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Aromatic amines: mechanisms of carcinogenesis and implications for risk assessment

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TABLE OF CONTENTS

1. ABSTRACT

Carcinogenic aromatic amines are widespread and need to be regulated. Genotoxic and non-genotoxic effects are both necessary for tumor development. The common mode of action includes metabolic activation, the reaction of metabolites with nucleic acids and cellular macromolecules as well as toxic effects. The dose-response relationship of irreversible DNA damage is linear down to background concentrations and a no-effect level (NEL) cannot be defined. The dose-response relationships of reversible toxic effects are often non-linear and have been used to derive no-observed adverse effect levels (NOAEL). However, this procedure does not account for background exposure, the activity of structurally related, and those structurally unrelated chemicals which compete for the same biochemical systems. Fixed limit values for acceptable risk are therefore unacceptably uncertain. The perspective should change from "risk" to the "contribution to risk". The ALARA principle is part of such an approach. It does not say how much exposure is acceptable. Scientific risk assessment and risk management should be kept distinct and the input of scientific data and expert judgement documented.

2. INTRODUCTION

The chemical and biochemical properties of aromatic amines as well as the primary lesions are very similar. If they are treated as a group several conclusions may be drawn with regard to their carcinogenic properties. Their acute and chronic effects can be explained by a common mode of action. If this is accepted it may be concluded that they all have a carcinogenic potential. Since the carcinogenic potency ranges from very weak to strong, a new look at the quantitative relationship should bring in new aspects to regulate some of these chemicals. The traditional approach does not account for the fact that different sources of the test chemical may exist and may contribute to a sizable background. Structurally related amines also contribute to this background insofar as the same biochemical endpoints are involved. The risk calculated for an aromatic amine based on experimental data may have little meaning in the real-life situation. In addition, the amine in question competes with other exogenous and endogenous compounds for biological endpoints of regulatory concern. In cases in which sufficient information is available to assess carcinogenic potency, it has been suggested that one should estimate not an "acceptable risk:, but, rather, a risk increment, i.e. the

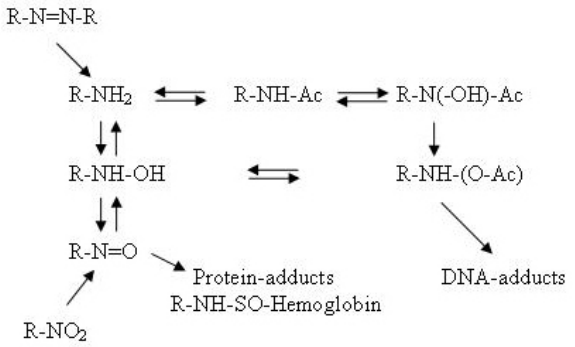

Figure 1. Basic metabolism of arylamines leading to DNA and protein-binding. R = aryl component (phenyl- ,naphthyl-, fluorenyl-, phenanthryl-)

extent to which an exposure contributes to overall carcinogenic risk. The exposure could be considered acceptable if it does not significantly increase the risk. Beyond this point it seems unavoidable to choose the ALARA principle, i.e., "As Low As Reasonably Achievable". Determination of what level is "reasonable" should be performed using all available scientific information, including evolving expert judgement.

3. FROM HAZARD TO RISK

3.1. Structure-activity relationships

Historically, the first task was to find out whether a certain aromatic amine has a carcinogenic potential and therefore, poses a hazard to humans. The International Agency for Research on Cancer (IARC) introduced a classification system (1). The aromatic amines were evaluated in this system as follows: Epidemiological evidence indicated that exposure to 2-naphthylamine, 4-aminobiphenyl or benzidine at the workplace was likely to produce bladder tumors in workers and this evidence was considered to be sufficient to classify these chemicals as carcinogenic to humans.(IARC category 1). In other cases, epidemiological evidence was less certain, but classification was supported by results from animal experiments showing carcinogenic potential, and it was concluded that such chemicals are probably carcinogenic in humans (IARC category 2A). On this basis, o-toluidine, 4-chloro-o-toluidine, and MOCA (4,4´-methylenebis(2-chloroaniline) were classified. If the information from epidemiology and animal experiments was held to be insufficiently certain, but suspicion remained, this was expressed by transferring the chemical into IARC category 2B ("magenta"). Major arguments in these cases usually came from *in vitro* testing and structural relationships. In some cases, it was decided that an agent was not classifiable as to its carcinogenicity to humans (IARC category 3).

Another classification system, which is also used in this discussion, was issued by the German "Commission for the Investigation of Health Hazards of Chemical Compounds in the Workplace" (2) and establishes MAK (maximale Arbeitsplatzkonzentration = maximum concentration in the workplace) and BAT (biologische Arbeitsplatztoleranz = biological tolerance values in the workplace) values.

These classification systems, like others, express the confidence with which it can be said that a particular chemical poses a hazard to humans. A yes or no answer is sought to the question of carcinogenic potential. This is a qualitative definition and tells nothing about the carcinogenic potency, i.e., the risk. More recently, consideration of information about the mode of action was permitted to support the classification. Structural relationships became important and the dominating question became, is the aromatic amine under discussion mutagenic?. Many test systems were developed to answer this question. The structure-genotoxicity (rather than structure-carcinogenicity) relationship was used to evaluate carcinogenic potential. Since dose-response relationships of genotoxic effects are linear down to extremely low doses, it was impossible to derive a threshold dose for carcinogens, below which no genotoxic effects can be expected. Classified carcinogens were therefore considered a hazard, but an acceptable risk could not be estimated on this basis.

3.2. Metabolic activation

Acute and chronic toxicity are characteristic properties of aromatic amines. Ever since certain aromatic amines were shown to be carcinogenic in humans, the question arose: how does the chemical structure determine the biological effects? Better understanding of this relationship could help us to assess the hazard and risk associated with the exposure to these chemicals. The common structural element is an amino group bound to an aromatic system. The chemical reactivity of this amino group depends on the mesomeric interaction with the aromatic system and is further modulated by substituents and steric factors (3, 4).

Both acute and chronic toxicity depend on the metabolic activation of the amino group. The key reaction (which may indeed be responsible for all the toxic activities of these compounds) is N-oxidation to aryl-N-hydroxylamines (5).

Either the free amine or the acetamide can be N-oxidized. Thus, an equilibrium exists between the two, which is determined by the competing activities of N-acetyltransferases and N-deacetylases (Figure 1). Frederick *et al.* (6) described the equilibria among benzidine, N-acetylbenzidine, and N,N´-diacetylbenzidine in liver slices (Figure 2). The positions of these equilibria are important, since acetylation of the amine to the acetamide is typically an inactivating reaction, as is the C-oxidation of the aromatic system. Dogs, for instance, develop bladder tumors following benzidine exposure more readily than do several other species, presumably because dogs, as "non-acetylator" animals, lack one of the inactivating metabolic steps (7, 8).

Both the N-hydroxylamine and the N-hydroxyacetamide may be further activated by enhancing the leaving group through conjugation of the N-hydroxy group with sulfate or acetate, the sulfate being usually a

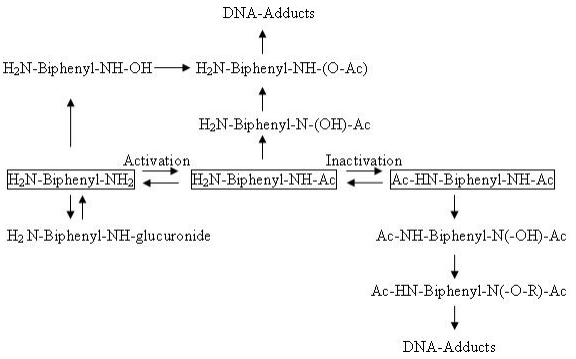

Figure 2. Major metabolic pathways to reactive benzidine metabolites: R = -Ac =acetyl, -SO3H = sulfate.

better mutagen than the acetate. Eventually, the biological activity depends on the bioavailability of a nitrenium ion, the ultimate reactive form which reacts with the cellular macromolecules, DNA, RNA, and proteins. The ultimate metabolites of most arylamines react *in vitro* and *in vivo* with the C-8 atom of guanine bases and the resulting adduct is primarily responsible for the resulting point mutations.

The metabolic activation of benzidine is an interesting example, because it shows the complexity which results from competing metabolic pathways and how the balance depends on the experimental system investigated. N-Hydroxy-N,N´-diacetylbenzidine (N-OH-DABZ) was used as the proximate carcinogen in *in vitro* experiments. Cytosolic sulfotransferase catalyzed the formation of a reactive metabolite which binds to proteins, and it was suggested that this pathway is involved in benzidine carcinogenesis (9). Later studies indicated that metabolic activation may take place at multiple locations in the body. Acid-labile glucuronides are formed in the liver and then distributed to the bladder. In the acidic urine, these glucuronides are hydrolyzed either to N-acetylbenzidine, which could be activated by peroxidases, or to N´-hydroxy-N-acetylbenzidine, which could be further activated (for instance) by O-acetylation. The formation of the resulting guanine-C-8-adduct was explained by the reaction of the nitrenium ion or benzidinediimine reactive species (10, 11, 12).

This short outline of an activating metabolism should demonstrate how sensitive the balance between numerous competing steps is, with regard to possible differences in individual susceptibility, nutritional habits, bladder voiding volume and frequency, etc. Bioavailability of the reactive metabolite is an essential prerequisite for biological activity, and it was expected that once, the relationships between chemical structure of the amine and these conditions are understood, it would be possible to explain diverse biological effects and account for quantitative differences in potency, based on the levels of reactive metabolites. Other bioactivation pathways have been proposed, such as the formation of reactive oxygen species, which may contribute to oxidative DNA damage and mutations induced, for instance, by 2-naphthylamine (NA, 13), 4-aminobiphenyl (ABP), and benzidine (14). However, this is also a general property of aromatic amine metabolism. Human lung chromosomes, for instance, contain high levels of arylamine peroxidase activity which readily activates ABP, benzidine, 4,4´-methylene-bis(2-chloroaniline) (MOCA), 2-aminofluorene (AF), and 2-naphthylamine (NA), as measured by DNA adduct formation (15). Prostaglandin H synthase activates benzidine (16)) and N-acetylbenzidine, leading to the typical guanine-C-8-acetylbenzidine-adduct (13). Peroxidase-mediated activation of aromatic amines can also be demonstrated by activating polymorphonuclear leukocytes with tumor promoters. Binding of metabolites to leukocyte DNA has been found with benzidine, 2-aminofluorene and methylaminoazobenzene (17).

The metabolism of aromatic amines was predominantly studied with 2-acetylaminofluorene (AAF) and somewhat less with 4-aminobiphenyl (ABP), 2-aminonaphthalene (AN) and benzidine (BZ). The ultimate goal of these studies – often performed in cell or tissue culture – was to find the critical metabolic pathway and biological lesion. Many positive correlations were found and quantitative differences in toxicokinetics seemed to explain many species and tissue specific effects of individual arylamines.

The role of metabolic activation for the individual susceptibility has particularly attracted interest for aromatic amines (18). One of the first examples for an enzymatic polymorphism was the acetylator genotype. Workers of the slow acetylator genotype who were occupationally exposed to benzidine were reported to be at greater risk to develop bladder tumors than workers of the rapid acetylator genotype (19, 20, 21, 22)

This was not confirmed in later studies which incorporated phenotypic and genotypic analysis (23, 24). The glutathione transferase M1 null genotype is associated with elevated bladder cancer risk in the general population (25) No increased bladder cancer risk due to the GSTM1-null genotype was reported in benzidine-exposed workers (26). However, the latter study was very limited in size (21 cases only) (27). On the other hand, an elevated bladder cancer risk for formerly benzidine-exposed workers in the Chinese dyestuff industry was associated with a homozygous mutant genotype of UDP-glucuronosyltransferase 2B7 (28). A larger subsequent study in Germany did not find this association (UDP-glucuronosyltransferase 2B7 C802T (His268Tyr) polymorphism in bladder cancer cases (29).

In summary, complex metabolic pathways and many polymorphisms of enzymes involved in the metabolism of aromatic amines are now known and the various equilibria between activating and inactivating steps must be influenced by the individual set up. One of the consequences is that epidemiologic effects are likely to show up only in particularly exposed rather homogenous populations. Therefore, epidemiology is not very helpful to decide between carcinogenic and non-carcinogenic potential or to establish a tolerable exposure.

3.3. The role of monocyclic aromatic amines

It was believed for a long time, that only the polycyclic aromatic amines, but not the monocyclic amines may have a carcinogenic potential. However, this did not hold true when it was detected that occupational exposure to 4-chloro-o-toluidine produces bladder tumors in workers. o-Toluidine had also to be classified as a carcinogen and eventually the experimental results with aniline were also not in line with this hypothesis. With each of a great variety of monocyclic aromatic amines N-hydroxylamines are metabolically formed under suitable conditions and reactions with DNA and mutagenic activity can be demonstrated (4). No criteria can as yet be defined, which allows to separate genotoxic from non-genotoxic or carcinogenic from non-carcinogenic monocyclic arylamines.

The role of genotoxicity has perhaps been overestimated. Not only with aromatic amines but also with other genotoxic carcinogens, the extent to which DNA lesions and initiated cells are formed does not necessarily correlate with carcinogenic potency. This was particularly evident when aniline and structurally related amines were recently compared (30). The discussion focused for a long time on the question: is aniline a genotoxic carcinogen, and if not, should it be classified at all as a carcinogen (IARC, limited evidence)? Tests for mutagenicity gave contradictory results and, because of the low genotoxic potency, the results were considered to be insufficient to explain the spleen tumors observed in rats (31, 32). It was concluded that these tumors must be caused by a non-genotoxic mechanism, with the possibility to establish a NOAEL (or “threshold” (33)). It was hypothesized that, with increasing dose, more damaged erythrocytes are eliminated in the spleen, which causes vascular congestion, pericapsular inflammation, fibrosis and eventually sarcoma and angiosarcoma of the spleen. This would represent a typical high-dose phenomenon. In addition, the argument used against classifying aniline as a suspected carcinogen was that spleen tumors in male rats may not be a relevant model for human risk,

The process of erythrocyte damage begins with N-oxidation of aniline to N-phenylhydroxylamine, in the liver. In the erythrocytes, phenylhydroxylamine is then co-oxidized to nitrosobenzene, and Fe2+-hemoglobin to Fe3+-methemoglobin (metHb). MetHb does not bind oxygen, and hypoxia develops. Both of these reactions are metabolically reversible: nitrosobenzene may be reduced back to phenylhydroxylamine and Fe3+ to Fe2+. This regeneration process depends largely on the availability of reduced glutathione, which keeps circulating metHb at a tolerable level. In the workplace, only blood metHb levels above 5% are considered to be adverse. Khan *et al.* suggested that detrimental effects occur only when the degradation of erythrocytes in the spleen is overloaded (34). One possible mechanism is that the membranes of damaged erythrocytes become less fluid, and (as with senescent erythrocytes) the cells are sequestered and degraded by the spleen. During this process, iron is released, which could activate oxygen and lead to DNA damage (35). This would constitute an indirect genotoxic mechanism. At the same time, lipids and proteins are oxidized and heme is excessively degraded. All these reactions contribute to cytotoxicity. Although some iron is already released within the erythrocytes during metHb formation, the intravasal degradation is not thought to play a significant role (36).

The example of aniline shows how intimately genotoxic and non-genotoxic effects may be interrelated and that analysis of genotoxicity alone will not answer the question of human health risk. Is it now possible to close the discussion and decide whether aniline has carcinogenic potential? Or, more precisely, can a threshold be defined, below which aniline does not contribute to carcinogenic risk? First of all, when metabolic activation and bioavailability of reactive metabolites are used as an end point, a no-effect level (NEL) was not reached at low doses in a 4-week study in male rats (37). It was therefore concluded that any exposure to aniline contributes to a background of metHb formation. A variety of endogenous and exogenous chemicals make up this background; other aromatic amines are particularly involved. In addition to metHb formation, erythrocytes are damaged by reactive metabolites which react with proteins and membranes. Nitrosobenzene, for instance, reacts with the SH-groups of cysteine in the ß-chain of hemoglobin. A stable sulfinamide-adduct is formed, which has been used as a biomarker of effect (38, 39, 40).

In summary, a number of toxic pathways contribute to the toxicity of aniline and genotoxic effects cannot be excluded. Acute toxic and genotoxic effects work together, and it seems that this pattern applies to aromatic amines in general. With aniline and other monocyclic amines, the promoting toxic effects may be more important for tumor production than with polycyclic amines, but the results support the conclusion that a common mode of action exists for aromatic amines. Consequently, any quantitative considerations have to take into account additive and synergistic effects for most steps in this process of tumor formation.

3.4. The role of aromatic nitro compounds

Aromatic nitro-compounds must be included with aromatic amines as members of a larger group of chemicals, whose correct collective name is ”N-substituted aryl compounds”. Reducing the nitro group yields the same N-hydroxylamine as does oxidizing the amine group. However, the reactions may take place at different locations. Nitro groups are reduced to nitroso derivatives primarily in the reductive environment of the intestine, whereas amines are oxidized predominantly in the liver. The ultimate metabolites may therefore be distributed differently. Johnson and Cornish studied the conversion of 1- and 2-nitronaphthalene to 1- and 2-aminonaphthalene in rats in 1978 (41). It is interesting to compare corresponding pairs of amino and nitro compounds, such as aniline and nitrobenzene (30, 42). The reactive metabolites - phenylhydroxylamine and nitrosobenzene - are identical, but the location of tumors in the rat is different. Aniline causes sarcomas, predominantly in the spleen, whereas nitrobenzene produces liver adenomas and carcinomas.

Both agents produce metHb (acutely) and anemia (chronically), and liver and kidney damage in the rat and mouse. Aniline and nitrobenzene were assigned the same biological tolerance value in the list of MAK and BAT values (43).

Aromatic nitro-compounds are ubiquitously present in the environment as combustion products. Wherever organic material is combusted, not only polycyclic aromatic hydrocarbons, but (in the presence of nitrogen) also polycyclic aromatic nitro-compounds are formed (44). Any risk estimate from exposure to an individual amine alone may be erroneous, unless the background of this amine and related amines, as well as the corresponding nitro arenes, are considered.

3.5. Mode of action

The concept of metabolic activation to ultimate reactive metabolites which react with cellular macromolecules was clearly supported by the finding that certain DNA-adducts are pro-mutagenic lesions. c-H-ras, the first oncogene that has been found in normal mouse liver as well as in mouse liver tumors, could be activated by a point mutation caused by the adduct that is formed by the reaction of the reactive 2-aminofluorene (AF) metabolite with the C-8-position of guanine (guanine-C-8-AF) (45). This activation was considered an early effect in the development of liver tumors in the mouse (46). Since that discovery, many more proto-oncogenes (which are activated in carcinogenesis) and tumor-suppressor genes (which are inactivated) have been identified. Do carcinogenic amines produce specific DNA lesions affecting these genes? Although aromatic amines produce typical DNA adducts, this is not tissue specific. Genotoxic carcinogens often produce mutations in codons, 12, 13 and 61 of the H-ras gene, and the corresponding mutations were found in different tissues, such as human lung and colon. However, the pattern of mutations is too different to establish a clear cause-effect relationship. For instance, it is not known why mutations of the H-ras-gene are seen in mouse liver tumors, but not in rat liver tumors (47). These mutations seems to represent tumor-initiating lesions in mouse skin and liver, and also in rat mammary tissue (48, 49).

Similar but distinguishable mutation profiles were seen with other amines, such as aminobiphenyl (ABP), 2-aminoanthracene and PhIP (2-amino-1-methyl-6-phenylimid-azo[4,5-b]pyridine) in the lacZ reversion assay (50). On the other hand, mutations in the ras oncogene from 2-acetylaminofluorene (AAF)-induced mouse lung and liver tumors were more specific than those from spontaneously occurring tumors in these tissues (51). Although both AAF and ABP produce the same type of DNA adduct, i.e., dG-C8-AF and dG-C8-ABP, the pattern of mutations is different. AF induces frameshift and base-substitution mutations (G-T transversions), but ABP only induces base-substitution mutations (G-A transversions). The mutagenic efficiency per adduct is greater for AF than for ABP (52) The dG-8-ABP adducts have been identified in human bladder-tumors (53), in bladder epithelial cells (54), in exfoliated bladder epithelial cells (55), and in many other human tissues, such as the mammary gland (56). The adduct levels correlate positively with cigarette smoking, type of tobacco, and slow acetylator phenotype. Do these results reflect bladder-specific or amine-specific effects?

Additional mechanisms have been suggested recently. Bladder cancer, for instance, is now proposed to be the result of gross chromosome aberrations rather than point mutations (57). Cells carrying chromosome instability (CIN) and microsatellite instability (MIN) have a selective growth advantage. Exposure to specific carcinogens can select for tumor cells with distinct forms of genetic instability (58).

Benigni and Pino (59) studied the tumor profiles of 536 rodent carcinogens in the experimental systems usually employed (rat, mouse; male, female). Aromatic amines and nitro arenes were among the classes most strongly represented in the database. The authors come to the conclusion that no obvious association exists between chemical/mode of action class and tumor profile. It appears, rather, that each class produces tumors at a wide range of sites. They suggest that differences in tumor profile depend on secondary events, which relate to the ultimate mechanism of reaction with DNA. Benigni and Passerini (60) evaluated several QSAR-models and concluded that the gradation of potency of aromatic amines depends, first, on their hydrophobicity, and second on electronic (reactivity, propensity to be metabolically transformed) and steric properties. So far, no endpoint has been discovered, which allows one to predict carcinogenicity or the potency of an aromatic amine. However, the models help to verify the proposed mode of action and in fact, supports it.

Obviously there are compound-specific effects, which can not yet be explained, but there are also enough corresponding findings to support the idea of a common mode of action for aromatic amines. The key steps outlined above are basically the same (61), as are metabolic activation, the kind of genotoxic lesions and, in many cases, an analogous pattern of target tissues - such as the generation of bladder tumors. When and where a tumor grows depends on the interaction of the chemical or its biologically active metabolites with a highly adaptable organism.

3.6. The role of toxicity

Genotoxicity is not the only biological effect of aromatic amines, as we have seen with aniline. A single mutation is not sufficient to generate a tumor, but two or three such critical lesions in combination are presumed to be able to control the multistep process of tumor formation (62). In case of large bowel tumors, up to eight irreversible alterations have been postulated. The underlying paradigm is that a genotoxic chemical, such as an aromatic amine, can transform a normal cell into a tumor cell, which gains increasing growth advantage and ultimately grows to a tumor. Numerous types of genotoxic lesions may contribute, chromosome instability included. All the knowledge about the spectrum of DNA lesions which are formed upon the administration of a single carcinogen as an initiator has not yet helped to identify the critical lesions

that predispose a cell to the development of neoplasia (63). Despite many positive correlations between genotoxic lesions and species and tissue specific effects, genotoxic effects are now considered to be necessary but not sufficient to explain the process of tumor formation. From early on, observations were communicated, which indicated that preneoplastic lesions in rat liver were only seen when carcinogen treatment produced a proliferative stimulus, partial hepatectomy being one of the possible triggers (64, 65, 66).

A well known example for non-correlation of genotoxicity and tumor formation came from a dose-response study, the so called "mega-mouse" experiment. Chronic administration of AAF to BALB/c mice produced liver as well as bladder tumors. The level of the typical guanine-C-8-AF DNA adduct (considered to be the relevant lesion) increased linearly with dose, in both tissues. The effective dose was even higher in bladders than in livers. Tumor incidence, however, increased linearly only in livers, starting at the level of spontaneous liver tumors. Despite the higher adduct levels in bladder, tumor incidence increased in this tissue steeply and non-linearly, only at higher doses. This increase was associated with an increase in cell proliferation. This means that, independent of the significantly higher DNA damage in the bladder, tumors grew only when cell proliferation was stimulated by the carcinogenic agent. In a corresponding experiment with ABP, bladder tumors were also obtained only when cell proliferation was increased. Adduct levels were 2 to 3 times higher in bladder than in liver. In this case, the rate of occurrence of liver tumors was rather low, which was explained by an increased formation of the transport from N-hydroxy-4-aminobiphenyl-N-glucuronide in the liver, which led to lower exposures in the liver and higher exposures to the reactive metabolite in the bladder, where the glucuronide is hydrolyzed. This example shows how pharmacokinetics can modify the genotoxic effect and how toxicity may determine tissue specificity (67).

In another revealing experiment, the carcinogenic effects of three polycyclic aromatic amides were compared: trans-4-acetylaminostilbene (AAS), 2-acetylaminophenanthrene (AAP), and 2-acetylaminofluorene (AAF). All three agents produced initiated, i.e., promotable cells in rat liver, but only one of them (AAF) produced liver tumors and therefore, is a complete carcinogen for this tissue. A fundamental difference between the three agents is that only the complete carcinogen is hepatotoxic. In this case, the adverse effect could be identified on the molecular level as non-genotoxic. AAF metabolites specifically uncouple the mitochondrial respiratory chain by abstracting electrons, an effect which opens the mitochondrial transition pore and interferes with the regulation of apoptosis (47). Inhibition of apoptosis may help damaged cells to escape cell death and acquire a tumorigenic phenotype (68). This could be interpreted as an indirect genotoxic mechanism, but experimental results allow one to explain the finding differently (cf. below).

These observations support the hypothesis that two different properties are required to make an aromatic amine a carcinogen for a target tissue: it must be both mutagenic and cytotoxic. Other end points, such as progressive loss of histone H4 lysine 20 trimethylation, and increased histone H3 serine 10 phosphorylation, which were detected in rat liver, but not kidneys and spleen, indicate also the importance of epigenetic changes in carcinogenesis (69).

Among the first authors who proposed a role for toxicity were Radomski *et al.* (70). 1-Naphthylamine (1-NA), in contrast to the 2-isomer, was considered to be non-carcinogenic and the rat resistant to the formation of bladder tumors. When the isomeric N-hydroxylamines (NOH-N) and the nitroso-derivatives NO-N) of the two isomers were tested directly by i.p. injection in rats, both oxidation products produced tumors (fibromas, fibrosarcoma and lymphosarcomas), but they also turned out to be hepatotoxic, such that the survival time was significantly reduced. Both 1-NOH-N and 1-NO-N were more carcinogenic than the 2-isomers, and both gave the same type of tumors. When administered to newborn mice, it was the other way around: 2-NOH-N was more carcinogenic than 1-NOH-N, and 2-NOH-N more efficient than 2-NO-N. The original testing of the amines for carcinogenicity was evidently insufficient and both isomers have a carcinogenic potential under suitable conditions. It also shows that the rat is not completely resistant to oral doses of 2-NA (71). Toxicity has strongly influenced the outcome of the test results.

The promoting effects of AAF have often been used in models of carcinogenicity testing and undefined toxicity was held to be responsible for this effect (72). Recent experiments explain this toxicity on the molecular level. In case of AAF, electrons are sequestered from the respiratory chain and changes in membrane polarity are transmitted to the mitochondrial transition pore, which controls apoptosis (73).

The cell´s adaptation program tries to normalize the situation. The threshold for the elimination of hepatocytes is elevated by opening of the transition pore. However, eventually, this mechanism is overwhelmed. Cells are lost and reparative substitution begins. In the stress situation, bile duct cells are increasingly produced, instead of hepatocytes, and a cirrhosis-like condition develops. Initiated cells begin to proliferate only when this stage is reached. In other words, the toxic effects create the environment in which the tumor develops.

It is important to realize that each of the regulatory signals may depend differently on dose and time, and it is therefore critical to assess relevant biomarkers. Other biochemical equilibria are also likely to be influenced.

3.6.1. Conclusions

Carcinogenic N-substituted aryl compounds are present not only at many workplaces but also in the general environment. If suitable conditions are chosen, it is possible to demonstrate, with practically all of them, the formation of corresponding ultimate metabolites, their reactions with

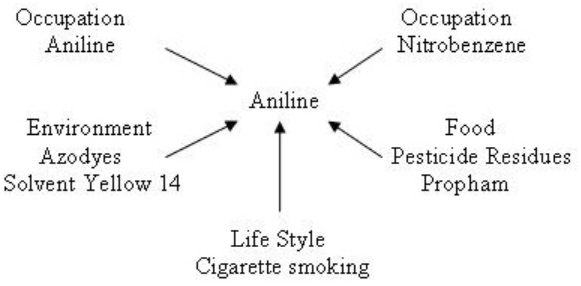

Figure 3. Various sources, which may contribute to aniline background exposure.

DNA, RNA and proteins, mutagenic activity, the formation of metHb, and other acute toxic effects. Only in a few cases, so far, was it possible to prove a causal relationship in humans and therefore to classify a particular amine in IARC category 1. However, is it necessary or even desirable to ask for positive animal tests to classify an aromatic amine in category IARC 2A, in order to conclude that it is probably carcinogenic in humans? What kind of information would be necessary to label an agent as hazardous or not hazardous to humans?

It appears to be impossible to exclude any suspicion of a carcinogenic potential for aromatic amines. Is it then possible to approach the problem of regulation by estimating the risk? A kind of relative risk is obtained if the agents are arranged according to their potency. However, eventually it is necessary to establish limit values. Traditionally, the risk associated with a certain exposure is calculated from animal experiments. If dose extrapolations, species differences and individual sensitivity with the respective defaults are considered, a point estimate of risk results, but with great uncertainty. This estimate does not yet include information of background exposures to this and related amines and nitro-compounds, which makes it even less certain (Figure 3). Moreover, irreversible genotoxic and reversible toxic effects of the agent both influence the process of tumor formation, with different dose-response relationships, which makes a limit value even more vulnerable to species and individual differences.

3.7. From hazard to risk

3.7.1. A new look at quantification

The classification of chemicals in one of the cancer categories reflects the weight of evidence to prove carcinogenic potential, but it does not imply any quantitative assessment of risk. Since genotoxicity is an essential property of carcinogenic aromatic amines, they fall info the class of non-threshold carcinogens. This means that a no-effect level cannot be scientifically established, and no MAK value can be assigned. However, quantitative assessment of potency is urgently needed for regulatory purposes, but all approaches to determine limit values for acceptable exposures are so far unsatisfying.

Typically, one either looks for a threshold, below which the carcinogen is ineffective, or the tumor incidence per dose is calculated from animal experiments using safety factors to account for dose dependence, species and individual variability. The exposure that leads to a tumor incidence of 10-5 or 10-6 is then declared to be acceptable. Such a point estimate is very uncertain and should be used only if the degree of uncertainty can be expressed.

Another approach calculates a unit risk that means additional cancer cases at a certain exposure (e.g., 1 microgram/m3 in the environmental air for 70 years). In practice, this requires animal data, the extrapolation to very low concentrations of the agent and the use of defaults. This calculation also yields very uncertain values.

As a new quantitative aspect to treat classified carcinogens, the term “non-appreciable contribution to risk” was introduced. Two new categories (4 and 5) were added to the classification scheme of carcinogens in the list of MAK and BAT values. In these two categories, carcinogens are listed for which an acceptable exposure, i.e., a MAK value, can be established. However, to account for the prevailing mode of action, non-genotoxic carcinogens are treated differently from genotoxic carcinogens. Tumor promoters, for instance, which show non-linear dose-response relationships, are assigned to category 4. “Substances with carcinogenic potential for which a non-genotoxic mode of action is of prime importance and genotoxic effects play no or at most a minor part provided the MAK and BAT values are observed. Under these conditions, no significant contribution to human cancer risk is expected.” Category 5 lists “Substances with carcinogenic and genotoxic effects, the potency of which is considered to be so low that, provided the MAK and BAT values are observed, no significant contribution to human cancer risk is to be expected.”(2, 74). These definitions account for the likelihood that pure tumor-initiating and pure tumor-promoting carcinogens will not exist. All of these approaches contain an element of evaluation, which does not mean scientific proof.

3.7.2. Aniline and the role of biomarkers

These new categories are particularly useful for aromatic amines, which produce tumors only if the genotoxic, tumor-initiating effects are supplemented by cell-proliferation stimulating, tumor-promoting effects. It is therefore necessary to evaluate the prevailing role case by case.

Aniline is a good example. DNA damage is expected to contribute little to the carcinogenic potency, whereas the diverse toxic effects are decisive. Before aniline was classified as a carcinogen, a MAK value hasdbeen assigned, which was based on the acute toxic effects with metHb as the end point, and the experience that health effects in humans are observed only with metHb levels in blood greater than 5%. Since aniline (or nitrobenzene) occurs ubiquitously, it contributes to the existing metHb background. This biomarker accounts for all the other metHb-forming chemicals. A reference value is in the range of 2-3%, which is exceeded at the workplace only with aniline concentrations greater than 2 ml/m3. Since the experimental results, particularly considering the behavior of structurally related monocyclic amines, did not

disprove a carcinogenic potential, aniline was assigned to category 4, with MAK value = 2 ml/m3.

To apply this assessment in practice, it is particularly important to have a good biomarker, to obtain information about the background and its potential increase. The Hb-adduct resulting from the reaction of nitrosobenzene with hemoglobin is such a biomarker. Corresponding to the MAK value, a BAT-value of 100 microgram aniline/L blood released from aniline-hemoglobin adduct is used as a limit value.

This protein adduct can be measured in blood samples and used as a biomarker of exposure and effect. It indicates that the N-hydroxylamine (or the nitroso-derivative) is distributed throughout the organism and, in agreement with the general experience, is available in most, if not all, tissues. Transplacental exposure during the pregnancy of smoking mothers, for instance, has been demonstrated (75).

The blood concentration of metHb is rather time-dependent, which means that it describes the momentary situation when the sample was taken. In contrast, the Hb adduct represents the average exposure over the prior 120 days – the life time of human erythrocytes. The advantage is that this biomarker can be measured in human individuals and accounts for the total uptake from breathing the air, eating contaminated food, absorption through the skin, etc., and measures the bioavailability of the reactive metabolite. Reference values can be obtained for a defined population. If workers are occupationally exposed to aniline in the workplace and if the blood level of the biomarker stays within the standard deviation of the background or reference value, then the occupational exposure is considered not to contribute significantly to the background risk. The term "significantly different" in the definition of category 4 and 5 must not be taken strictly statistically. It may rather be used in the sense of "appreciably different" from the background and may leave some room for a case-to-case evaluation.

3.7.3. The ALARA principle

The experience with Hb adducts as a biomarker clearly demonstrates that the general population is exposed to many monocyclic and polycyclic aromatic amines (76, 77). The biomonitoring results also make clear that acute toxicity follows the same principles in humans and in experimental animals. Moreover, Hb adduct levels correlate well with the formation of dG-C8-adducts,, which are considered closer to the genotoxic effects, but not necessarily to the carcinogenic potency (51, 78)

Usually, properties of a particular chemical are characterized by results obtained under controlled, predominantly experimental conditions. Any risk calculated on this basis neglects two modifying unknowns: the existence of background exposure to the chemical under consideration and the simultaneous presence of structurally related aromatic amines. Since the effects with numerous amines should be additive, or even synergistic, this "monocausal" approach is highly questionable and the use of calculated absolute risks may not mean very much in real life.

In this situation, it appears to be almost inevitable to ask for alternatives and to refer to the ALARA principle, which recommends that the exposure to carcinogens should be "As Low As Reasonably Achievable". It is important to emphasize that this does not mean that only zero exposures may be acceptable, but a different approach to the problem is necessary. Acceptable exposures should be based on human data, without making default assumptions. The question should not be, how high is the risk associated with the exposure to a particular agent, but how much does it contribute to the risk? Rather than endless discussions about zero tolerance based on non-existent thresholds, information about avoidable and unavoidable exposures will help to decide what can reasonably be achieved (79). Biomarkers of effect can be used to define background exposures. If, in a given situation (for instance, at the workplace), the exposure to hazardous chemicals is controlled, the biomarker may indicate that the level had increased. If background data, or (even better) reference values exist, the measurement shows whether these parameters have been exceeded. An advantage is that the individual can serve as his or her own control. Sensitive individuals may reach the upper bound of the reference value at lower exposures and are therefore equally well protected.

4. SUMMARY AND PERSPECTIVES

The biological effects of aromatic amines are all related to one structural element, the amino group attached to an aromatic system. This amino group is metabolically activated and the reactive metabolites react with (among others) proteins and nucleic acids. The reaction with DNA produces pro-mutagenic lesions, and the resulting mutations are considered to be essential for the formation of tumors. These lesions are irreversible and they accumulate. The dose-response relationship of DNA damage is linear down to concentrations in the range of background exposures and a no-effect level cannot be defined. That is the reason why limit values were not assigned to genotoxic carcinogens.

To assess the mode of action and the carcinogenic potency, it has now become mandatory also to analyze the toxic properties of these carcinogens. This area has been neglected in the past and only a few mechanisms can be explained on the molecular level. As the understanding of the complex network of cellular regulation improves, it is expected that any uptake of an exogenous chemical interferes with the balance of various physiological endpoints, and it is difficult to identify the critical pathway. Most likely, more than one is relevant. It is also difficult to predict when and where adaptive reactions are exhausted and the effect becomes adverse. As demonstrated with some typical tumor promoters, non-linear dose-response relationships of reversible effects occur and the points of deviation from linearity have been used to derive "no observable

adverse effect" levels (NOAEL) or a no-effect level (NEL). However, such values cannot be translated into acceptable exposures under real-life conditions, where isolated exposure to one particular compound in the low-dose range obviously does not occur. The same chemical may come from different sources, which contribute to the background, as well as most, if not all, the structurally related amines, which compete for the same physiological endpoints. In biomonitoring studies, it has been shown that many aromatic amines are present simultaneously in the environment. In addition, structurally unrelated chemicals may add to the chemical stress, if common targets are involved. Examples include hemoglobin in erythrocytes, mitochondrial respiration, and stress of the defense capacity (for instance, by depletion of reduced glutathione).

The more we learn about the role of signaling networks, the less confidently can we trust the mono-causal approach to evaluate the potency of chemicals. What we see in our experiments is caused by high doses in rather homogeneous populations. What we do not see, is the interaction of the chemical with the many reactions of the organism to adapt to chemical stress at low doses.

We may accept that the whole group of aromatic amines is characterized by a common mode of action, which consists of the same metabolic activation pathways, the role of mutagenic and acute toxic effects, and the interaction with the signaling network. Consequently, we must acknowledge that it will not be possible to define an acceptable exposure by a fixed limit value, which describes a risk that is then declared to be acceptable. The ALARA principle indicates a way out, by changing the perspective from "risk" to "contribution to risk." The ALARA principle does not pretend to have a scientific answer to the question, What exposure is acceptable? But it asks the regulator to discuss why he or she considered the decision to be reasonable, and to explain the extent to which scientific data and expert judgment were involved in reaching this decision. This concept may be a promising possibility to link scientific risk assessment and risk management.

5. ACKNOWLEDGEMENTS

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Abbreviations: ALARA: as low as reasonably achievable, BAT: biological tolerance values at the work place, DFG: Deutsche Forschungsgemeinschaft, IARC: international agency of research on cancer, MAK: maximale Arbeitsplatzkonzentration (maximum concentration at the workplace), NEL: no effect level, NOAEL: no observable adverse effect level

Key Words: ALARA Principle, Aromatic Amines, Carcinogenic Potency, Dose-Response Relationships, Hazard, Nitro Arenes, Threshold, Risk Assessment, Review

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