic effect of a DNA damaging genotoxic tumour initiator in contrast to dose-nonlinearity for this effect of non-genotoxic tumour promoters. Theoretically, dose-linearity of the effect of a genotoxic carcinogen implies per definition the lack of a threshold. However, this assumption is seemingly an over-simplification disregarding the possibility of practically instantaneous detoxification of the ultimately DNA damaging species as well as regulatory mechanisms downstream of the primary DNA-adduct formation. Therefore, we and others recently proposed to challenge the classical dogma of dose-linearity for genotoxic carcinogens [1,2]. The existence of a threshold for tumour promoting agents is also discussed highly controversially [reviewed in 3, 4].

In this chapter, we review data on dose-response relationships in animal models of carcinogenesis. For the sake of clarity, we will focus on two classes of model compounds, i.e. genotoxic PAHs and non-genotoxic “dioxins”.

Genotoxic PAHs

A linear dose-response relationship without the existence of a threshold is often assumed for the tumour initiating activities of DNA damaging genotoxic compounds, such as PAHs. This is based on the amount of primary DNA-lesions (below saturation of the respective carcinogen-activating enzymes) increasing with increasing concentrations of the compound. However, this assumption disregards regulatory mechanisms downstream of the primary DNA-adduct formation, such as DNA repair and cell cycle checkpoints. Such regulatory mechanisms are extremely relevant, especially at lower doses of PAHs. Hence, mutations are manifested when intracellular regulatory mechanisms are saturated resulting in linear dose-response curves above a certain threshold [5].

Another aspect which determines the shape of the dose response curve is that the correlation between DNA adducts, mutagenesis and carcinogenesis is not necessarily stringent. When comparing DNA adduct levels and tumour development in mice that have been treated orally or by intraperitoneal injection with benzo[*a*]pyrene, similar amounts have been observed in tumour target and non-target tissues [6]. For instance, injection of a single dose of 375 μg of B[*a*]P induces 1023 fmol adducts/mg DNA in liver, 840 fmol adducts/mg DNA in forestomach and 1851 fmol adducts/mg DNA in lung. However, tumour development is only detected in liver [6]. When given orally, a total dose of 6510 μg of B*a*P induces 905 fmol adducts/mg DNA in liver and 446 fmol adducts/mg DNA in forestomach, but tumors only occur in forestomach. In accordance, high levels of benzo[*a*]pyrene DNA adducts were detected in organs without any sign of tumour development (e.g. kidneys) [7]. In an oral feeding study with B*a*P (125 mg/kg/day for 5 consecutive days) using the Muta™ Mouse, similar amounts of mutations were found in the target organs forestomach and spleen and the non-target organs colon and glandular stomach [8]. However, an obvious link between tumour formation and increased cell proliferation was seen in the development of several tumours, such as forestomach and liver. Accordingly, work by Culp and coworkers [9,10] indicates that coal tar-induced cytotoxicity and cell proliferation were the final determinants — in addition to DNA binding — for the tumour induction in the small intestine of the mice. In a 4 week feeding study of a coal tar mixture the authors show that DNA adduct levels increase up to a concentration of 0.6% (13.4 ppm B[*a*]P), but then decrease, so that the adduct level in the mice fed with 1% coal tar mixture (22 ppm B*a*P) is similar to those found with the 0.3% dose (6.6 ppm B*a*P). However, tumours of the small intestine are only observed when feeding the mice with 0.6% or 1% of coal tar mixture. These concentrations of coal tar mixture, but not 0.3%, induce a nearly 50% increase in cell proliferation. Hence, additional factors to DNA adduct formation are essential for benzo[*a*]pyrene-induced tumour development (and also other PAHs-dependent tumours). Moreover, even the situation exists where DNA lesions (investigated lesion: strand breaks/alkali labile sites) are produced by reactive genotoxins (investigated genotoxin: styrene 7,8-oxide) to an observable extent only after a practical threshold caused by practically immediate detoxication is exceeded [11].

So far, no data are available defining precise threshold levels for PAHs in animal models. However, computational modeling of *in vitro* data sets would predict nonmonotonic dose-response relationships also *in vivo* [12,13]. Indeed, nonmonotonic dose-response relationships have been detected for tumour induction by the genotoxic compounds 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline and diethylnitrosamine [14,15].

Non-genotoxic "dioxins"

There is good experimental evidence that the effects of dioxins, a prototype of which is TCDD, and dioxin-like compounds are receptor-mediated, i.e. require binding of the compound to the AhR. Although the binding of a compound to a given receptor is a bi-molecular reaction leading to a biological outcome, which is directly proportional to receptor occupancy [reviewed in 4], this simple assumption does not take into account that multiple downstream effects post-ligand binding generally occur in a living cell. For instance, it is still not clear whether nuclear translocation of the AhR requires a certain threshold concentration of the ligand. Furthermore, depending on the experimentally defined end-points, different dose response curves may exist. For instance, DeVito and coworkers compared different effects of repeated low level exposure to TCDD in mice and revealed different dose-response curves for the induction of Cyp1A1/1A2 and tyrosine phosphorylation in liver [16]. A significant increase in EROD-activity, a marker for CYP1A1 activity, was already detected at the lowest dose of 1.5 ng/kg/d. Even at the highest dose of 150 ng/kg/d the maximum of the dose response curve was not reached. While a significant increase in tyrosine phosphorylation in liver was also observed at the lowest dose of 1.5 ng/kg/d, maximal effects occurred at a dose of 4 ng/kg/d [16]. In several initiation-promotion studies, the promoting activity of TCDD has been analysed quantitatively. One of the first detailed dose-response studies was carried out by Kociba and coworkers [17]. They analysed the emergence of enzyme-altered foci, preneoplastic nodules and hepatocellular carcinomas in rats. Interestingly, enzyme-altered foci were observed at a dose of 0.01 μg/kg/d whereas higher doses were needed for the induction of preneoplastic nodules and hepatocellular carcinomas (0.1 μg/kg/d). In contrast to studies in mice, female rats appeared to be much more sensitive than male rats. Interestingly, an opposite gender specificity was described in mice [reviewed in 4].

Several studies on the dose-response relationship using an initiation-promotion protocol have followed using the appearance of enzyme altered foci as experimental endpoint [18–23] and have been modelled thereafter.

Portier and coworkers [24] provided a mathematical model of an experimental study in which four different TCDD doses (3.5, 10.7, 35.7, 125 ng/kg/d) were administered in DEN-initiated rats [20]. The data were consistent with dose-linearity at least for the smaller doses, and surprisingly suggested that TCDD enhanced the production of enzyme altered foci and hence acts as a tumour initiator, a conclusion recently supported also by Stinchcombe and coworkers [25]. However, based on the assumption of two differernt

types of initiated cells, Conolly and Andersen predicted a U-shaped dose-response curve in the low-dose range (0.1–10 ng/kg/d) [26,27], a model in line with the above mentioned Kociba-study.

A U-shaped dose-response curve indicates an inhibitory effect on tumourigenesis in the low dose range and might be explained by the existence of two different types of initiated cells (see above): cells of one type show a decrease in their proliferation in response to TCDD in the low dose range while cells of another type show an increase in proliferation in the high dose range. Indeed, Teeguarden and coworkers [23] have revealed an inhibition of proliferation of non-transformed liver cells in the rat at low TCDD concentrations (0.1 ng/kg/d) while proliferation increased at high doses (10 ng/kg/d) correlating with liver toxicity [summarised in 26]. In addition, Fox and coworkers have shown zonal differences in the TCDD-response with mitoinhibitory effects of TCDD in the centrilobular regions and proliferative effects in the periportal region in rat liver [28]: TCDD was administered by using a dose loading/maintenance regimen to achieve rapid quasi-steady-state TCDD liver concentrations of 0.03, 30 or 150 ng/g liver. At the dose of 150 ng/g liver, a significant elevation of BrdU labelling index was found in the periportal region, while a slight decrease in BrdU labelling index was observed in the centrilobular region. This raises the question if oval cells which are located periportally are also cellular targets of TCDD. Moreover, a number of studies performed in transformed rat hepatoma and oval cells and primary mouse thymocytes have demonstrated that TCDD, via acting through AhR, can dependent on the cell type either increase or decrease cell proliferation [29–31].

In summary, the shape of dose-response-relationship and the existence of thresholds for the tumourigenesis-related effects of TCDD and related compounds remain largely unresolved. Based on current understanding, receptor binding is not likely to be a threshold-related event. However, multiple intracellular signalling events downstream of the AhR result in complex biological responses as demonstrated by mito-inhibitory, proliferative and anti-apoptotic effects. These effects depend on the dose, zonal region and cell type. The molecular mechanisms underlying these pleiotropic effects remain to be elucidated.

Conclusion and perspectives

As outlined above, there is a great demand for elucidation of the cellular signalling pathways which are evoked by exposure to PAHs and TCDD in order to better understand dose-response relationships. Especially in the case of TCDD, the cellular effects in rodent liver are greatly dependent on the dose, zonal region and, as concluded from results with different liver cells in culture, also from the different intracellular equipment of different cell types. The same applies to disturbances by TCDD of developing tissues. Another open question is the role of the AhR, ARNT and the recently discovered AhRR (AhR repressor) as mediators of cellular effects of PAHs

and TCDD. Downregulating these mediators by siRNA-technology in cell culture and tissue specific conditional knock-out mice with individual or combined deletions in these genes will aid the clarification of the signalling pathways induced by PAHs and TCDD. Even more complexity is given by the fact that humans are generally exposed to mixtures of PAHs and TCDD. Almost no information is available about the cellular effects or signalling pathways that are induced by such mixtures.

To close this gap, the characterization of the intracellular signalling cascades induced by PAHs and TCDD is needed. In order to better understand possible cross-talk(s) between these two pathways, the signalling pathways have to be analysed, first independently. Establishing dose-response relationships by correlating gene expression profiles with the observed cellular effects, such as proliferation/survival and apoptosis — at different doses of the compounds — will be of great importance. Furthermore, comparison of gene expression profiles in different cell culture models (liver cells, cells from developing tissues including stem cells) will help to better understand and quantitatively relate dose responses to the in part already known cell type specificity of the effects of TCDD. Building on all of this the establishment of the mutual cross-talks of components of TCDD/PAH mixtures will be important.

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Summary

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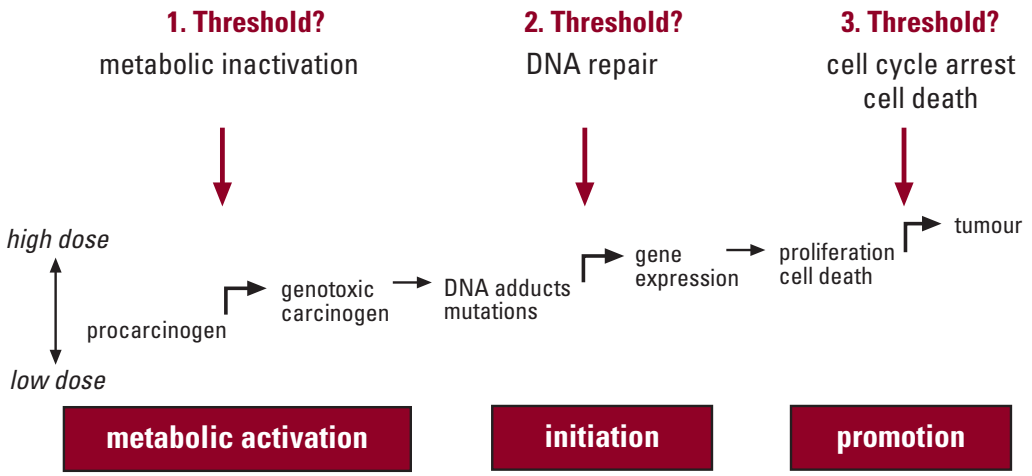

This review describes the present state of knowledge, research gaps and perspectives on dose response relationships in chemical carcinogenesis with emphasis on benzo[*a*]pyrene (B[*a*]P) as a prototype DNA damaging carcinogen and dioxin (2,3,7,8-tetrachlorodibenzo-p-dioxin, TCDD) as a prototype non-genotoxic carcinogen. The goal for collecting and presenting the data comprised in this review was to improve the basis for cancer risk assessment by insights into mechanisms of chemical carcinogenesis, allowing for a better definition of dose response as linear, non-thresholded non-linear or even having practical or absolute thresholds and to show the present limitations.

For the prototype DNA-damaging carcinogen B[*a*]P — although not unambiguously demonstrated — there is at least the reasonable expectation for the existence of a finite dose of the carcinogen (a threshold) to be required for the induction of a carcinogen-activating enzyme (CYP1A1) which at least in the organ responsible for most of the mammalian organism's metabolism (the liver) is not expressed in absence of induction. Moreover, even the situation exists where DNA lesions (investigated example: DNA alkali-labile sites and strand breaks) are produced by reactive genotoxins (investigated example: styrene 7,8-oxide) to an observable extent only after a practical threshold caused by practically immediate detoxication is exceeded. However, for all DNA damaging carcinogens, for which exploitable data in rodents (rats and mice) were found in the literature, there was no positive evidence for the existence of thresholds for the formation

of DNA adducts, although owing to the high sensitivity of the method extremely low adduct levels (in several cases down to 10^{-10}) could be determined. The investigation of potential thresholds for the formation of mutations is limited by the sensitivity of the available experimental systems being 10,000 fold less than the most sensitive methods for detecting DNA adducts, this lower sensitivity of mutation assays being primarily determined by the frequency of background mutations which reflect spontaneous events occurring prior to or during DNA replication. Assuming a near random distribution of adducts in the genome would imply the need for a minimum number of DNA adducts to be present in the genome before it can statistically be expected that a mutation in a specific gene becomes observable. Thresholds for carcinogen-induced changes in the expression of many genes as investigated by gene array analysis is little investigated and the available experimental systems suffer from relatively low sensitivity combined with high costs not inviting many repeat experiments for increasing the statistical resolving power. Loss of proliferation control as well as induction of cell death (by apoptosis or necrosis) are easy to associate conceptually with the requirement of finite doses of carcinogen and hence to be thresholded. Indeed, several studies were reviewed in this position paper which show that DNA adducts were found in all tissues, with remarkably high levels in organs devoid of any tumour development; but in the organs where tumours developed increased cell proliferation was also seen. In several studies, cytotoxicity and cell proliferation were the final discriminatory factors rather than DNA binding alone for the tumour induction. This shows that factors additional to DNA adduct formation are also critical for tumour development (reviewed in this position paper for B[*a*]P and other PAHs). Also, computational modelling of *in vitro* data sets predict nonmonotonic dose-response relationships (i.e. thresholds or even hormetic dose reponses, that is inhibition of spontaneous tumourigenesis at very low doses of carcinogens) *in vivo*. Indeed, nonmonotonic dose-response relationships have been detected for tumour induction by the genotoxic compounds 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline and diethylnitrosamine.

Thus not only for non-genotoxic carcinogens but also for some DNA-damaging carcinogens rudimentary evidences for non-linear dose responses and even for thresholds for their tumourigenicity and for various steps on the way to cancer exist as outlined above. However, in order to make these data safely exploitable for improved risk assessment, they urgently await more thorough investigation and analysis. The most crucial improvement needed is that for DNA-damaging carcinogens quality and reporting form of data are required which make a reliable discrimination possible whether a threshold dose exists below which no genotoxicity or no tumourigenicity occurs or whether the non-observation of these effects at low doses are just the result of limiting sensitivity under the experimental conditions used.

Definitions

For the sake of unambiguity in the following the way how some crucial terms are intended to be understood in this review are defined:

Genotoxic:

The term "genotoxic" is used in this review to indicate the induction of genome changes, including DNA adducts, strand breaks/alkali labile sites, gene mutations, structural chromosome aberrations or numerical chromosome changes (aneuploidy).

Mutations:

— The term **"gene mutations"** is used in this review to indicate variations at the gene level, which result in the alteration of coding or non-coding sequences as a result of substitution, addition, or deletion of one or more bases within those sequences.

— The term **"chromosome mutations"** is used in this review to indicate variations in chromosome structure and arrangement, followed by either the loss or rearrangement of the genetic material.

— The term **"genome mutations"** is used in this review to indicate numerical chromosome changes giving rise to aneuploidy. During mitotic cell division aneuploidy may occur by a variety of malsegregation events, the most important of them being chromosome loss, mitotic non-disjunction and telomere shortening.

Monotonic versus non-monotonic dose responses:

— The term **"monotonic dose response"** is used in this review to indicate either only increases or only decreases over an entire dose-range.

— The term **"non-monotonic dose response"** is used in this review to indicate dose-response curves which are at least biphasic, indicating possible thresholds for effects (NOEL), or effects at low concentrations which are opposite (significantly higher or lower) to those at higher concentrations (hormesis).

Threshold:

The term "threshold" is used in this review to indicate either no effect where the sensitivity of the method would clearly show an effect if present or in the light of the experimentally determined variability most conservative dose-response curve extrapolation crossing the abscissa at a finite concentration (i.e. zero effect at a concentration above zero).

The present review addresses the question whether particular mechanisms induced by carcinogens can act via non-linear but not thresholded dose responses or can even have practical or absolute thresholds.

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