Determining the Potential of Aromatic Amines to Induce Cancer of the Urinary Bladder

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Aromatic amines have been associated with the induction of cancer of the urinary bladder. Commercial production started over 100 years ago in Europe, with the synthesis of a mauve pigment from aniline. The discovery of other pigments by combining aniline with various chemicals initiated the aniline dye industry. By the turn of the century, a correlation between working in the dyestuff industry and the development of bladder cancer was established. Initially thought to be the result of exposure to aniline, various investigators identified benzidine, beta-naphthylamine, and 4-aminobiphenyl as the causative agents. Evaluations of various aromatic amines in rats, mice, guinea pigs, rabbits, dogs, and monkeys showed significant species differences, with the dog and monkey being the most sensitive species. Several laboratories related these species differences to differences in the respective routes by which the various species metabolized aromatic amines. Excellent correspondence was shown between metabolic activation of benzidine, 4-aminobiphenyl, and beta-naphthylamine in dogs and primates and the induction of bladder cancer. Rodents were shown to be unresponsive to human bladder carcinogens. The need to use data developed in the most sensitive species, the dog, is essential to accurately predict the carcinogenic potential of aromatic amines.

Health hazards associated with workplace exposure to chemicals was first systematically described in Bernardino Ramazzini’s *Discourse on the Diseases of Workers* published in 1700. Considered by many to be the father of occupational medicine, Ramazzini described adverse health effects associated with mining, printing, potting, and other industries. A clear relationship between occupation and cancer was described by Percivall Pott, an eminent English physician who in 1775 reported the association between chimney sweeps and cancer of the scrotum. Lung cancer in uranium miners, lung cancer in the asbestos industry, and liver cancer from vinyl chloride monomer are examples of other occupationally related cancers first identified in humans and subsequently confirmed in animal tests.

The induction of bladder cancer by certain aromatic amines was also first observed in the workplace. Commercial production of aromatic amines began in the mid 19th century. In 1856, William Henry Perkin attempted to make quinine from impure aniline sulfate, but instead obtained a dark material which he discovered could dye silk a bright mauve. Asked to make large quantities by a dye company, Perkin established a business which was the start of the aniline dye industry (Perkin 1856). His aniline was made by the reduction of crude nitrobenzene and contained many impurities. It was later shown that it was the impurities in the crude aniline that created the desired color. Perkin’s “meuveine” was used for many years to dye textiles and paper.

Perkin’s profitable discovery stimulated experimentation with aniline. Soon, Natansen in Poland produced a new purple dye by reacting aniline and ethylene dichloride. In Germany, Hofmann created a similar pigment with aniline and carbon tetrachloride. In France, Freres combined stannic chloride and aniline to obtain a purple dye and commercialized his process. He named his dye magenta to commemorate the French victory in the Battle of Magenta in 1859. At about the same time, German chemists began manufacturing a dye which they called fuchsin. As with Perkin’s mauveine, the shade of fuchsin produced depended on the impurities in the aniline used. Impurities that were important for color formation include the toluidines and xylidines. Crude aniline that was used in the manufacture of dyestuff also contained 4-aminobiphenyl (Hofmann 1862) and beta-naphthylamine (Jackson 1875). Commercial production of benzidine began about 1853. It was used in the production of several azo dyes, including Palatine Orange and Congo Red. At that time the human health hazards associated with aromatic amines were not recognized.

In 1895, Rehn, a German surgeon, reported bladder cancer in three of his patients, all of whom worked in the same factory, in the “fuchsin room.” Rehn’s fourth bladder cancer patient worked at another factory that also manufactured fuchsin. Rehn examined the manufacturing process and concluded that...
the cancers were the result of inhaling aniline vapor. Because the tumors were attributed to aniline exposure, bladder cancers in the dyestuff industry were referred to as “aniline cancers.” In 1898, about 18 years after beta-naphthylamine production began in Germany, Leichtenstern reported a correlation between beta-naphthylamine exposure and bladder tumors. A 1904 report by Rehn described an additional 20 cases of bladder tumors, all arising in workers involved in the manufacture of aniline, fuchsine, benzidine, and naphthylamine. In 1920, a German physician, Schwerin, reported personally diagnosing 38 cases of occupationally related bladder cancer, confirming Rehn’s conclusion that bladder tumors were directly related to exposure to crude aniline. After World War I, the dyestuff industry spread from Germany to many other countries, as did the induction of bladder tumors in the workforce.

Although aniline had been thought to be a urinary bladder carcinogen, this could not be confirmed in animal studies. In experiments by Yamazaki and Sato (1937), Berenblum and Bonser (1937), Goldblatt (1947), Gehrmann, Foulger, and Fleming (1949), and others, aniline failed to induce bladder tumors in the various animal species in which it was tested. A thorough, well-documented epidemiology study by Case et al. (1954) of workers handling aniline for many years showed no increase in bladder tumors. A second epidemiology study, by Evans et al. (1937) found no bladder tumors in 65 workers exposed to aniline for 20 years. In addition to these reports, studies by Barsotti (Barsotti and Vigliani 1949), Maguigan (1950), Scott (1962), Vigliani (Vigliani and Barsotti 1962), DiMaio (1937), Druckrey (1950), and Gehrmann, Foulger, and Fleming (1949) concluded, as the result of industrial experience and their examination of workers exposed to aniline, but not exposed to known carcinogenic amines, that pure aniline did not present a carcinogenic risk in the workplace.

By the early 1950s, there was clear evidence that dyestuff workers experienced a relatively high incidence of bladder cancer and all indications pointed to the aromatic amines used in the production processes. Other occupations that used aromatic amines also showed an increased incidence of bladder cancer. This included workers that manufactured rubber using beta-naphthylamine (McMichael, Andjelkovic, and Tyroler 1976), medical technicians and nurses that used benzidine to test for occult blood (Holland et al. 1974), and tire remolders who were exposed to aromatic amine vapors when the tires were heated (Garner, Martin, and Clayson 1984). In addition, patients that received beta-naphthylamine mustard as a treatment for polycythemia died of bladder cancer 2 to 11 years after beginning of treatment (Thiede and Christensen 1969).

IDENTIFICATION OF CARCINOGENIC AROMATIC AMINES

The large epidemiology study conducted by Case et al. (1954) correlated the incidence of bladder cancer in the British chemical industry to aromatic amine exposure that occurred between 1921 and 1950. The authors examined the work history of about 4000 men who had handled aniline, benzidine, beta-naphthylamine, and alpha-naphthylamine. Case et al. confirmed that workplace exposure to aniline did not result in an increased incidence of bladder cancer. They did show a significantly higher tumor incidence in workers exposed to benzidine and beta-naphthylamine. The small amount of the beta isomer in alpha-naphthylamine was thought to be responsible for the slightly higher incidence of tumors observed in the group exposed to alpha-naphthylamine.

Several reports published in the 1930s described a correlation between workplace exposure to beta-naphthylamine and bladder tumors. Soon after, evidence began to accumulate, indicting benzidine as a human bladder carcinogen. In 1955, Melick et al. reported the induction of bladder tumors in workers handling 4-aminobiphenyl and determined that the lag time (time between exposure and tumor onset) was between 5 and 19 years. After bladder tumors were observed in workers handling these three aromatic amines, animal tests confirmed their carcinogenic activity. Although Perlmann and Staehle (1932) described a study in which they injected rabbits with beta-naphthylamine and observed bladder tumors in 6 of 31 animals, Berenblum and Bonser (1937) tried but failed to confirm these results when they injected rabbits and rats for many months. In this study and in several other studies, both rabbits and rats were shown to be a poor choice as an animal surrogate for evaluating the carcinogenic potential of aromatic amines because they gave inconsistent results.

Hueper, Wiley, and Wolfe in 1938 reported the induction of bladder tumors in dogs with beta-naphthylamine. This was successfully repeated by Bonser in 1943 and by Poole-Wilson in 1953. These and other studies showed the dog to be the most suitable animal species for assessing the carcinogenic potential of beta-naphthylamine. Diagnostic pathology confirmed that the bladder tumors observed in dogs closely resembled the bladder tumors seen in dyeworkers. Other aromatic amines that induce human bladder cancer, including benzidine and 4-amino-biphenyl, have been shown to consistently produce bladder tumors in dogs.

Rodents have also been used to assess the carcinogenic potential of benzidine, 4-aminobiphenyl, and acetylaminofluorene. Boyland et al. (1964), Clayson, Lawson, and Pringle (1967), Bonser et al. (1956), and others have shown that rodents used in tests of aromatic amines known to cause bladder cancer in humans (1) give inconsistent results, (2) usually do not develop bladder tumors, (3) require relatively high doses for long periods, (4) primarily develop liver, lung, and mammary gland tumors, and (5) show a significant resistance to this class of carcinogens as compared to dogs and humans. These studies confirm that rodents are an inappropriate animal model for determining the carcinogenic potential of aromatic amines. In addition, rodents can provide misleading information. For example, although 4,4’-methylene-bis(2-methylaniline) caused mammary and lung tumors in rats, there is no evidence that it causes bladder cancer in any other species (Stula et al. 1975).
IDENTIFICATION OF AN APPROPRIATE ANIMAL MODEL

In 1952, Walpole, Williams, and Roberts suggested that the “aniline tumors” reported by Rehn and others were caused by the impurities in crude aniline. They tested two of the impurities, 4-aminobiphenyl and 3,2'-dimethyl-4-aminobiphenyl, in rats. A few of the rats developed tumors of the mammary gland, salivary gland, liver, and intestine, but no tumors were found in the urinary bladder. In contrast, urinary bladder tumors were induced by both chemicals in dogs. In 1954, Walpole, Williams, and Roberts reported that 4-aminobiphenyl induced bladder tumors in dogs at relatively low doses (5 to 20 mg/kg/day) in less than 3 years. In contrast to the rodent studies, in dogs, 4-aminobiphenyl did not cause tumors in the liver, lungs, mammary glands, or in the many other tissues examined.

Deichmann administered 4-aminobiphenyl to dogs at doses as low as 1 mg/kg/day and observed bladder tumors in less than 3 years (Deichmann et al., 1965). The studies by Deichmann and by Walpole showed that bladder tumors induced in dogs by benzidine, beta-naphthylamine, and 4-aminobiphenyl are histologically very similar to the bladder tumors found in humans. Subsequent studies have further confirmed the dog as the most appropriate animal model for testing the carcinogenic potential of aromatic amines. Recognizing that the dog is the most sensitive animal species with regard to bladder carcinogens and that it responds most like humans, Deichmann determined the relative potency of benzidine, 4-aminobiphenyl, and beta-naphthylamine in dogs by testing the three carcinogens simultaneously. Comparing dose, time to onset of tumor (“lag time”), and tumor incidence as measures of carcinogenic potency, the study showed 4-aminobiphenyl to be the most potent, followed by beta-naphthylamine and then benzidine. In contrast to the results obtained in dog studies with these known human carcinogens, other aromatic amines tested, including o-toluidine, m-toluidine, p-toluidine, and aniline did not produce any form of bladder abnormality when administered to dogs (Deichman, 1967; Gehrmann, Foulger, and Fleming, 1949). The cumulative doses of the toluidine isomers given to the dogs in these studies considerably exceeded the doses required to produce bladder tumors in dogs from exposure to 4-aminobiphenyl and betanaphthylamine.

METABOLIC ACTIVATION

By 1938, Hueper, Wiley, and Wolfe had evaluated aromatic amines in five animal species—mice, rats, guinea pigs, rabbits, and dogs—before selecting the dog as the species of choice for testing possible bladder carcinogens. They hypothesized that the dog metabolizes aromatic amines like humans do. Others speculated that the resistance of rodents in developing bladder tumors was because of a difference in the manner in which they metabolized aromatic amines. Early work by James and Elizabeth Miller (1967) and others (Gutmann et al., 1972; Brill and Radomski, 1971) showed that N-hydroxylation is a prerequisite for carcinogenic activity. Aromatic amines that are readily N-hydroxylated, such as benzidine, beta-naphthylamine, and 4-aminobiphenyl are able to induce bladder cancer in dogs and humans (Figure 1). Structural analogs, such as 2,4'-diaminobiphenyl, alphanaphthylamine, and 2-aminobiphenyl that are not easily N-hydroxylated, do not cause bladder cancer (Figure 2). This was confirmed by Radomski and Brill (1970) who showed that although alpha-naphthylamine was poorly hydroxylated and did...
not cause bladder tumors in dogs, synthesized $N$-hydroxy metabolite, when administered to dogs, caused bladder cancer. Not all $N$-hydroxylated amines are carcinogenic, as exemplified by $N$-hydroxy-$N$-fluorenylacetamide.

It was initially unclear as to whether aromatic amines are activated in the liver and then transported to the urinary bladder or were activated upon reaching the bladder mucosa. Several studies have shown that $N$-hydroxylation occurs primarily in the liver. The dog bladder has very low $N$-hydroxylase activity (Poupko, Hearn, and Radomski 1979). In the liver, the $N$-hydroxy metabolites form $N$-glucuronides which are stable at the neutral pH of blood and are transported to the kidney where they are excreted into the urine (Poupko et al. 1979). The mildly acidic urine causes hydrolysis of the glucuronide, freeing the carcinogenic $N$-hydroxy metabolite in the target organ. The $N$-hydroxy amine is further activated by the bladder epithelium to highly reactive nitrenium ions that bind to DNA (Flammang and Kadlubar 1986). This is shown in Figure 3.

In most species, including the rat, mouse, hamster, and human, glucuronidation, acetylation, and oxidation (hydroxylation) are competing reactions. The $N$-acetylation of aromatic amines, which occurs in the liver, is thought to be a detoxification pathway. Acetylation converts the amine to a less reactive amide which is not activated to a carcinogenic metabolite. Because the

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**FIGURE 3**

Metabolic activation of carcinogenic aromatic amines.
dog cannot N-acetylate and thus detoxify aromatic amines, it is the animal species most sensitive to their carcinogenic activity (Lower and Bryan 1973). In rodents, N-hydroxy metabolites are N-acetylated and the N-hydroxy-N-acetylarylamines are thought to be responsible for the liver tumors induced by aromatic amines in rats and mice. Because dog liver is unable to N-acetylate, liver tumors have not been found in dogs treated with carcinogenic aromatic amines. Lower and Bryan showed a relationship between liver tumor induction and N-acetylation by administering carcinogenic N-hydroxy-N-acetyl metabolites to dogs that subsequently developed both bladder tumors and hepatomas.

Humans apparently form very little of the N-hydroxy-N-acetylarylamines and this accounts for the lack of liver tumors in humans exposed to carcinogenic aromatic amines. Liver N-acetyltransferases can act as O-acetyltransferases and convert the N-hydroxy metabolite to the corresponding acetoxy ester. This appears to be a relatively minor metabolic reaction. Acetylation of the primary amine by N-acetyltransferase is the predominant detoxification pathway.

METABOLISM AS A BASIS FOR TEST SPECIES SELECTION

To adequately test a class of chemicals for carcinogenic activity, an understanding as to how laboratory animals and humans metabolize that class of chemicals is highly desirable. It allows for the selection of a test species that activates and detoxifies the chemicals via pathways used by humans. As early as the 1930s, dogs were used to demonstrate the ability of known human bladder carcinogens to induce the same tumor in this species. Similar evaluations in rodents resulted in a variety of cancers, inconsistently induced, but no bladder tumors. In rodents, the target organs in which tumors developed and the tumor incidence therein varied from study to study. Further, certain aromatic amines that induced liver, mammary, and other tumors in rodents failed to cause bladder tumors in dogs. In contrast, dogs consistently developed bladder tumors when administered benzidine, 4-aminobiphenyl, beta-naphthylamine, and other human bladder carcinogens. By 1970, sufficient target organ and tumor incidence data were available to conclude that a significant species difference in the metabolism of aromatic amines must exist.

Dogs and humans N-hydroxylate aromatic amines to form metabolites responsible for the induction of bladder cancer. Humans also N-acetylate the primary amine as a major detoxification pathway. N-glucuronides have also been identified as major metabolites in dogs and humans. Although the N-glucuronide of the N-hydroxy metabolite retains the carcinogenic activity of the hydroxylated amine, the carcinogenic potential of the N-glucuronide of the primary amine (parent compound) remains unclear. Some evidence exists that suggests these glucuronides are also hydrolyzed by urine and the free aromatic amine is hydroxylated (activated) by the bladder urothelium of both dogs and humans. Hydrolysis of these N-glucuronides is rapid in acidic (pH 5-6) urine (Babu et al. 1992; 1996) as found in dogs and humans, as compared to the less acidic urine of rats (Kadlu- bar et al. 1981; Kadlubar 1986). In contrast, the major activation pathways in rodents appear to be N- and O-acetylation (Swaminathan and Hatcher 1992). Rodents also N-acetylate as a detoxification pathway. Therefore, both the relatively lower production of N-glucuronides in rats and the higher urinary pH in this species apparently act together to reduce their susceptibility to develop bladder cancer.

Recognizing the need to use the most appropriate animal species to properly assess the carcinogenic potential of new pharmaceutical agents, a Food and Drug Administration (FDA) panel established in 1971 to define carcinogenicity testing standards recommended that all chemicals having structural similarity to carcinogenic aromatic amines be tested in the dog. The panel supported its recommendation by stating that only the dog develops aromatic amine induced bladder cancer similar to human bladder cancer (FDA 1971).

HUMAN EXPOSURE TO AROMATIC AMINES

Humans continue to be exposed to aromatic amines from a variety of sources. Bryant (1988), Yu (1995), Vineis (1992), and others have shown that tobacco smoke contains 4-aminobiphenyl and other human bladder carcinogens, and that smokers have a significantly increased risk of developing bladder cancer. Several pharmaceutical products in common use contain aromatic amines, including dapsone, procaine, p-aminosalicylic acid, sulfanilimide, and minoxidil. Exposure to aromatic amines also occurs as a result of diet. Some are present as natural components of food, whereas others are formed during the cooking process. Workplace exposure still occurs during the manufacture of dyes, rubber, pharmaceuticals, and a variety of other products. N-acetylation appears to be the primary detoxification mechanism for humans exposed to aromatic amines (Beland and Kadlubar 1986).

A genetic polymorphism for N-acetylation has been identified in humans, making some people more susceptible than others. Clinical studies in the 1950s evaluating the anti tubercu- losis drug isoniazid revealed that the study populations contained both slow and fast acetylators. The rate of acetylation is under genetic control, which determines the level of N-acetyltransferase activity in the liver. N-acetyltransferases are controlled in humans by two genes called NAT1 and NAT2 (Grant et al. 1997; Cascorbi et al. 1999). The products of these genes have very similar amino acid sequences, but they are independently expressed and kinetically distinct. Polymorphism in NAT2 as a result of point mutations in the coding region results in individuals who have mutant alleles and are slow accelerators. NAT1, which has recently also been found to be polymorphic, shows selectivity for compounds such as p-aminosalicylic acid. One NAT1 variant that produces increased enzyme activity and increased carcinogen-DNA adduct binding in bladder has been identified. However, the impact of NAT1 polymorphism is less understood at present than that of NAT2.

There is epidemiological evidence that acetylator phenotype affects an individual’s susceptibility to aromatic amine–induced bladder cancer. Slow acetylators that have a genetic mutation in
the NAT2 gene and thus a significant decrease in the ability to N-acetyltransferase (Grant et al. 1997) appear to be at greater risk when compared to fast acetylators in developing bladder cancer from occupational exposure to aromatic amines and from cigarette smoke (Kaderlik and Kadlubar 1995; Daly et al. 1993). It has been shown that cigarette smoking is the major cause of bladder cancer in men in the United States. Because N-acetylation is the major detoxification pathway for carcinogenic aromatic amines, smokers that are slow acetylators are less able to detoxify aromatic amines and have been shown to have a higher incidence of bladder cancer (Yu et al. 1995).

**DISCUSSION**

In determining the carcinogenic potential of a chemical, it is very important to use the most appropriate animal species. The aromatic amines are one of the most rigorously studied chemical groups, especially with regard to metabolic activation, detoxification, species differences, and the induction of bladder cancer. The scientific literature confirms that in assessing the risk of bladder cancer from exposure to an aromatic amine, data obtained in dogs should far outweigh data from rodent studies. Surprisingly, the results of early rodent studies are still occasionally used as the basis for concluding that an aromatic amine has carcinogenic activity, even when chronic dog data for the chemical shows otherwise.

An epidemiology study conducted by the National Institute for Occupational Safety and Health (NIOSH) concluded that exposure to o-toluidine and aniline caused the bladder cancers observed in a workforce which used 110 other chemicals (Ward et al. 1991). In support of this conclusion, a chronic rodent study was cited, which reported tumors in female rats (National Cancer Institute 1978; 1979). There was no mention of the dog studies conducted by Deichmann and others that showed chronic exposure to o-toluidine or aniline did not result in bladder abnormalities. Also, it is important to note that there are no published reports of bladder cancers in the work force of the manufacturers of these chemicals. A reexamination of the chemicals used 10 to 25 years ago by the cohort examined in the Ward study found the workers had used diphenylamine (Freudenthal and Anderson 1995). In the 1960s and 1970s, diphenylamine was contaminated with significant levels of 4-aminobiphenyl (Safe et al. 1977), considered the most potent of the aromatic amines capable of inducing bladder cancer. Further, in a 1996 letter to NIOSH, the company acknowledged that exposure to 4-aminobiphenyl in the 1960s was the likely cause of the bladder cancers in its workers (Sherman 1996). The latency period in these workers fits the time frame of their exposure to the contaminated diphenylamine. Consideration of the data from the animal model most relevant for predicting human bladder cancer, along with a careful review of company records, would have lead to the conclusion that 4-aminobiphenyl, not o-toluidine or aniline, was the causative agent.

The discovery of bladder cancer in humans from exposure to aromatic amines and the recognition that the dog also develops bladder tumors after exposure to these chemicals has identified the dog as a sensitive animal model for detecting these carcinogens. Metabolism studies have increased our understanding of the activation and deactivation reactions of aromatic amines in rodents, dogs, and humans. They allow us to differentiate between an adequate animal model and inappropriate animal models based on differences in metabolic pathways. The older scientific literature containing the results of chronic dog studies is often less accessible than more recent rodent studies. Nevertheless, a review of the full literature supports the FDA’s conclusion of 1971, that when the disease in question is bladder cancer, dog studies of aromatic amines should be relied upon over rodent studies in predicting effects in humans.

**REFERENCES**


