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Primary aromatic amines and cancer: Novel mechanistic insights using 4-aminobiphenyl as a model carcinogen

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ABSTRACT

Aromatic amines are an important class of human carcinogens found ubiquitously in our environment. It is estimated that 1 in 8 of all known or suspected human carcinogens is or can be converted into an aromatic amine, making the elucidation of their mechanisms of toxicity a top public health priority. Decades of research into aromatic amine carcinogenesis revealed a complex bioactivation process where Phase I and Phase II drug metabolizing enzymes catalyze *N*-oxidation and subsequent conjugation reactions generating the highly electrophilic nitrenium intermediate that reacts with and forms adducts on cellular macromolecules. Although aromatic amine-DNA adducts were believed to be the main driver of cancer formation, several studies have reported a lack of correlation between levels of DNA adducts and tumors. Using genetically modified mouse models, our laboratory and others observed several instances where levels of conventionally measured DNA adducts failed to correlate with liver tumor incidence following exposure to the model aromatic amine procarcinogen 4-aminobiphenyl. In this review we first provide a historical overview of the studies that led to a proposed mechanism of carcinogenesis caused by aromatic amines, where their bioactivation to form DNA adducts represents the central driver of this process. We then highlight recent mechanistic studies using 4-aminobiphenyl that are inconsistent with this mechanism which suggest novel drivers of aromatic amine carcinogenesis.

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1. Introduction and historical perspective

The German physician Rehn first reported a suspected link between dye exposure and bladder cancer as early as 1895 based on his observation that two of his bladder cancer patients had worked in the same dye factory (Dietrich & Dietrich, 2001; Rehn, 1895). This link was further substantiated in 1912 when Leuenberger analyzed all cases of bladder tumors in Basel, Switzerland and reported that approximately one half were from dye factory workers (Leuenberger, 1912). Since aromatic amines were the most commonly used chemicals in the dye industry at the time, these early epidemiological observations hinted at a potential link between aromatic amines and human cancer. The bladder carcinogenicity of aromatic amines in animal models was confirmed in 1937 by Hueper et al., who reliably produced tumors in dog bladders by administering 2-naphthylamine (Hueper, Leming, & Wolfe, 1937).

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In humans, the carcinogenicity of 2-naphthylamine became apparent when a large proportion of patients treated with the chemotherapeutic agent chlornaphazine, which is metabolized to 2-naphthylamine, developed bladder cancer (Thiede & Christensen, 1969). Even though aromatic amines were initially established as chemical carcinogens using dogs, subsequent studies into mechanisms of aromatic amine carcinogenesis have mostly been carried out using rodents. This review aims to present the evolving mechanisms of aromatic amine carcinogenesis, and mainly focuses on rodent studies in which aromatic amines predominantly produce liver tumors (National Toxicology Program, 2011).

More than one in eight of all known or suspected human carcinogens is an aromatic amine or a chemical that can convert into one, making aromatic amines a major class of human carcinogens (National Toxicology Program, 2011). Although evidence for the carcinogenicity of various aromatic amines has been produced in a variety of test systems and *in vivo* animal models (reviewed elsewhere (Radomski, 1979; Vineis & Pirastu, 1997; Wogan, Sun, Kim, Skipper, & Tannenbaum, 2009)), the mechanism(s) by which they cause cancer is still not entirely understood. According to the conventional three-stage model of chemical carcinogenesis, normal cells acquire mutations during the tumor initiation stage and proliferate to form tumors in the subsequent tumor promotion and progression stages (MacKenzie & Rous, 1941; Rous & Kidd, 1941). In this context, aromatic amines represent procarcinogens that undergo sequential bioactivation by enzymes that catalyze oxidation and conjugation reactions to generate electrophiles that bind covalently to DNA and cause subsequent mutations, initiating the process of chemical carcinogenesis (Butler, Guengerich, & Kadlubar, 1989). Based mostly on *in vitro* studies, the major enzymes that have been thought to catalyze oxidation and conjugation reactions to bioactivate aromatic amines are the cytochrome P450 isoform CYP1A2 followed by one or both of the two arylamine *N*-acetyltransferases (NATs). Even though studies performed *in vivo* generally support the role of aromatic amine-DNA adducts in aromatic amine carcinogenesis, they also point to the possibility of alternative bioactivation and tumor promotion/progression pathways for aromatic amines (Kimura et al., 1999; Sugamori et al., 2012). Given this disparity between *in vitro* and *in vivo* findings, the goals of this review are: 1) to provide an overview of studies that established the roles of bioactivation enzymes and DNA adducts in aromatic amine carcinogenesis; 2) to highlight studies where levels of aromatic amine DNA adducts have failed to correlate with carcinogenesis; and 3) to present recent studies that may shed light on novel pathways and mechanisms that drive aromatic amine carcinogenesis. In this review we focus on studies using 4-aminobiphenyl (ABP) as a model aromatic amine carcinogen, since they provide some of the most recent mechanistic insights into aromatic amine carcinogenesis. Chemical structures of a subset of primary aromatic amines that are discussed in this review, particularly ABP, are shown in Fig. 1.

2. Proposed models of aromatic amine bioactivation and detoxification

2.1. Aromatic amines as 'procarcinogens'

Through initial studies with the *N*-acetylated aromatic amine 2-acetylaminofluorene (AAF) in the rat, Cramer et al. was the first to demonstrate that hydroxylation of its nitrogen atom represents a major metabolic reaction *in vivo* (Cramer, Miller, & Miller, 1960). Other non-acetylated primary aromatic amines such as ABP and 2-naphthylamine were shown to undergo a similar *N*-hydroxylation reaction, and it was thought that the formation of *N*-hydroxylated metabolites contributes greatly to the carcinogenicity of these compounds (Radomski, 1979). Thus aromatic amines came to be recognized as 'procarcinogens' that undergo competing bioactivation and/or detoxification reactions that govern their carcinogenicity. Classically, these 'bioactivating' reactions are thought to be governed by enzymes found to catalyze two main reactions: 1) *N*-hydroxylation, followed by 2) *O*-esterification of the *N*-hydroxylated compound (Hein et al., 1993;

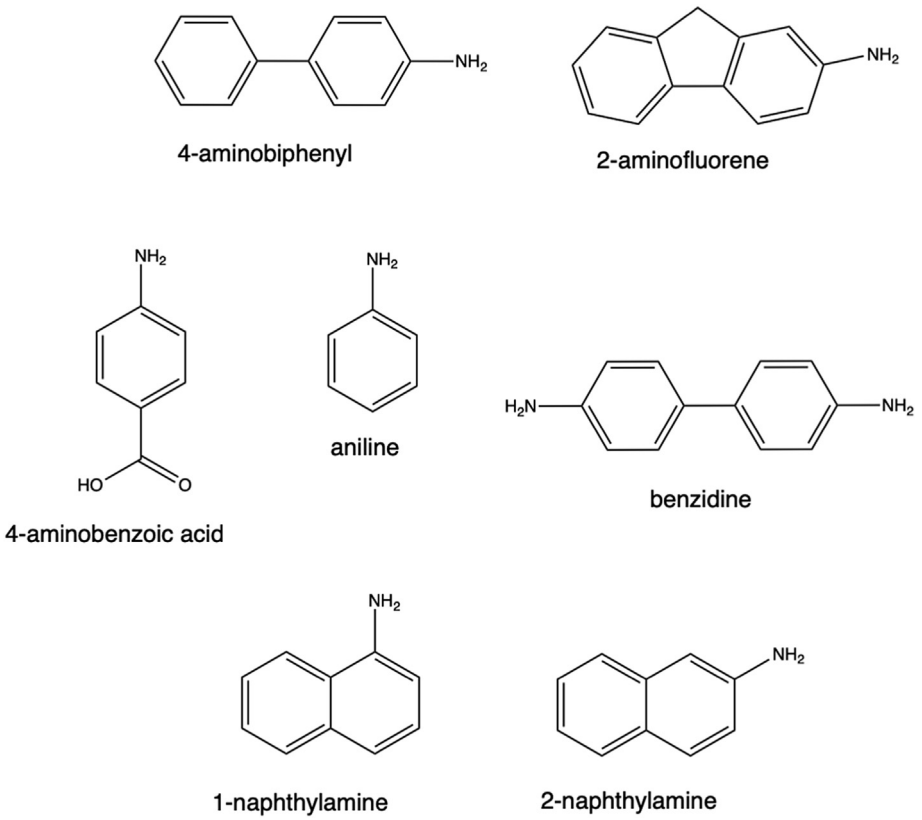

Fig. 1. Chemical structures for a subset of common primary aromatic amines.

Land et al., 1989). The final product of these reactions is an ester conjugate that is unstable, and readily decomposes to form a reactive electrophile called the arylnitrenium ion that covalently binds to nucleophilic sites on proteins, DNA and RNA, forming adducts (Fig. 2) (Josephy & Novak, 2013; Kadlubar et al., 1991; Kerdar, Dehner, & Wild, 1993; McClelland, Davidse, & Hadzialic, 1995; Novak, Kahley, Eiger, Helmick, & Peters, 1993).

It is generally accepted that the formation of aromatic amine DNA adducts is the initial event required for the generation of mutations that initiate tumor growth (Hang, 2010; Kadlubar et al., 1991; Poirier, 2004). This is particularly important if these events occur in genes that regulate cell cycle progression. In fact, ABP has been found to cause mutations in the H-*ras* gene in livers of exposed mice (Parsons, Delongchamp, Beland, & Heflich, 2005). Although adducts have been reported to form at all DNA bases, guanine is the most frequent target, with adducts forming largely on the C8 and N2 atoms (Kadlubar, Unruh, Beland, Straub, & Evans, 1980; Kim & Guengerich, 2005; Poirier, Santella, & Weston, 2000). The *N*-(deoxyguanosine-8-yl)-adduct is a stable and major adduct formed following ABP exposure, and is also found following exposure to other aromatic amines such as AAF (Heflich & Neft, 1994; Hsu et al., 2004; Neumann, 1986). As such, the *N*-(deoxyguanosin-8-yl)-ABP (dG-C8-ABP) adduct is commonly used in many *in vitro* and *in vivo* studies as a measure of ABP exposure in target tissues of biological models (Bendaly et al., 2009; Millner, Doll, Cai, States, & Hein, 2012; Sugamori et al., 2012). ABP adducts are also often used as molecular tools to measure exposure in humans. However, detection of ABP-DNA adducts, as a measure of exposure in target organs, is invasive and technically challenging. Therefore the detection of ABP-hemoglobin adducts in the blood, which form through an oxidation mechanism, is used more frequently to gauge levels of ABP exposure (Pathak, Chiu, Amin, & Turesky, 2016). For example, much higher levels of ABP-hemoglobin adducts are found in smokers than in non-smokers (Chen, Zhang, & Vouros, 2018; Guo et al., 2018; Sarkar et al., 2006; Shertzer, Dalton, Talaska, & Nebert, 2002).

2.2. N-hydroxylation—the first step in aromatic amine bioactivation

As mentioned above, *N*-hydroxylation of aromatic amines is often considered as the first step in procarcinogen bioactivation, occurring through enzyme-mediated oxidation by monooxygenases. Primary aromatic amines may undergo *N*-hydroxylation catalyzed primarily by member(s) of the CYP superfamily of enzymes, but also by flavin-containing monooxygenases and peroxidases (Butler, Guengerich, & Kadlubar, 1989; Butler, Iwasaki, Guengerich, & Kadlubar, 1989; Flammang et al., 1989; Frederick, Mays, Ziegler, Guengerich, & Kadlubar, 1982). Several CYP isoforms have been reported to *N*-hydroxylate primary aromatic amines, including CYP1A, CYP2A, CYP2B and CYP2C family members.

In vitro studies using rat liver microsomes exposed to various CYP isoform-selective inducers suggested that many primary aromatic amines undergo *N*-hydroxylation catalyzed largely by members of the CYP1A family, particularly CYP1A2 (Hammons, Guengerich, Weis, Beland, & Kadlubar, 1985; Kamataki et al., 1983). By correlating with well-known CYP1A2 substrates, Butler et al. established CYP1A2 as a

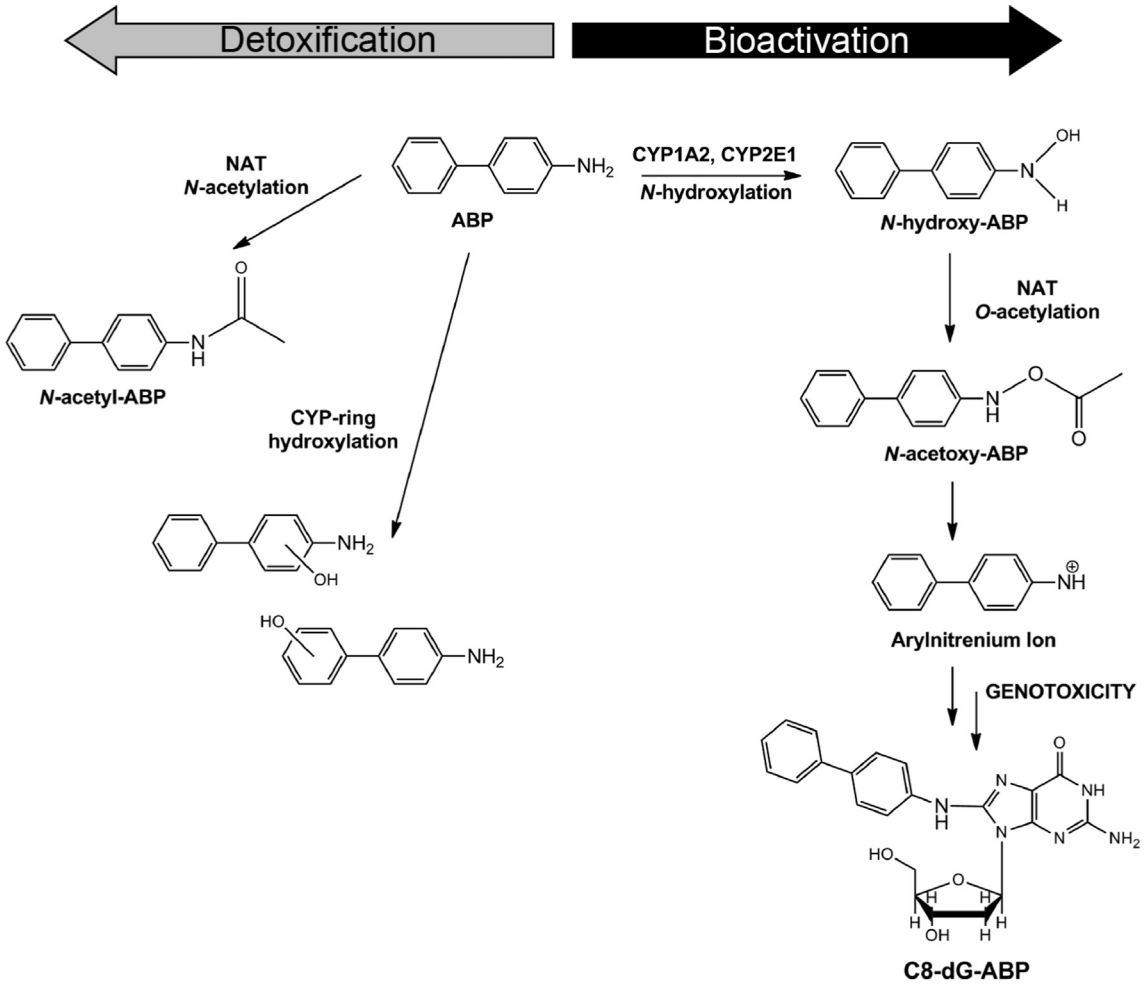

Fig. 2. 4-Pathways of ABP bioactivation and detoxification. Only a subset of known ABP metabolic pathways are shown, since other CYP enzymes, as well as SULTs and UGTs may also be involved.

major player in the *N*-hydroxylation of ABP in human liver (Butler, Iwasaki, et al., 1989). Other CYP enzymes such as CYP1A1, CYP1B1, CYP2A6, CYP2A13, CYP2B1, and CYP2C11 have also been shown to catalyze ABP *N*-hydroxylation *in vitro*, but their roles *in vivo* are less clear because of low abundance and/or extrahepatic protein expression (Butler, Iwasaki, et al., 1989; Fukami, Nakajima, Sakai, Katoh, & Yokoi, 2007; Kobayashi et al., 1999; Nakajima et al., 2006). Although much of the existing *in vitro* evidence using human liver microsomes strongly implicates CYP1A2 as the major mediator in ABP activation, this does not appear to be the case in mouse models. In fact, liver microsomes from *Cyp1a2 (−/−)* mice still retain roughly 50% of their total ABP *N*-hydroxylation activity (Kimura et al., 1999). This lack of agreement between human and mouse findings has also been observed with the heterocyclic amine 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), and it strongly suggests the involvement of other enzymes that also mediate this reaction (Cheung et al., 2005). The presence of a major alternative ABP *N*-hydroxylating enzyme in mice was demonstrated in two recent publications from our laboratory. Using both chemical inhibitors of CYP2E1 and CYP2E1-deficient mice, we identified CYP2E1 as a major contributor to ABP *N*-hydroxylation, with activity comparable to that of CYP1A2 in both neonatal and adult mouse liver microsomes (Wang, Bott, Tung, Sugamori, & Grant, 2015; Wang, Sugamori, Tung, McPherson, & Grant, 2015). The contribution of CYP2E1 to the *N*-hydroxylation of aromatic amines may have been previously masked by the use of dimethyl sulfoxide, an inhibitor of CYP2E1, as the typical solvent of choice to solubilize aromatic amine substrates in *in vitro* enzyme activity assays. Since CYP2E1 activity is often associated with changes in redox states, in Section 4.1 we present further evidence from our laboratory that explores potential consequences of ABP bioactivation mediated by CYP2E1. The extent to which CYP2E1 may contribute to the *N*-hydroxylation of other aromatic amines remains to be determined.

2.3. Mechanisms of aromatic amine detoxification and further activation

2.3.1. Aromatic ring oxidation

Although oxidative metabolism of aromatic amines is classically described with emphasis on *N*-hydroxylation and resulting activation of these compounds, *C*-oxidation of the aromatic ring also occurs, and competes with *N*-hydroxylation, to serve as a mechanism for oxidation-mediated detoxification (Hammons et al., 1985). The extent to which aromatic amines undergo ring *versus* *N*-hydroxylation is largely compound-dependent. Both *in vitro* and *in vivo* studies in experimental animal models have shown that ABP can undergo ring oxidations at various positions on each of its aromatic rings (Booth & Boyland, 1964; Butler, Guengerich, & Kadlubar, 1989; Hammons et al., 1985; McMahon, Turner, & Whitaker, 1980). However, ABP oxidative detoxification plays a minor role in ABP metabolism, since *N*-hydroxylation is the preferred reaction catalyzed by CYP enzymes (Butler, Iwasaki, et al., 1989; Kadlubar, Fu, Jung, Shaikh, & Beland, 1990; McMahon et al., 1980). Studies with the ABP isomers 2-aminobiphenyl and 3-aminobiphenyl have shown that the extent of ring *versus* *N*-hydroxylation is also isomer dependent, since both 2-aminobiphenyl and 3-aminobiphenyl undergo significantly more aromatic ring oxidation and less *N*-hydroxylation than ABP (Bayraktar, Kajbaf, Jatoe, & Gorrod, 1987; El-Bayoumy, LaVoie, Tulley-Freiler, & Hecht, 1981). These findings are also consistent with the importance of *N*-hydroxylation as a critical step for the carcinogenicity of aromatic amines, since both 2-aminobiphenyl and 3-aminobiphenyl are only weakly carcinogenic (Weisburger & Weisburger, 1966). Furthermore, the weak carcinogenicity of 3-aminobiphenyl may arise through an unknown mechanism that is independent of *N*-hydroxylation, since its *N*-hydroxylated metabolite is not mutagenic in *in vitro* systems (Ioannides et al., 1989).

Other aromatic amines also display differential preferences for ring- *versus* *N*-hydroxylation reactions, which can influence their carcinogenic potential. For example, both 1-naphthylamine and 2-naphthylamine undergo ring oxidation, but only 2-naphthylamine undergoes *N*-oxidation (Hammons et al., 1985; Poupko, Radomski, Santella, & Radomski, 1983; Radomski, Deichmann, Altman, & Radomski, 1980). The consequence of exclusive ring oxidation in 1-naphthylamine is a complete lack of bladder carcinogenicity in dogs (Radomski et al., 1980). Compelling evidence supporting *N*-hydroxylation as the key determinant of aromatic amine carcinogenicity came from rodent studies using *N*-hydroxy-1-naphthylamine, where all rats administered *N*-hydroxy-1-naphthylamine developed sarcomas at the site of administration (Dooley, Beland, Bucci, & Kadlubar, 1984). These observations strongly support the importance of *N*-hydroxylation as the first step in procarcinogen activation and of ring oxidation as a potent mechanism of oxidative detoxification. As such, the measurement of relative rates of *N*-hydroxylation and ring oxidation may allow for better prediction of aromatic amine activation potential and resultant carcinogenicity *in vivo*.

2.3.2. Conjugating reactions

A number of different enzyme systems can catalyze the conjugation of aromatic amines and their metabolites. In particular, arylamine *N*-acetyltransferases (NATs), sulfotransferases (SULTs), UDP-glucuronosyltransferases (UGTs), and deacetylases can catalyze reactions that either detoxify parent aromatic amines or bioactivate their *N*-hydroxylated derivatives (Kim & Guengerich, 2005). Although the role of SULTs and UGTs are important for the detoxification and bioactivation of aromatic amines, the bulk of this review focuses on NAT-mediated metabolism. NATs serve as a line of defense by catalyzing *N*-conjugating reactions as a mechanism for detoxification, to generate *N*-acetylated metabolites that are less likely to undergo further bioactivation. For example, some *N*-acetylated aromatic amine derivatives undergo subsequent CYP-mediated ring oxidation rather than *N*-hydroxylation reactions (Bayraktar et al., 1987; El-Bayoumy et al., 1981). However, these same NAT enzymes can also be deleterious because they can produce reactive ester derivatives that have undergone initial *N*-hydroxylation followed by NAT-catalyzed *O*-acetylation or intramolecular *N,O*-acetyl transfer. The unstable acetoxy ester then spontaneously decomposes to form an electrophilic DNA-damaging arylnitrenium ion (Flammang & Kadlubar, 1986; Tsuneoka et al., 2003).

ABP metabolites can also undergo other conjugation reactions. Studies with hepatic cytosolic and microsomal fractions have shown that *N*-hydroxy-ABP can undergo glucuronide conjugation to form relatively stable products (Kadlubar, Miller, & Miller, 1977; Poupko et al., 1983; Tsuneoka et al., 2003). In primary rat hepatocytes *N*-acetylation is the major route of ABP metabolism followed by *N*-hydroxylation (Chou, Lang, & Kadlubar, 1995; Orzechowski, Schrenk, Schut, & Bock, 1994). The role of *N*-acetylation as a mechanism of aromatic amine detoxification is further supported by the observation that *N*-acetylated ABP is a poor tumor inducer in rats (Kriek, 1971). One proposed mechanism to explain the apparent tissue selectivity of aromatic amines for inducing tumors of the bladder in humans involves initial *N*-hydroxylation followed by *O*-conjugation (or *N*-glucuronidation) in the liver, to derivatives that migrate in the blood to the bladder. Under the low pH conditions in the urine these conjugated derivatives undergo hydrolysis back to their *N*-hydroxylated forms as well as *N*-glucuronidated derivatives, and are further metabolized again to their ultimate reactive forms (Kadlubar et al., 1977; Kadlubar et al., 1991; Tsuneoka et al., 2003). Species differences in aromatic amine induction of bladder cancer may be partially attributed to the prolonged residence time of urine in the bladders of humans and dogs compared to rodents (Kadlubar et al., 1977). In addition, the pertinent enzymes are subject to species differences (Martignoni, Groothuis, & de Kanter, 2006; Sharer, Shipley, Vandenbranden, Binkley, & Wrighton, 1995), an important consideration for extrapolating findings from animal models to humans. For example, the *N*-(deoxyguanosin-8-yl)-AAF adduct is the major adduct observed in both *N*-hydroxy-AAF exposed mice and rats, yet SULT

inhibition prevents this adduct from forming in mice but not in rats (Lai, Miller, Miller, & Liem, 1985; Meerman, Beland, & Mulder, 1981). This is because in mice, *N*-hydroxy-AAF is deacetylated before esterification by SULTs to generate *N*-sulfoxy-AAF, whereas in the rat NAT-dependent acetoxy ester formation prevails prior to deacetylation, leading to the formation of *N*-acetoxy-AAF (Flammang & Kadlubar, 1986; Lai, Miller, Miller, & Liem, 1988).

The relative contributions of SULTs, UGTs and NATs to the conjugation of *N*-hydroxylated metabolites to generate the reactive arylnitrenium ion are unclear, and suggest that species and chemical structure differences play important roles. For instance, dogs lack NAT expression in either liver or bladder, yet are still prone to aromatic amine-induced bladder cancer (Flammang & Kadlubar, 1986; Kadlubar et al., 1991). This is most likely attributed to a significant level of redundancy that exists among different conjugation pathways. It was therefore surprising when we observed that mice deficient in functional NATs were protected from ABP-induced liver tumors, despite seeing no reduction in ABP-induced DNA damage or mutations (Sugamori et al., 2012; Wang et al., 2012). In fact, we now believe that the protective effect of NAT deficiency might not be due to the classical role that NATs play in ABP metabolism, but rather to an alternate, novel role that NATs may play in tumor promotion and progression (see Section 4.2). As such, the role of NATs in aromatic amine metabolism shall be further discussed.

2.4. The role of arylamine N-acetyltransferases in aromatic amine metabolism

2.4.1. Arylamine N-acetyltransferase both activate and detoxify aromatic amines

In humans, two functional NAT enzymes, expressed in the cytosol, are encoded by two gene loci (*NAT1* and *NAT2 genes*) (Fretland, Doll, Gray, Feng, & Hein, 1997). Both human *NAT1* and *NAT2* have high homology to *Nat1* and *Nat2* from a number of different animal species such as the mouse, rabbit, Syrian hamster, and rat (Hein, 2002; Hein et al., 1997). For the remainder of this review, we will focus on NAT studies conducted primarily in mice, which has become the most frequently used animal model due to cost-effectiveness and ease of genetic manipulation. In mice, three genes (*Nat1, Nat2* and *Nat3*) encode three NAT proteins (Fretland et al., 1997; Kelly & Sim, 1994; Martell, Vatsis, & Weber, 1991). Based on substrate selectivity, human NAT1 (hNAT1) and mouse NAT2 (mNAT2) are functional orthologues, as are human NAT2 (hNAT2) and mouse NAT1 (mNAT1). hNAT1 and mNAT2 are found ubiquitously in all tissues, while hNAT2 and mNAT1 are more liver-restricted (Cornish et al., 2003). Mouse NAT3 plays little to no role in the *in vivo* clearance of classical NAT substrates (Sugamori et al., 2007). Primary aromatic amines, heterocylic aromatic amines and hydrazines that are not native to cells are the best characterized NAT substrates (Butcher & Minchin, 2012). Little is known about endogenous NAT substrates. *p*-Aminobenzoylglutamate, a catabolite of folate metabolism, is the only known endogenous substrate of NATs (hNAT1) (Minchin, 1995; Upton et al., 2000). Mice lacking the *Nat2* gene have undetectable levels of urinary *N*-acetylated-*p*-aminobenzoylglutamate, which can also be detected in human urine (McPartlin, Courtney, McNulty, Weir, & Scott, 1992; Wakefield, Cornish, Long, Griffiths, & Sim, 2007). More recently, NATs have been suggested to control cellular levels of acetyl coenzyme A (CoASAc), which is further discussed in Section 4.2.

NATs catalyze three different types of acetylating reactions: *N*-acetylation of primary aromatic amines, *O*-acetylation of arylhydroxylamines and arylhydroxamic acids, and intramolecular *N,O*-acetyl transfer (Fretland et al., 1997; Mattano, Land, King, & Weber, 1989; McQueen, Chau, Erickson, Tjalkens, & Philbert, 2003). As mentioned above, the latter two reactions are involved in metabolic activation of *N*-hydroxylated and *N*-hydroxy-*N*-acetylated procarcinogens. Studies of recombinant human NATs expressed in *E. coli* have shown that both hNAT1 and hNAT2 can catalyze ABP *N*-acetylation, *N*-hydroxy-ABP *O*-acetylation and intramolecular *N,O*-acetyl transfer on *N*-hydroxy-4-acetyl-ABP (Hein et al., 1993; Mattano et al., 1989). hNAT2 has significantly higher affinity for ABP than hNAT1, and activity ratios and clearance calculations suggest that hNAT2 has a key role in acetylation reactions concerning ABP activation/detoxification in liver (Hein et al., 1993; Minchin et al., 1992). Similar studies in mice show that mNAT1 has a greater affinity to catalyze the *N*-acetylation of ABP and other aromatic amines, but with a lower maximal velocity than mNAT2. Despite this, the intrinsic clearance (V_{max}/K_m) is similar for mNAT1 and mNAT2 (Fretland et al., 1997). However, *in vivo* studies in *Nat2 (−/−)* mice suggest that mNAT2 is exclusively responsible for both the *N*- and *O*-acetylation of ABP (Loehle et al., 2006). This contrasts with what would be expected since mNAT1 is generally considered to be the functional orthologue of hNAT2, the ABP *N*-acetylation enzyme in humans. These results allude to important differences in substrate specificity between mouse and human NATs.

2.4.2. N-Acetyltransferase polymorphisms and influence on genotoxicity

Both NAT1 and NAT2 are highly polymorphic in a number of organisms. In humans, at least 24 *NAT1* and 26 *NAT2* alleles have been reported and give rise to rapid, intermediate, and slow acetylator phenotypes (for a detailed review, see (Hein, 2002) and (Sim, Abuhammad, & Ryan, 2014)). In mice, polymorphisms in the *Nat2* allele in inbred strains are responsible for rapid (C57BL/6, C3H/HeJ, BALB/c) and slow (A/J, A/HeJ) acetylator phenotypes (Kelly & Sim, 1994; Martell et al., 1991). Polymorphisms in the *Nat1* gene have also been discovered in the inbred *Mus spretus* strain (Boukouvala, Price, & Sim, 2002). In NAT recombinant expression models, no differences are observed between mNAT2 allelic variants in their ability to *N*-acetylate ABP, and both variants carry out ABP *N*-acetylation at much higher rates than mNAT1 (Fretland et al., 1997). On the other hand, *in vitro* studies using mouse liver cytosols demonstrate higher ABP *N*-acetylation rates in rapid acetylators than in slow acetylators. Despite this, no difference was seen in hepatic levels of dG-C8-ABP DNA adducts after *in vivo* exposure of mice to ABP (McQueen et al., 2003). The reasons for this may be that mNAT2 has reduced expression and stability in slow acetylators, or that ABP may be activated by other competing reactions (McQueen et al., 2003). Although ABP may not be sensitive to acetylator status *in vivo*, other aromatic amines like AAF are influenced by acetylator status, which is reflected by higher AAF-DNA adducts in mice with the rapid acetylator allele (Levy & Weber, 1989).

Differences in ABP *N*- and *O*-acetylation rates have also been observed in primary human hepatocytes. Rapid acetylators produce higher levels of *N*-acetyl-ABP from ABP, and also higher levels of dG-C8-ABP DNA adducts when exposed to *N*-hydroxy-ABP (Doll, Zang, Moeller, & Hein, 2010). The same has been observed in Chinese hamster ovary cells transfected with hNAT2 rapid, intermediate or slow acetylator alleles (Bendaly et al., 2009). NAT polymorphisms are also found to correlate with the occurrence of human cancers. For example, several studies have shown that hNAT2 slow acetylators are at higher risk of bladder cancer following aromatic amine exposure than rapid acetylators, an effect that was attributed to the fact that hNAT2 *N*-acetylation competes poorly with CYP-mediated *N*-hydroxylation in slow acetylators (Hein et al., 2000; Moore et al., 2011). Since NATs operate to both activate aromatic amines and detoxify them, to date there is no consensus with regard to whether NATs exert an overall protective or tumorigenic role in human cancers, with the role of NATs probably being compound- and cancer type-dependent.

3. Levels of aromatic amine-DNA adducts do not always correlate with carcinogenesis

3.1. Early experiments that reported a lack of correlation between aromatic amine-induced DNA damage and tumors

As early as 1951 Richardson et al. observed that co-administration of 3′-methyl-4-dimethylaminoazobenzene with 3-methylcholanthrene,

an established inducer of the CYP1 enzymes, actually inhibited 3′-methyl-4-dimethylaminoazobenzene-induced liver tumors in rats (Richardson, Stier, & Borsos-Nachtnebel, 1952). This observation seems to be at odds with the roles of CYP1 enzymes in bioactivating aromatic amines, forming DNA adducts, and initiating liver carcinogenesis. Using 16 different aromatic amines, Parodi et al. failed to detect a correlation between each compound's ability to damage DNA, generate mutations, and form liver tumors, whereas a correlation was detected for a group of carcinogenic hydrazines (Parodi et al., 1981; Parodi et al., 1981; Parodi, Zunino, Ottaggio, De Ferrari, & Santi, 1983). These are the earliest reports that hint at the existence of a non-genotoxic component in aromatic amine carcinogenesis.

In the Parodi et al. study, the authors measured *in vivo* DNA fragmentation and sister-chromatid exchange as biomarkers of DNA damage but not aromatic amine-DNA adducts directly (Parodi, Taningher, et al., 1981). When techniques finally became available for the measurement of bulky DNA adducts *in vivo*, such as DNA ^{32}P-postlabelling and mass spectrometry (Andrews, Vouros, & Harsch, 1999; Randerath, Reddy, & Gupta, 1981), a number of studies directly compared the levels of aromatic amine-DNA adducts with tumors (Poirier & Beland, 1994). In general, aromatic amine exposure is associated with both aromatic amine-DNA adducts and tumor formation, but when examined in detail, levels of aromatic amine-DNA adducts often do not correlate with levels of tumors. This is perhaps best illustrated by a comprehensive series of studies comparing the structurally similar aromatic amines *trans*-4-acetylaminostilbene (AAS) and AAF. When radiolabelled AAS or AAF was fed to rats, AAS generated a higher level of DNA adducts in the liver than AAF, but only AAF produced liver tumors (Baur & Neumann, 1980; Ruthsatz & Neumann, 1988). When provided with a tumor promoting environment, however, AAS led to more liver tumors than AAF (Hilpert, Romen, & Neumann, 1983). When both AAS and AAF were administrated to rats, the sequence of administration affected tumor levels such that more liver tumors were detected with AAF administered after AAS, even though similar total levels of DNA adducts were detected regardless of administration sequence (Kuchlbauer, Romen, & Neumann, 1985; Ruthsatz & Neumann, 1988). Furthermore, this disconnect between the ability of each chemical to form DNA adducts and generate liver tumors cannot be explained by the persistence of each adduct, as both AAS- and AAF-DNA adducts showed similar rates of elimination from rat liver (Ruthsatz & Neumann, 1988).

Additional studies that demonstrated a lack of correlation between aromatic amine-DNA adducts and tumors arose from comparisons made between different tissues and with different exposure levels. Despite finding the highest level of AAS-DNA adducts in the liver, the administration of AAS to rats led to the formation of mammary but not liver tumors (Poirier, Fullerton, Kinouchi, Smith, & Beland, 1991). In another study, a lack of correlation was reported between the levels of DNA adducts and tumors across different tissues in rats following the administration of nitrosofluorene (a major toxic metabolite of AAF) (Cui, Torndal, Eriksson, & Moller, 1995). In mouse liver, one study detected dose-dependent increases in ABP-DNA adducts in both sexes but a dose-dependent increase in liver tumors only in females (Poirier, Fullerton, Smith, & Beland, 1995). In mouse bladder, despite the observation of a dose-dependent increase in AAF-DNA adducts, only high doses of AAF led to bladder tumors (Poirier et al., 1991). Nevertheless, the overall correlation between levels of aromatic amine-DNA adducts and tumors seems to be stronger in the bladder than in the liver, which may be attributed to the increased rate of cellular proliferation in the bladder epithelium that more efficiently converts transient DNA adducts into mutations driving tumor formation (Besaratinia & Tommasi, 2013; Yoon, Kim, Tommasi, & Besaratinia, 2012).

Although much ignored, these studies reporting a lack of correlation between levels of aromatic amine-DNA adducts and tumors offer important mechanistic insights into aromatic amine carcinogenesis - they were the first to suggest the existence of additional, non-DNA adduct-related factors exerting a crucial influence over aromatic amine carcinogenesis. Further evidence that points to the existence and importance of these non-genotoxic factors in aromatic amine carcinogenesis arose from more recent studies using genetically modified mice that lack key bioactivation enzymes.

3.2. Knockout mouse models support the existence of additional, non-DNA adduct factors controlling aromatic amine carcinogenesis

While a large body of knowledge accumulated on the role of bioactivation enzymes in chemical carcinogen metabolism *in vitro*, relatively little was known regarding the contribution of these enzymes to chemical carcinogenesis in intact animals – that is, until the development and widespread use of genetically modified mouse models. Using mice deficient in putative bioactivation enzymes, researchers were finally able to investigate the *in vivo* contribution of these enzymes not only towards acute toxicity but also towards ultimate carcinogenesis following exposure to a chemical carcinogen (Gonzalez & Kimura, 1999). For example, mice deficient in either CYP1B1 or microsomal epoxide hydrolase, enzymes suggested by *in vitro* studies to bioactivate the polycyclic aromatic hydrocarbon 7,12-dimethylbenz[*a*]anthracene (DMBA), were protected from DMBA-induced lymphomas and skin tumors (Buters et al., 1999; Miyata et al., 1999). These studies not only validated the DMBA bioactivation paradigm established *in vitro* but also supported the formation of DMBA-DNA adducts as a rate-limiting step in DMBA carcinogenesis.

As described in Section 2, earlier *in vitro* studies had pointed to CYP1A2 as the key bioactivation enzyme that carries out the initial *N*-hydroxylation of aromatic amines. In the same study that demonstrated significantly reduced ABP *N*-hydroxylation activity in CYP1A2 deficient mice, Kimura et al. failed to detect any reduction in ABP-induced DNA adducts or liver tumors in CYP1A2 deficient mice (Kimura et al., 1999). These findings prompted Kimura et al. to repeat their carcinogenicity study using another prototypical *in vitro* substrate for CYP1A2, the heterocyclic aromatic amine 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). In this study, mice deficient in CYP1A2 were protected from DNA adducts but not liver tumors induced by PhIP (Kimura et al., 2003; Snyderwine, Yu, Schut, Knight-Jones, & Kimura, 2002). Given the recent data from our laboratory that supports a role for CYP2E1 as an alternative ABP *N*-hydroxylating enzyme (Section 2), it will be interesting to test whether CYP2E1 single knockout or CYP1A2/CYP2E1 double knockout mice are protected from ABP-induced liver DNA adducts and tumors. Since NAT1 and NAT2 have also been suggested to bioactivate ABP *in vitro*, our laboratory measured ABP-induced liver DNA adducts in mice deficient in both NAT1 and NAT2 using the same tumor-inducing protocol as Kimura et al. In our studies, mice deficient in NAT1/2 did not have lower levels of ABP-induced liver DNA adducts, even though they were protected from ABP-induced liver tumors (Sugamori et al., 2012).

One major challenge in correlating levels of specific aromatic amine DNA adducts to tumors resides in the complexity of DNA damage exerted by aromatic amines. For example in addition to dG-C8-ABP, ABP adducts have also been found on the N2 position of guanine and on the C8 position of adenine among others, making it difficult to profile precisely all DNA damage inflicted by ABP (Guo et al., 2018). To overcome this challenge, we reasoned that for any DNA damage to contribute to tumorigenesis, it must be fixated in the genome in the form of DNA mutations, which we were able to measure *in vivo* using a reporter mouse line – the Muta™Mouse (Gossen et al., 1989). As expected, ABP exposure produced mutations *in vivo*, but like previous studies with DNA adducts, mutation frequencies failed to correlate with strain and sex differences in tumor formation rates (Wang et al., 2012).

Together these studies support the existence of a previously uncharacterized, non-DNA damage-related factor(s) that exerts a significant influence over ABP carcinogenicity.

4. Novel modulators of aromatic amine carcinogenesis

4.1. A potential role for oxidative stress in aromatic amine carcinogenesis

With accumulating evidence pointing to a lack of correlation between levels of DNA adducts and liver tumors induced by ABP (Kimura et al., 1999; Sugamori et al., 2012), our laboratory searched for additional factors that may influence ABP carcinogenesis. *In vitro* studies from other laboratories had reported that *N*-hydroxy-ABP, the major *in vivo* oxidative metabolite of ABP (Orzechowski et al., 1994), was capable of redox cycling and producing reactive oxygen species in the presence of electron donors that are commonly found in cells (Makena & Chung, 2007; Murata & Kawanishi, 2011; Murata, Tamura, Tada, & Kawanishi, 2001; Pathak et al., 2016; Siraki, Chan, Galati, Teng, & O'Brien, 2002). Two *in vivo* studies detected a depletion of hepatic thiols, indicative of increased oxidative stress, in mouse liver following a single dose of ABP (Shertzer et al., 2002; Tsuneoka et al., 2003).

To investigate the potential involvement of oxidative stress in ABP-induced liver carcinogenesis, we measured levels of oxidative stress both in a mouse hepatoma cell line (Hepa1c1c7) and *in vivo* in mouse liver following ABP exposure. In Hepa1c1c7 mouse hepatoma cells, we confirmed previous *in vitro* findings that *N*-hydroxy-ABP but not the parent compound ABP is a potent inducer of oxidative stress (Wang, Sugamori, et al., 2015). In these cells, the *N*-hydroxylation of ABP by heterologously expressed CYP2E1 but not CYP1A2 led to oxidative stress, through a mechanism that may involve mitochondrially-expressed CYP2E1 (reviewed in (Hartman, Miller, & Meyer, 2017)). *In vivo*, ABP produced significant oxidative stress in mouse liver after the same exposure conditions that lead to liver tumor formation in a neonatal tumor bioassay, and this oxidative effect of ABP was abrogated in CYP2E1 deficient but not CYP1A2 deficient mice, mirroring our results obtained in cell culture (Wang, Sugamori, et al., 2015). In the same study, a higher level of oxidative stress was found in male mice compared to females, which correlates with the increased liver tumor incidence observed in males, whereas a stronger NRF2-mediated antioxidant defense response was found in females, which may contribute to their relative protection against liver tumor growth. On the other hand, ABP exposure failed to produce either acute (Hanna, Riedmaier, Sugamori, & Grant, 2016) or chronic (unpublished results) cytotoxic or inflammatory responses, processes which are thought to contribute to liver tumor development (Naugler et al., 2007). Our results raise the intriguing possibility that oxidative stress may play a significant role in ABP carcinogenesis, at least in mouse liver (Fig. 3).

Our laboratory was not the first to observe an oxidative effect of aromatic amines. Driven by the lack of correlation between the genotoxic properties of AAF and carcinogenesis, studies from the Neumann laboratory reported increased resistance to apoptosis in rat liver following AAF treatment, which they attributed to the production of reactive oxygen species in the mitochondria *via* redox cycling between its *N*-hydroxy and nitroso forms (Klöhn, Brandt, & Neumann, 1996; Klohn, Massalha, & Neumann, 1995; Klöhn & Neumann, 1997). Similar redox cycling between different metabolites have been reported for other aromatic amines including 3,5-dimethylaminophenol and aniline *in vitro* and in cell culture (Chao et al., 2015; Chao et al., 2018; Pathak et al., 2016).

AAF and ABP represent two of the best-studied genotoxic aromatic amine carcinogens, yet both also possess oxidative properties that may contribute to their carcinogenicity. Whether oxidative stress represents a novel modulator of aromatic amine carcinogenesis awaits further studies that investigate its involvement in the carcinogenicity of other aromatic amines.

4.2. N-acetyltransferases in health and disease

Findings over the past decade have shown novel tumor promoting roles of NATs that go beyond their well-accepted involvement in xenobiotic metabolism (Sugamori & Grant, 2017). Accumulating epidemiological evidence supports the mis-regulation of hNAT1 in cancers of the breast, prostate, skin, lung, brain, colon and renal system (for a detailed review see (Butcher & Minchin, 2012)). hNAT1 overexpression alone in breast cancer cells resulted in increased cell growth and conversely, hNAT1 inhibition decreased cell growth and invasiveness both in breast and colon cancer cells, potentially through a mechanism where hNAT1 stabilizes the gain-of-function form of p53 (Adam et al., 2003; Tiang, Butcher, Cullinane, Humbert, & Minchin, 2011; Tiang, Butcher, & Minchin, 2010; Tiang, Butcher, & Minchin, 2015; Wang, Minchin, & Butcher, 2018). These studies suggest that hNAT1 may

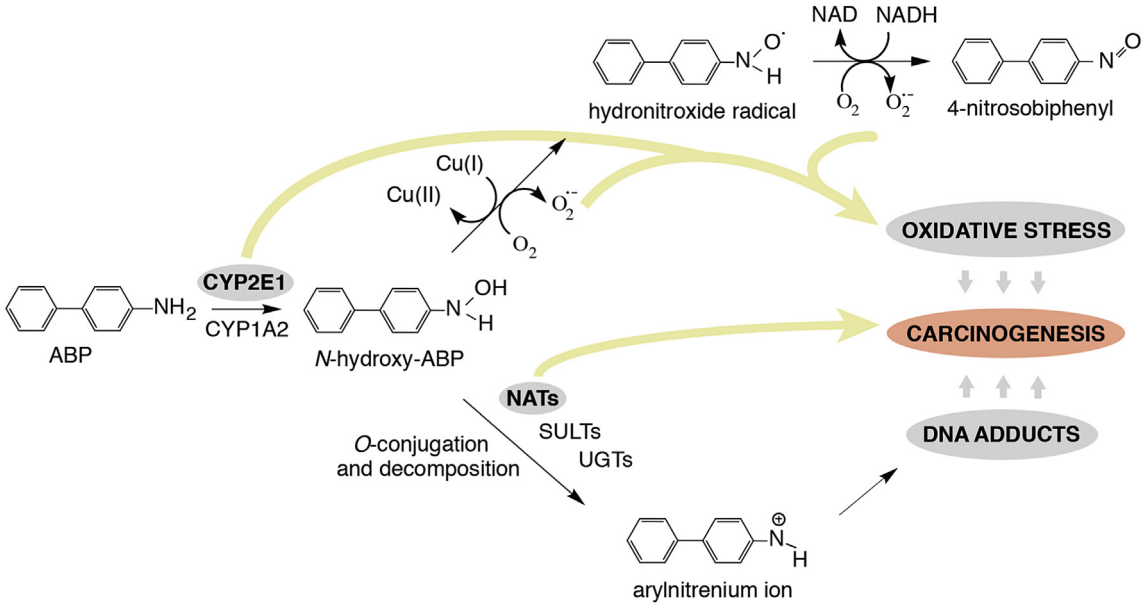

Fig. 3. Novel, non-DNA adduct-dependent drivers of 4-aminobiphenyl-induced rodent liver carcinogenesis (indicated by yellow arrows). CYP2E1 can produce tumor-promoting oxidative stress through the *N*-hydroxylation of ABP to the more redox active *N*-hydroxy-ABP metabolite and/or by leaking reactive oxygen species through a decoupling mechanism intrinsic to this enzyme (Caro & Cederbaum, 2004). *N*-hydroxy-ABP has been proposed to further oxidize to the hydronitroxide radical and 4-nitrosobiphenyl in the presence of catalysts found ubiquitously in cells such as copper (Cu) and nicotinamide adenine dinucleotide (NADH). In addition, NATs can contribute to ABP carcinogenesis by directly altering cell proliferation and/or indirectly by affecting cellular energetics.

play other carcinogenic roles apart from its classical role as a metabolizing enzyme for aromatic and heterocyclic amines (Fig. 3).

Aside from its role in folate metabolism, hNAT1 was recently proposed to act as a key mediator of cellular energetics. Acetyl coenzyme A (CoASAc) is an essential cofactor that acts as the acetyl group donor for the NAT-catalyzed acetylation of aromatic amines. CoASAc is also an important contributor to many cellular processes such as fatty acid metabolism, mitochondrial energetics and epigenetic chromatin modifications, among others. Recent studies have shown that hNAT1 catalyzes the direct hydrolysis of CoASAc using folate as a catalyst *in vitro*, expanding the potential endogenous roles of NATs to the regulation of global CoASAc levels (Laurieri et al., 2014; Stepp, Mamaliga, Doll, States, & Hein, 2015). Not only has hNAT1 been implicated in cellular energy metabolism, hNAT2 was recently identified as an insulin sensitivity gene from a genome-wide association study (Knowles et al., 2015). In both cell culture and in mouse models, deficiency of mNAT1 (the functional orthologue of hNAT2) led to insulin insensitivity in terms of decreased glucose uptake and increased lipolysis, through an incompletely understood mechanism that may involve the mitochondria (Camporez et al., 2017; Chennamsetty et al., 2016; Knowles et al., 2015). Together, these studies suggest a novel role for NATs in energy homeostasis that may indirectly impact carcinogenesis (Fig. 3). Indeed, a recent study suggests that the anti-tumorigenic effects of hNAT1 knockdown in breast cancer cells may be mediated through its effect on mitochondrial energetics (Carlisle et al., 2018). Whether the endogenous tumor-promoting roles of NATs are mediated through regulation of global CoASAc levels, and how this might influence aromatic amine carcinogenesis, remains to be determined.

5. Concluding remarks

The inevitable exposure to chemical procarcinogens in our environment makes it important to understand the hazards they pose and the mechanisms by which they manifest their carcinogenic effects in people. Aromatic amine research conducted over the past 70 years has shed light on many aspects of procarcinogen metabolism and carcinogenesis, such as the roles of oxidation and conjugation enzymes in their bioactivation and detoxification, the influence of both tissue and species differences, and the necessity but insufficiency of DNA adducts and mutations in carcinogenesis. However, our understanding of aromatic amine carcinogenesis is still incomplete as illustrated by the recent discovery of the role of CYP2E1 in ABP bioactivation and the contribution of non-genotoxic effects of aromatic amines, such as oxidative stress, to tumor promotion (Fig. 3). Moreover, the emerging intrinsic tumor-promoting properties of NATs should prompt us to reassess ways that NATs and other oxidation and conjugation enzymes interact with exogenous aromatic amines and with endogenous substrates and cofactors to affect energy metabolism and perhaps even synergize in carcinogenesis (Fig. 3). Lastly, much about aromatic amine carcinogenesis has been learned through animal studies, but the extent to which these findings can be extrapolated to humans remains unclear and represents a major challenge in the field of chemical carcinogenesis. Towards this goal, “humanized” mouse models (mice expressing human drug metabolism enzymes) that are being developed in many laboratories including ours has the potential to greatly improve our understanding of aromatic amine carcinogenesis in humans.

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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