
Importance of Metabolism. Mechanistic Considerations Relevant for Toxicological Regulation

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Abstract

Most xenobiotics do not remain unchanged in the human organism (and in other organisms), but rather are metabolized. The change in chemical characteristics of the thereby produced metabolites as compared with the chemical characteristics of the corresponding parent compounds usually leads to changes in both the desired properties, e.g., therapeutic efficacy, and also in undesired properties, i.e., xenobiotic metabolism usually is toxicologically not neutral, but rather leads in most cases to toxication or detoxication of the respective compound (overview see Oesch-Bartlomowicz and Oesch 2007). Thereby xenobiotic metabolism becomes one of the most important factors controlling the toxicity of the respective compound. This, in turn, makes xenobiotic-metabolizing enzymes to control factors for xenobiotic toxicity.

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Fundamentals

These xenobiotic-metabolizing enzymes drastically differ quantitatively and in many instances even qualitatively between animal species, organs, cell types, developmental stages, and physiological states such as health and individual diseases and most often even between strains and genders (for a succinct review, see Hengstler and Oesch 1999). For an extrapolation of toxicity findings in experimental systems to humans, cognizance of differences in xenobiotic-metabolizing enzymes between the systems used and humans is therefore critical.

Quantity and also chemical identity of the formed metabolites depend on many factors. This complexity leads to difficulties to predict from experimental systems which metabolites and how much of them will be generated in humans. Difference in xenobiotic metabolism between species is one of the factors which most profoundly limit the extrapolation of toxicological results obtained in experimental systems to humans (for a comprehensive review, see Hengstler et al. 1999). The later considerations in this chapter will therefore especially take this interspecies parameter into account. However, in order to be comprehensive, it must also be stated that in addition to these crucial species differences, further important differences exist also within a given species. This includes genetic differences in xenobiotic metabolism between strains and between individuals. In humans genetic polymorphisms in xenobiotic-metabolizing enzymes known to date account for up to 40 % of cytochrome P450 (CYP)-dependent xenobiotic metabolism (Modak 2010). The generally used pharmaco/toxicokinetic default uncertainty factor of 3.2 to account for human interindividual differences in the extrapolation of toxicity data to human may need to be enlarged if it does not encompass human polymorphisms from poor to extensive metabolizers of the xenobiotic compound in question (Schroeder et al. 2011). Moreover, differences caused by different gene expressions during development or disease states may drastically influence xenobiotic metabolism, most profoundly if the liver, the organ responsible for the largest portion of the mammalian xenobiotic metabolism, is involved. In addition, environmental factors, nutrition, and drug treatment can profoundly modulate xenobiotic metabolism by enzyme induction, repression, activation, or inhibition (for an overview see Oesch and Arand 1999; updated version in German: Arand and Oesch 2004). These numerous factors may interact with each other, generating a high complexity of xenobiotic metabolism control and consequent toxicities. For instance, very early on, it had already been shown that differences in nutritional status profoundly influenced drug-metabolizing enzyme induction (e.g., by DDT) and the consequent toxicity of a third compound (e.g., carbon tetrachloride) (McLean and McLean 1966).

In order to improve the water solubility and excretability of xenobiotics, the organism most often uses conjugation with endogenous water-soluble building blocks such as glutathione, glucuronic acid, or sulfate. Such conjugations need the preexistence of suitable substituents in the xenobiotic compound in question which, if not preexisting in the parent compound, first have to be introduced or

liberated. This step in xenobiotic metabolism is called phase I, and the subsequent conjugation is called phase II (the then frequently following active excretion of the generated water-soluble metabolite from the cell of origin is often called phase III).

The phase I metabolites possess at the site to be conjugated electrophilic (such as epoxides, α,β -unsaturated carbonyls) or nucleophilic (such as hydroxyl, sulfhydryl, amino, carboxyl) structural components. Depending on their relative chemical reactivities, electrophilic moieties can have high toxicological potential by reacting with nucleophilic moieties of endogenous compounds, toxicologically most significant if they thereby modify the structures of macromolecules such as proteins, RNA, and – especially important – DNA, the latter potentially leading to significant genotoxicity. In contrast to this, nucleophilic metabolites usually do not covalently react with endogenous molecules and therefore usually are toxicologically less problematic. However, they can, in some cases, have affinity to receptors and thereby lead to desired therapeutic or undesired toxic interactions.

The conjugating phase II reactions in most cases lead to a large increase in the water solubility of the compound in question, to its efficient excretion, and to termination of its biological activity, be it beneficial (therapeutic) or undesired (toxic). However, some exceptions exist, for instance, some glucuronides (e.g., of morphine) possess high biological activities and some conjugates (e.g., of vicinal halogenated alkanes) with glutathione possess higher genotoxic potential than the parent compound (for an overview see Oesch-Bartlomowicz and Oesch 2007).

The enzymes catalyzing phase I reactions include oxidoreductases and hydrolases. Oxidoreductases relevant for xenobiotic metabolism include cytochromes P450 (CYP), flavin-containing monooxygenases (FMO), monoamine oxidases (MAO), and cyclooxygenases (COX). In most cases these oxidoreductases introduce oxygen into xenobiotic molecules or abstract electrons. CYPs are quantitatively especially often involved in xenobiotic metabolism. Thus, two-thirds of the top 200 drugs prescribed in the United States (year of survey: 2002) are cleared through metabolism that involves CYPs (Williams et al. 2004). Further important xenobiotic-metabolizing oxidoreductases include dehydrogenases and reductases such as alcohol dehydrogenases (ADH), aldehyde dehydrogenases (ALDH), and carbonyl reductases. They abstract or add hydrogen atoms. Diverse xenobiotic-metabolizing hydrolases catalyze the hydrolysis of esters, amides, glucuronides, sulfates, or epoxides.

In the phases II reactions, electrophilic substrates are conjugated by glutathione *S*-transferases (GST), nucleophilic substrates by UDP-glucuronosyltransferases (UGT), sulfotransferases (SULT), *N*-acetyltransferases (NAT), acyl-CoA-amino acid-*N*-acyltransferases, and methyltransferases (for an overview see Oesch and Arand 1999; updated version in German: Arand and Oesch 2004).

A correct prediction of toxicity is especially important in cases of long latencies such as cancer, since a wrong prediction leads to accumulation of numerous

Table 1 Reactive metabolites: some important prototypes

Parent compounds	Reactive metabolites	Enzymes involved in the control
Aromatic/olefinic hydrocarbons	Epoxides	Cytochromes P450
		Glutathione S-transferases
		Epoxide hydrolases
Aromatic/heterocyclic amines	Reactive esters	Cytochromes P450
		Sulfotransferases
		Acetyltransferases
		Glutathione S-transferases
		UDP-glucuronosyltransferases
Dialkylnitrosamines	Carbonium ions	Cytochromes P450
	Electron deficient alkyl groups	
Vicinal dihaloalkanes	Episulfonium ions	Glutathione S-transferases

irreversible damages before the error becomes manifest. For such toxicities electrophilically reactive metabolites are especially important which frequently have a short life span and are formed in low quantities. For such cases cognizance of reactive metabolites and xenobiotic-metabolizing enzymes responsible for their control (formation, detoxication, sequestration into alternative pathways) is especially important. Examples of some important electrophilically reactive metabolites and xenobiotic-metabolizing enzymes involved in their control are given in Table 1. An important consequence of the fact that quantitatively minor metabolites may be responsible for toxic (especially for genotoxic) effects is that species-specific divergent pathways leading to such minor but toxicologically important metabolites may become crucial. When a human-only metabolite is not formed in the experimental species chosen for toxicity testing, an incomplete xenobiotic safety assessment may result leading to an underestimation of toxicological risk. The FDA/CDER guidance on safety testing of drug metabolites (U.S. Department of Health and Human Services 2008) therefore states that a unique human metabolite must itself be tested for toxicity when the metabolite level reaches >10 % parent systemic exposure at steady state.

Some overall approximations in relatively high similarities of some xenobiotic-metabolizing enzymes or their response to exogenous stimuli between certain experimental animal species and humans may be attempted. Although the different animal models have many differences in the ligand-binding domain of the respective nuclear receptors involved in the control of xenobiotic-metabolizing enzymes induction compared with humans (Mohutsky et al. 2010), induction responses compared with humans appear to be most similar in rats and mice for CYP1A; in rats, mice, and pigs for CYP3A; in monkeys for CYP2C; and in dogs for CYP2D (Martignoni et al. 2006; Zuber et al. 2002; Bogaards et al. 2000). However, some exceptions of outstanding practical importance highlight the fact that a priori acceptance of these overall relatively high similarities may be dramatically misleading for an

individual xenobiotic compound under consideration. Thus, rifampicin does not induce CYP3A in rats or mice, but does so in humans (leading to unwanted pregnancies in combined use of contraceptives and rifampicin) and in rabbits (Kocarek et al. 1995; Back et al. 1988). Inversely, pregnenolone-16 α -carbonitrile (PCN), which strongly induces CYP3A in rats and mice, causes no induction in humans or rabbits, and CYP3A induction by 5 α -pregnane-3,20-dione is seen only in humans and mice, but not in rats or rabbits (Mohutsky et al. 2010). For improved predictions animal models have been genetically modified in which some nuclear receptors controlling induction of a xenobiotic-metabolizing enzyme of that species have been knocked out and replaced by the corresponding human gene (Scheer et al. 2008; for a similar approach see Ma et al. 2007).

Examples of Metabolism-Associated Toxicity

Having discussed the basic aspects of drug metabolism, the following chapter will focus on examples of compounds where drug metabolism plays an important role for risk assessment. Usually risk assessment is based on animal experiments. For identification of acceptable human exposures, NOAELs (see chapter “► [Examination of Acute and Chronic Toxicity](#)”) from laboratory animals are used and multiplied with safety factors (see chapter “► [Extrapolation Factors and Safety Factors in Toxicology](#)”). Usually this procedure identifies exposure levels that are safe for humans. However, working with safety factors, e.g., a fixed safety factor of ten to consider possible interspecies differences of metabolism, may under certain circumstances lead to mistakes. This is the case when interspecies differences in metabolism between humans and the relevant animal species are huge. To illustrate this problem, some examples of well-characterized compounds will be discussed in the following paragraphs (from: Hengstler et al. 1999 and Hengstler et al. 2003) and references cited herein. It should be considered that they represent extreme and rare cases. Nevertheless, they are important to illustrate how mistakes in risk assessment can be avoided.

MeIQx (2-Amino-3,8-Dimethylimidazo[4,5-f]Quinoxaline)

MeIQx represents an intensively studied heterocyclic amine found in fried as well as cooked meat. It is formed by a heat-dependent reaction between muscle creatinine and amino acids. MeIQx is a strong colon carcinogen in rats and mice. However, it does not cause colon cancer in cynomolgus monkeys. Therefore, a critical question is whether human risk assessment should be based on the rodent or monkey data. Because of the small evolutionary distance, one might be tempted to favor the monkey for this purpose. However, a relatively simple experiment demonstrates that risk assessment must be based on the more susceptible rodents. An Ames mutagenicity test using microsomes from livers of human, rat, and

cynomolgus monkeys as a metabolizing system reveals major interspecies differences. Human and rat microsomes strongly activate MeIQx to a mutagen, whereas microsomes from cynomolgus monkeys are almost inactive (Fig. 1). This corresponds to the mechanism of metabolic activation of MeIQx to a carcinogenic nitrenium ion (Fig. 2). Human and rat cytochrome P4501A2 form a hydroxylamine that is further metabolized to a reactive *N*-acetoxyester. In contrast, cynomolgus monkeys lack an activity corresponding to human or rat cytochrome P450 1A2. However, it should be considered the cynomolgus monkey represents an exception with respect to MeIQx metabolism. Even other monkey species, such as marmosets, form the hydroxylamine from MeIQx and are therefore susceptible to its carcinogenic effect. In conclusion, humans are similarly susceptible to MeIQx-induced carcinogenicity as rats and do not represent a resistant species, such as cynomolgus monkeys. Therefore, risk assessment must be based on the more resistant species.

Aflatoxin B1

Aflatoxin B1 is one of the most potent liver carcinogens for humans and rats. However, the TD50 (the dose that induces tumors in at least 50 % of the animals) shows large interspecies differences, ranging between 1 and 6 $\mu\text{g}/\text{kg}/\text{day}$ for different rat strains, whereas even doses of 2,000 μg AFB1/ kg/day did not yet cause liver tumors in 50 % of the C57/BL6 mice. Therefore, the interspecies differences between rats and mice are larger than a factor of 1,000, a difficult scenario for human risk assessment.

To study whether humans are as susceptible to AFB1 as rats or rather as resistant as mice, genotoxicity assays were performed using liver microsomes of all three species as a metabolizing system (Fig. 3). Sister chromatid exchanges (SCE) in human lymphocytes were analyzed as a genotoxic end point. Incubation of AFB1 (10 μM) with liver microsomes of all three species caused a clear increase in SCE when NADPH was added to the incubation mixture, whereby NADPH acts as a cofactor of the cytochrome P450-mediated metabolic activation of AFB1. However, metabolic activation by mouse liver microsomes was stronger compared to human and rat. It should be considered that lower AFB1 concentrations (only 1 μM for mice compared to 10 μM for human and rat) were used. This seems to be in contrast to the aforementioned carcinogenicity studies where mice appeared to be more resistant than rats. However, this discrepancy could be explained by an additional *in vitro* experiment (Fig. 3). In microsomal preparations the cofactors of phase II metabolism, such as glutathione (GSH), are too diluted to allow an *in vivo* like phase II metabolism. Therefore, GSH and cytosol of the corresponding species (containing, e.g., glutathione *S*-transferases) were added to the microsomal incubations. These experiments showed a strongly reduced SCE induction when mouse cytosol was added (Fig. 3). In contrast, addition of cytosol and GSH did not reduce genotoxicity of human and rat microsomal incubations. Therefore, mouse liver microsomes have a higher capacity to activate AFB1 to a genotoxic species

Fig. 1 Activation of the heterocyclic amine MeIQx (2-amino-3,8-dimethylimidazo(4,5-f)quinoxaline) to a mutagenic metabolite by liver microsomes of humans, rats, and cynomolgus monkeys. The dashed line shows the number of spontaneous revertants in the Ames test (Davis et al. 1993; review: Hengstler et al. 1999)

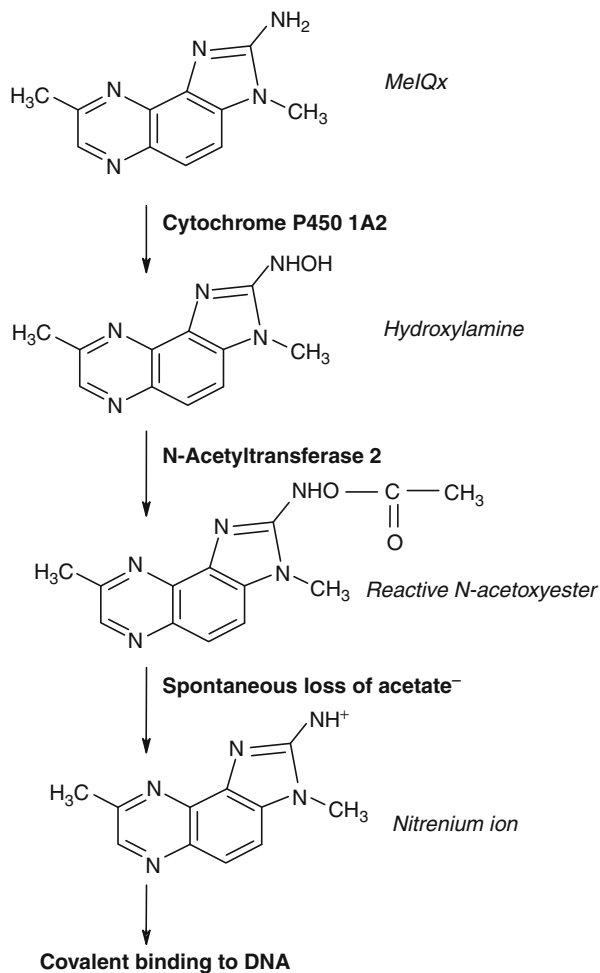
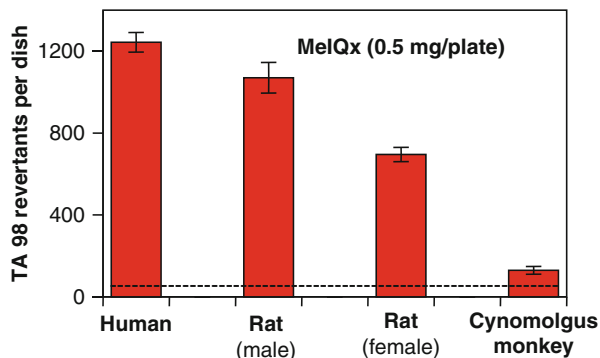


Fig. 2 Mechanism of metabolic activation of MeIQx to a DNA-binding nitrenium ion by cytochrome P450 1A2 and N-acetyltransferase 2. Humans and rats efficiently form the hydroxylamine. However, cynomolgus monkeys lack a corresponding activity

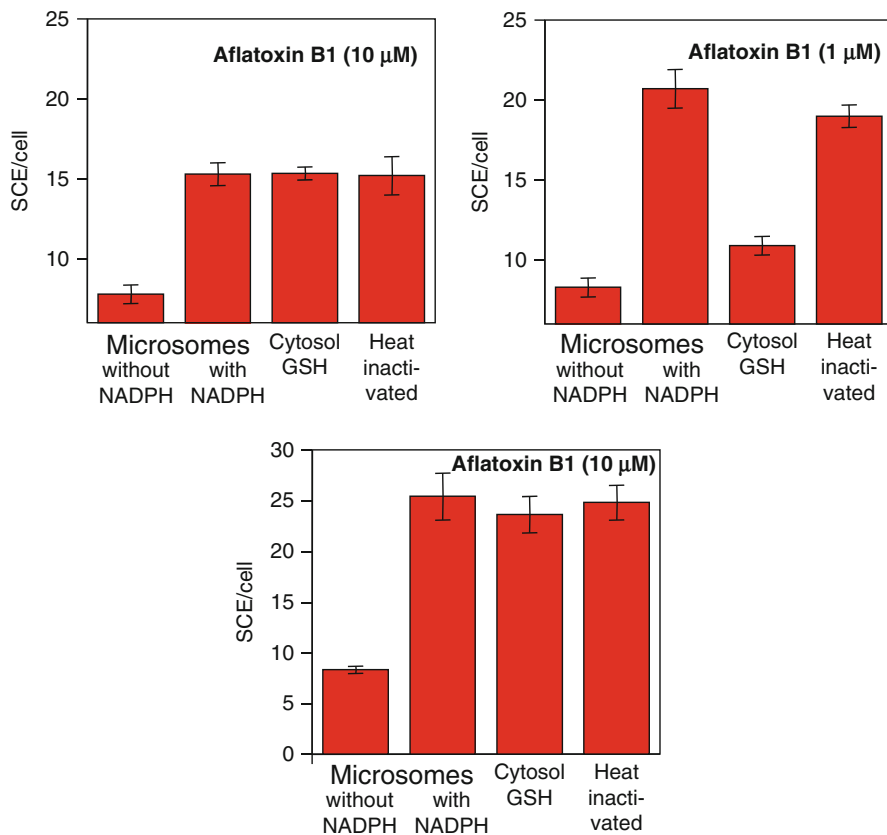


Fig. 3 Influence of phase I and phase II metabolism on the genotoxicity of aflatoxin B₁ (AFB₁), as evidenced by the sister chromatid assay in human lymphocytes. Venous blood of a volunteer was incubated with AFB₁ in the presence of human, mouse, and rat liver microsomes. Concentrations of 10 μM AFB₁ were used for incubations with human and rat microsomes. For incubations with mouse microsomes, only 1 μM AFB₁ was used since higher concentrations were no longer in the linear concentration-effect range. *First column:* negative controls with microsomes but without the cofactor NADPH. *Second column:* influence of phase I metabolism in the presence of microsomes and NADPH. *Third column:* combined influence of phase I metabolism and glutathione *S*-transferases in presence of microsomes, NADPH, cytosol, and 2.5 mM glutathione. *Fourth column:* negative control, with the same conditions as for the third column but using heat-inactivated cytosol (Wilson et al. 1997; review: Hengstler et al. 1999)

compared to humans and rats. On the other hand, the cytosolic compartment of mice also shows a higher capacity to detoxify AFB₁.

Today, the mechanisms underlying these observations are known. Activation of AFB₁ to a genotoxic carcinogen, namely, AFB₁-exo-8,9-epoxide, is catalyzed mainly by cytochrome P450 1A2 and 3A4 (human). The extremely efficient inactivation of AFB₁-exo-8,9-epoxide in mouse liver cytosol is catalyzed by the glutathione *S*-transferase isoenzyme mGSTA3-3 (synonym, mGST-Yc).

Fig. 4 DNA binding of ^3H -aflatoxin B_1 in cultivated hepatocytes of humans (three donors), rats (Sprague Dawley, male and female), and mice (CD-1, male) after incubation for 24 h (Cole et al. 1988; review: Hengstler et al. 1999)

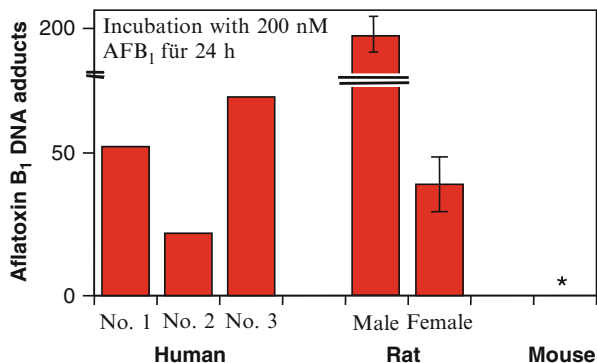
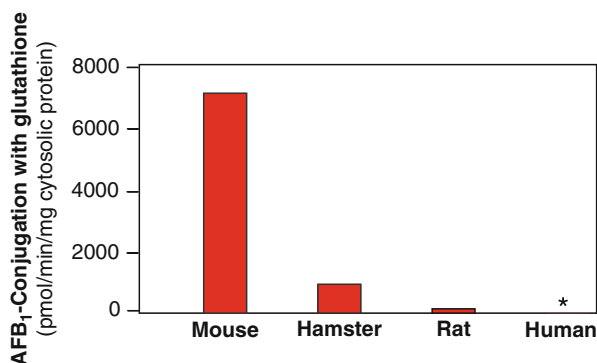


Fig. 5 Conjugation of aflatoxin B_1 -exo-8,9-epoxide (generated by incubation with microsomes) with glutathione by cytosol of livers from mice, hamsters, rats, and humans (Slone et al. 1995). *Below detection limit



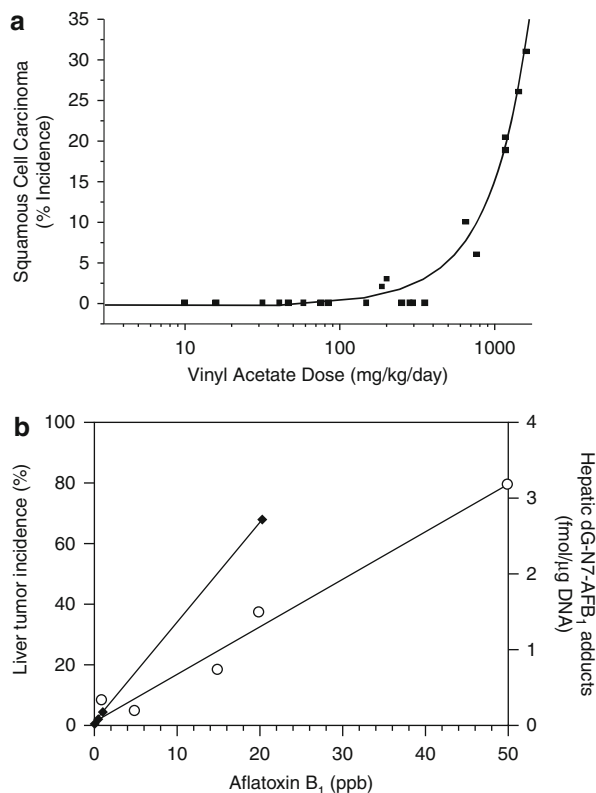
In contrast, humans and rats do not express phase II enzymes with a similarly high capacity to detoxify AFB₁-exo-8,9-epoxide.

An adequate technique for identification of the interspecies difference in AFB₁ susceptibility is the analysis of DNA adducts in primary hepatocytes (Fig. 4). While an approximately similar extent of DNA adducts was formed in human and rat hepatocytes, the corresponding data of mice were below the detection limit. Finally, the glutathione conjugation capacity can directly be analyzed, illustrating the low capacity of human liver cytosol to detoxify AFB₁-exo-8,9-epoxide compared to mice (Fig. 5). In conclusion, humans are more susceptible to AFB₁-mediated carcinogenesis than mice. Therefore, human risk assessment should be based on rat data.

Vinyl Acetate: The Relevance of Practical Thresholds

The examples of MeIQ_x and AFB₁ have illustrated the importance of basing risk assessment on toxicity data of speZcies that resemble the human situation. This is particularly relevant in case of huge interspecies differences of metabolic activation

Fig. 6 (a) Dose–response relationship between exposure to vinyl acetate in the drinking water and induction of carcinomas in rats and mice. Since rats and mice respond similarly to vinyl acetate on a mg/kg/day basis, data for rats and mice were summarized in one figure (details in Hengstler et al. 2003). Carcinomas induced by vinyl acetate under these conditions are tumors of the oral cavity, esophagus, and forestomach. The data show an increase of tumor incidence only at doses higher than 100 mg/kg/day. (b) Induction of liver tumors (○) and DNA adducts (◆) by aflatoxin B₁. In contrast to vinyl acetate a linear dose–response relationship was obtained by aflatoxin B₁ (review: Hengstler et al. 2003)



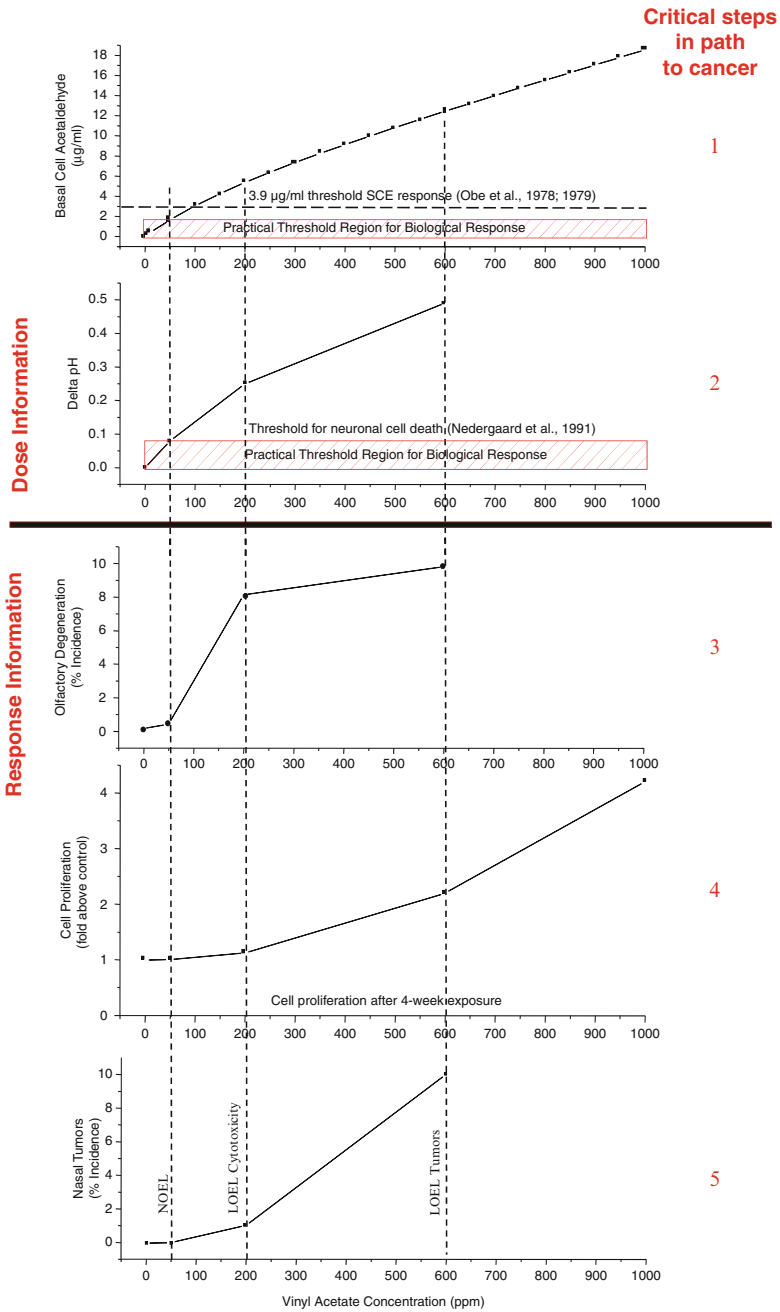


Fig. 7 Five critical steps on path to cancer induced by vinyl acetate in the olfactory epithelium (From Hengstler et al. 2003). *Panel 1*: concentrations of acetaldehyde in the basal cells of the olfactory epithelium, the cells of origin of nasal tumors. *Panel 2*: reduction of pH in relation to

cytotoxicity by decreasing the pH value. Decreases of the pH value of less than 0.15 units usually remain without toxic consequences. However, a further decrease in pH may cause cytotoxicity and replacement proliferation. This will promote carcinogenesis resulting from acetaldehyde-induced DNA lesions.

For risk assessment it is important to consider that both metabolites of vinyl acetate, acetaldehyde, and acetic acid also are endogenously formed in the organism. Acetaldehyde is formed in threonine metabolism. Endogenously, acetaldehyde is formed in concentrations of approximately 0.3 $\mu\text{g/ml}$ blood. Exposure to vinyl acetate at levels that increase acetaldehyde and acetic acid within this endogenously occurring range does not induce tissue damage or carcinogenesis, which will be shown below. Therefore, it can be concluded that the organism has established protective mechanisms that avoid tissue damage at physiological levels of both vinyl acetate metabolites. Exposure to vinyl acetate should be acceptable if the resulting increase in acetaldehyde and acetic acid at the highest exposed cells of the organism is lower than endogenously formed concentrations.

Although the aforementioned theoretical considerations may seem plausible, the assumption of a "practical threshold" is only acceptable when proven by experimental data. Dose–response experiments for vinyl acetate-induced carcinogenicity show a wide dose range without increased tumor incidence (Fig. 6a). However, a clear increase is observed at doses higher than 100 mg vinyl acetate/kg body weight/day. The shape of the dose–response curve of vinyl acetate clearly differs from that of the no-threshold carcinogen AFB₁, for which both tumor incidence and DNA adducts do not show any evidence for a threshold (Fig. 6b).

The threshold of the dose–response relationship for vinyl acetate is due to the fact that critical concentrations of acetaldehyde and acetic have to accumulate up to certain concentrations where mechanisms relevant for carcinogenesis are activated. For the olfactory epithelium, five steps of vinyl acetate-mediated carcinogenesis seem to be critical (Fig. 7). According to our PBPK model exposure of 50 ppm, vinyl acetate leads to acetaldehyde concentrations of 1.7 $\mu\text{g/ml}$ (step 1 in Fig. 7). Moreover, the resulting acetic acid causes a pH reduction of 0.08 units in basal cells of the olfactory epithelium, the cells of origin of carcinogenesis (step 2). This pH decrease is less than the critical value of ΔpH 0.15 which may cause cytotoxicity. Therefore, degeneration of olfactory cells (step 3) and replacement proliferation (step 4), steps critical on path to cancer, do not yet occur at 50 ppm vinyl acetate. However, increasing vinyl acetate exposure to 200 or even 600 ppm will activate these mechanisms. Vinyl acetate exposure of 600 ppm will lead to a concentration of 12.4 $\mu\text{g/ml}$ acetaldehyde in basal cells (step 1). The pH value will decrease by 0.49 units (step 2) which will cause degeneration of the olfactory epithelium (step 3) and will lead to replacement proliferation (step 4) of basal cells. Therefore, all steps critical for carcinogenesis are active at 600 ppm vinyl acetate. This leads to



Fig. 7 (continued) vinyl acetate exposure. Reduced pH is responsible for cytotoxicity in the olfactory epithelium (*Panel 3*), which causes replacement proliferation (*Panel 4*) and finally promotes induction of nasal tumors (*Panel 5*)

a clear increase of tumor incidence (Fig. 7). The model demonstrates that the mechanisms critical for carcinogenesis (steps 1-4) become active only when threshold concentrations of acetaldehyde and acetic acid are exceeded. These threshold concentrations will only be exceeded when vinyl acetate exposure occurs above certain levels (Fig. 7). In conclusion, two metabolites, acetaldehyde and acetic acid, are responsible for the toxic and carcinogenic effects of vinyl acetate. Both metabolites also occur endogenously. Only above certain threshold concentrations carcinogenicity can be expected. Therefore, risk assessment of vinyl acetate has to take into account quite different principles as for, e.g., aflatoxin B₁ or heterocyclic amines where similar threshold mechanisms are not known.

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