Structure and Regulation of the Drug-Metabolizing Enzymes Arylamine *N*-Acetyltransferases

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Abstract: Arylamine *N*-acetyltransferases (NAT) are xenobiotic-metabolizing enzymes responsible for *N*-acetylation of many arylamines. They are also important for *O*-acetylation of *N*-hydroxylated heterocyclic amines. These enzymes play thus an important role in the detoxification and activation of numerous therapeutic drugs and carcinogens. Two closely related polymorphic isoforms (NAT1 and NAT2) have been described in humans and interindividual variations in NAT genes have been shown to be a potential source of adverse drug reaction. In addition, NAT1 and/or NAT2 phenotypes may modulate the risk of certain cancers in people exposed to aromatic amine carcinogens. Recent advances on the regulation of human NAT1 activity has shown that hydroxylamine and/or nitroso intermediates of NAT1 substrates inhibit the enzyme through direct irreversible interaction with its catalytic cysteine residue. Oxidative molecules such as hydrogen peroxide, S-nitrosothiols and peroxynitrite have also been shown to inactivate reversibly or irreversibly the enzyme in a similar manner. In this review, after summarizing the general background on human NAT enzymes, we focus on the recent developments on the regulation of the activity of these drug-metabolizing enzymes by substrate-intermediates and by oxidant molecules. The recent findings reviewed here provide possible mechanisms by which these non genetic determinants inhibit NAT1 activity and thereby may affect drug efficacy/toxicity.

Keywords: *N*-acetyltransferases,xenobiotics, polymorphism, splice variants, catalytic mechanisms, covalent modifications, oxidative stress.

INTRODUCTION

Most xenobiotic-metabolizing enzymes (XME) affect the bioavailability of drugs, making them important determinants in pharmacogenomics. The arylamine *N*-acetyltransferases (NATs) are XME that participate in the metabolism of numerous primary arylamines and hydrazine drugs and carcinogens. In addition to their *N*-acetylation catalytic activity, NAT enzymes have also *O*-acetylation activity towards *N*-hydroxyarylamines. The NAT2 phenotype, discovered 50 years ago, was one of the earliest hereditary traits modulating drug metabolism to be identified. Based on preliminary observations, drugs such as isoniazid and sulfamethazine were described as "polymorphic" (efficacy different depending on individuals) whereas others, such as *p*-aminosalicylic acid, were described as "monomorphic". This dichotomy was due, at least in part, to low levels of the NAT2 isoform in 50-60% of individuals of european descent, these individuals being described as "slow acetylators". In contrast, polymorphism associated with the NAT1 isoform was recognized long after these initial findings, with the advent of molecular genetics. It is now recognized that the human *NAT1* and *NAT2* loci are highly polymorphic, with more than 25 alleles identified at each locus. A historical account of acetylation pharmacogenetics can be found elsewhere [1]. Knowledge of these polymorphisms has been used for etiological purposes, including the investigation of drug responses and non-idiopathic disorders. However, neither basic mechanisms of NAT expression nor functional properties of the enzymic forms are fully understood. Other genetic and environmental factors have been shown to modulate NAT1 expression and its catalytic activity is prone to covalent inactivation.

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NAT AND DRUG RESPONSE

NAT2 polymorphism has been linked with various adverse drug reactions (ADR). In particular, many studies have shown that most patients with ADR for drugs metabolized by the NAT2 enzyme are slow acetylators. This suggests that some of these ADR are probably at least partly linked to the accumulation of non-acetylated xenobiotics [2]. Isoniazid (INH), a drug metabolized by NAT2, has been widely used in frontline chemotherapy for tuberculosis since the early 1950's and was used in the first pharmacogenetics studies. When used at standard doses, the toxic effects of INH, including neuropathy [3] and hepatotoxicity [4], have been associated with low levels of *N*-acetylation of INH and thus with the NAT2 slow acetylator genotype [5, 6]. Given the scarcity of ADR in general populations of patients, the participation of other biotransformation pathways is likely in most cases.

Particular attention has been paid to the formation of reactive drug metabolites in cases of idiosyncratic toxicity. In such cases, insufficiently acetylated drugs may accumulate and be converted to reactive forms by oxidative enzymes. NAT2 may therefore reduce the risk of idiosyncratic toxicity by reducing the probability that such reactive drug metabolites will be formed [2]. Idiosyncratic toxicity has been observed in patients treated with hydralazine, procainamide and sulfonamides, which have been shown to induce NAT-related ADR [7, 8]. Sulfamethoxazole, an effective antimicrobial agent, has been reported to induce

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signs of hypersensitivity, including cutaneous rash or fever. In a very small proportion of patients, toxic epidermal necrolysis (Lyell syndrome) or Stevens-Johnson syndrome has been reported. The slow NAT2 phenotype [8] and NAT2 genotypes [9] have been shown to be associated with sulfonamide-induced ADR [10]. The incidence of ADR for sulfonamides has been reported to be higher in AIDS patients than in other subjects [11]. However, in this pathological situation, both the NAT2 phenotype [12] and the putative genetic impairment of NAT2 are matters of debate [13, 14]. In HIV-infected subjects, the observed discordance between the acetylator phenotype deduced from the genotype and that determined through substrate administration may reflect the impact of metabolic changes on NAT2 [12]. In general, susceptibility to NAT-related ADR involves complex molecular and cellular mechanisms [15] that probably depend on the pathophysiological status of the patients.

NAT EXPRESSION AND THE RISK OF CANCER

The NAT enzymes conjugate a variety of aromatic amine carcinogens such as benzidine (occupational exposure) or -naphthylamine (tobacco-associated compound). They are also important for *O*-acetylation of *N*-hydroxylated heterocyclic amines such as 2-amino-1-methyl-6-phenylimidazopyridine (PhIP, grilled or fried food carcinogen) [16]. The biotransformation of these compounds by *N* and/or *O*-acetylation has been linked to carcinogenesis through activation and generation of electrophilic derivatives and the subsequent formation of DNA adducts [17, 18]. Like most ADR, NAT-related carcinogenesis involves several metabolic pathways including phase I and phase II enzyme-mediated reactions. However, in contrast to what is observed for ADR, cancer risk is the outcome of a multifactorial process that generally depends on the exposure of the organism to a set of undefined and unquantifiable xenobiotics over a certain period of time. The use of animal models, such as the congenic and transgenic rodent strains developed by Hein's group, has made it possible to demonstrate that NAT2 activity may be involved in detoxification and/or the activation of bladder [16] or prostate [19] carcinogens. Caution is required when extrapolating results obtained in animal models to human health, but slow and rapid NAT2 acetylators have been associated with modest increases in susceptibility to certain cancers, including bladder and colorectal cancers, respectively [20]. The risk of colorectal tumors has been linked to high dietary intake of heterocyclic amines, the carcinogenicity of which is thought to result from the *O*-acetylation of their *N*-hydroxylated metabolites [16]. Conflicting results have been reported [21], but NAT2 phenotype probably modulates the risk of certain cancers in people exposed to aromatic amines derived from tobacco smoke, food and/or environmental pollution [22, 23]. With regards to association between NAT1 genotype and risk of cancer, the results are less consistent. However, a number of studies have shown association between rapid NAT1 and/or NAT2 acetylator genotype and colorectal cancer. Further genotype/phenotype studies are needed to confirm these findings [16]. Given the heterogeneity of NAT allele frequencies in different ethnic groups, it seems likely that different populations display different susceptibility mechanisms upon exposure to aromatic compounds [24]. Moreover, the possible modifying effects of different levels of exposure to carcinogens [25] and the involvement of other XME variants [26-28] should be taken into account when assessing the association between NAT1/2 phenotypes and cancer risk.

NAT enzymes are encoded by two separate genes located on 8p22, a chromosomal region commonly deleted in certain human cancers such as bladder tumour [29-31]. This suggests that the absence of NATs or their inactivation may contribute to carcinogenesis and/or tumor progression [30, 32, 33]. Recent findings concerning the regulation of *NAT* expression are consistent with this hypothesis (see below). The functional characterization of mice with targeted disruptions of *NAT* genes by single [34] or double [35] knock-out may provide insight into the putative oncogenic consequences of a lack of *NAT* expression.

NAT GENETIC POLYMORPHISMS

The slow and rapid NAT2 phenotypes were first characterized in the early 1950's, with the use of several drugs including isoniazid and sulfamethazine [36, 37]. NAT2 polymorphism has since been demonstrated at the DNA level, by means of RFLP analysis of DNA from healthy individuals independently phenotyped [38-41]. Expression of the *NAT2* gene was thus clearly shown to be involved in acetylation polymorphism; the slow acetylator trait was demonstrated to display autosomal recessive transmission. The common procedure for NAT2 phenotyping is based on the determination of urinary caffeine metabolite ratios after caffeine intake [42-44]. In contrast, no common drug can be used for NAT1 phenotyping and the NAT1 enzyme has long been considered to be monomorphic. However, studies in the 1990's revealed the existence of polymorphic variants at the *NAT1* locus [45]. They also showed a heterogeneous distribution of acetylation activity in individuals phenotyped with NAT1-selective probes such as *p*-aminosalicylic acid [46, 47]. To date, the *NAT1* and *NAT2* loci have been shown to express at least 26 and 36 alleles, respectively, these alleles resulting from the existence of numerous single-nucleotide polymorphisms (SNPs) (up to five SNPs per allele). A consensus allele nomenclature was published in 1995 [48] and subsequently updated by the international scientific community. The reference *NAT1* and *NAT2* alleles (respectively *NAT1*4* and *NAT2*4*) have both been associated with rapid acetylator phenotypes. A detailed description of other *NAT* alleles can be found on a dedicated website at http//www.louisville.edu/medschool/pharmacology/NAT.html.

Population studies with NAT2-selective probe drugs and *in vitro* analysis of NAT2 variants have led to the characterization of at least 24 of the 36 known allelic variants as slow-type acetylator enzymes [49]. These variants (*NAT2*5*, **6*, **7*, **14*) are associated with amino-acid substitutions (Ile^{114}→Thr, Arg^{197}→Gln, Gly^{286}→Glu and Arg^{64}→Gln, respectively) [49]. Major ethnic differences in *NAT2** allele distribution have been observed. For example, the G^{857}→A variation, corresponding to the Gly^{286}→Glu substitution (alleles *NAT2*7A* and *NAT2*7B*) is frequent in Asians (12%) but rare in persons of european descent and

Africans (1-2%). The G^{191}→A variation, corresponding to the Arg^{64}→Gln substitution (present in alleles *NAT2*14A*, **14B*, **14C*, **14D*, **14E*, **14F* and **14G*), originally identified in African Americans [50] and native Africans [51, 52], is much more frequent among Africans (7-19%) than among persons of european descent and Asians (1%). To date, 16 SNPs and one nucleotide deletion have been reported at the *NAT2* locus, all located within the NAT coding region. The relationships between polymorphic substitutions (12 amino acid substitutions have been reported) and the activity of the variant enzymes have been investigated in eukaryotic and prokaryotic expression systems. These studies suggested that the slow acetylator phenotype might in some cases result from low levels of immunoreactive NAT2 protein [53]. Large differences in the levels of production of recombinant protein variants have been reported in a yeast expression system for NAT2 variants displaying Arg^{64}→Trp, Ile^{114}→Thr, Gln^{145}→Pro or Arg^{197}→Gln substitutions [53, 54]. In other cases, adifferent mechanism is likely to be responsible for conferring the slow NAT2 phenotype. Differences in protein stability may thus account for the low levels of activity of variants displaying Arg^{64}→Trp, Arg^{64}→Gln or Gly^{286}→Glu substitutions [53, 54]. Conversely, four silent variations (T^{111}→C, C^{282}→T, C^{481}→T, C^{759}→T) and a conservative missense substitution (Lys^{268}→Arg) have been associated with rapid acetylation [55]. Overall, despite the observed heterogeneity in acetylation activity, the alleles *NAT2*5*, *NAT2*6*, *NAT2*7*, *NAT2*14* and *NAT2*17* resulting in the Ile^{114}→Thr, Arg^{197}→Gln, Gly^{286}→Glu, Arg^{64}→Gln and Gln^{145}→Pro substitutions, respectively, may be considered to be associated with the slow NAT2 phenotype [53]. However, further studies aiming to investigate the effect of combinations of SNP variations on enzyme activity are required to decipher the molecular bases of NAT2 acetylation polymorphism [53].

Whereas much is known about NAT2 polymorphism, the characterization of NAT1 polymorphism began much more recently and is less advanced. To date, 26 alleles containing SNPs (24 SNPs including 19 SNPs within the coding region), deletions or insertions (both located within the 3'-UTR region) have been reported. The reference *NAT1*4* allele (70% in persons of european descent) and *NAT1*10* which contains two SNPs outside the coding region (20% in persons of european descent) are common in all ethnic groups studied to date. The functional properties of most alleles are largely unknown. The NAT1 variants encoded by *NAT1*14B* (Arg^{187}→Gln), *NAT1*15* (Arg^{187}→Stop), *NAT1*17* (Arg^{64}→Trp), *NAT1*19* (Arg^{33}→Stop) and *NAT1*22* (Asp^{251}→Val) have been shown to generate levels of protein below the detection limits of western blotting [56]. Low intrinsic stability may also account for the weak activity of the variants encoded by *NAT1*17* and *NAT1*22* [56]. From a mechanistic point of view, NAT1 14, NAT2 15, NAT1 17 and NAT1 22 variants have been shown to be polyubiquitinated and targeted for proteasomal degradation [57]. Minchin's group has provided a plausible model for the rapid degradation of these variants. They hypothesized that NAT1 exists in the cell in either a stable acetylated state or an unstable non-acetylated state. The variant allozymes may contain

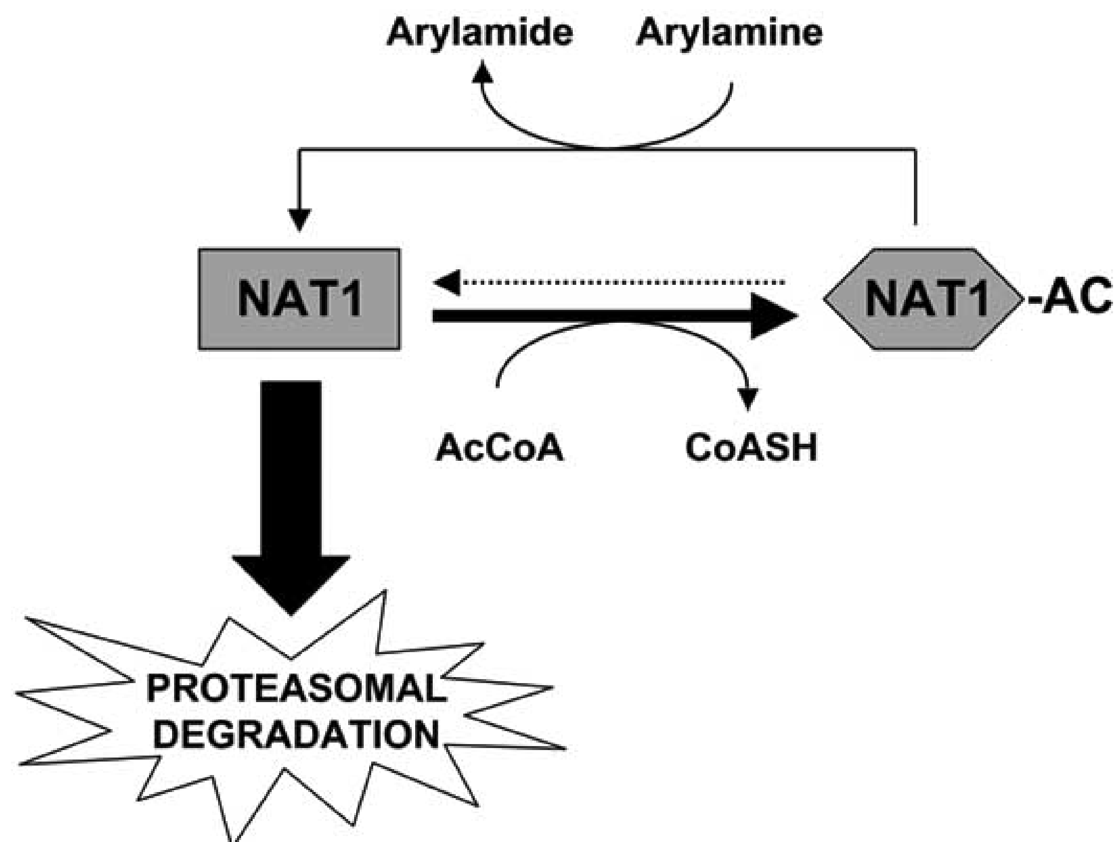

Fig. (1). Model of the proteasomal degradation of NAT1. NAT1 would exist in the cell in either a stable acetylated or a non stable unacetylated state. The latter forms are rapidly ubiquitinated and degraded. For details see [57].

structural features that prevent the catalytic formation of an acetylthiol ester and hence favor the formation of non-acetylated state of enzymes. These forms are thus rapidly ubiquitinated and degraded [57] (Fig. (**1**)). Further work is needed to confirm these original mechanisms. Slow acetylator alleles account for less than 3% of the NAT1 alleles found in persons of european descent [20] but up to 25% in certain populations [58]. Nevertheless, further studies are required to determine the effect of nucleotide variations outside the NAT1 coding region — especially those affecting the 3'-UTR — and to characterize allelic variants more fully in terms of enzyme activity.

REGULATION OF *NAT* EXPRESSION

NAT1 and NAT2 differ markedly in terms of organ and tissue distribution. NAT2 protein is present mainly in the liver [37] and gut [59]. However, a wider expression of NAT2 at the mRNA level has been described [60]. NAT1 is found in a wide range of organs and tissues, including the liver [61], colon [62], blood [63], bladder [64], placenta [65], skin [66], gingiva [67], and skeletal muscle [68]. NAT1 has also been detected in cancer cells, in which it may play a role in the development of tumor and/or its resistance to cytotoxic agents such as 2-(4-aminophenyl)benzothiazole [69] or etoposide [70]. NAT2 expression has not been detected in fetuses during prenatal period. Conversely, NAT1 activity has been reported in several fetal tissues, including the lungs, kidneys, and adrenal glands [71]. Lastly, it has been shown by RT-PCR that *NAT1* is expressed in pre-implantation embryos at the blastocyst stage, and in the placenta after as little as 5.5 weeks of gestation. Indeed, we cannot rule out the possibility that *NAT1* is expressed even earlier in gestation [72]. *NAT1* appears to be one of the first XME genes expressed during development in humans [72] and mice [34, 73]. The age-related pattern of expression of *NAT* genes in mice is consistent with the data obtained for humans [74]. The potential role of this expression pattern in developmental differences in arylamine genotoxicity was recently addressed by McQueen and colleagues [75, 76]. Studies in transgenic mice have suggested that *NAT* expression is not essential for embryonic development [34, 35]. However, Minchin suggested that human NAT1 plays a role in folate metabolism, by acetylating the folate catabolite *para*-aminobenzoylglutamate [77]. As maternal folate supplementation is known to protect against neural tube defects in the fetus, *NAT1* expression may be involved in the correct developmental patterning of the neural tube [72].

Although interindividual genetic variation is clearly important in acetylation heterogeneity, other determinants are known to control the activity of NAT enzymes. Minchin's group has demonstrated that NAT1 is downregulated by substrates in several human cell lines [32]. The substrate-dependent down regulation of NAT1 is consistent with a shift of equilibrium towards an unstable non-acetylated state of the enzyme [57] (Fig. (**1**)).

Recent studies have shown that the expression in various tissues of murine *NAT2* seems to involve the alternative splicing and/or differential utilization of more than one promoter [78]. However, until recently, the regulation of *NAT* expression at the transcriptional level had not been assessed in humans. A promoter of the human *NAT1* gene was recently identified and the functional features of a 20-base pair regulatory sequence located 245 bases upstream from the coding region were analyzed [79]. This basal promoter has been investigated by means of electrophoretic mobility shift assays, which demonstrated the involvement of AP-1 and YY-1. Mitchell and Warshawsky investigated whether tissue-specific factors regulate human NATs [80]. They transiently transfected cells with constructs containing putative proximal control regions for both the *NAT* genes and found that both the *NAT1* and *NAT2* regions were induced by 4-aminosalicylic acid or sulfamethazine in liver cells but that only one of the *NAT1* regions was inducible by both xenobiotics in bladder cells. Although these preliminary data gave some informations about NAT1 and NAT2 promoter regions, further work is needed to clearly identify and understand the role of these regions on NAT expression. Recently, Hein and co-workers have reported the first comprehensive study of nine non-coding exons of human NAT1 [81]. Their findings support the existence of four NAT1 transcription start sites. They have also demonstrated a strong promoter activity for a DNA fragment spanning a major transcription start site located 11.8 kb upstream of the single coding exon (Fig. (2)). These data

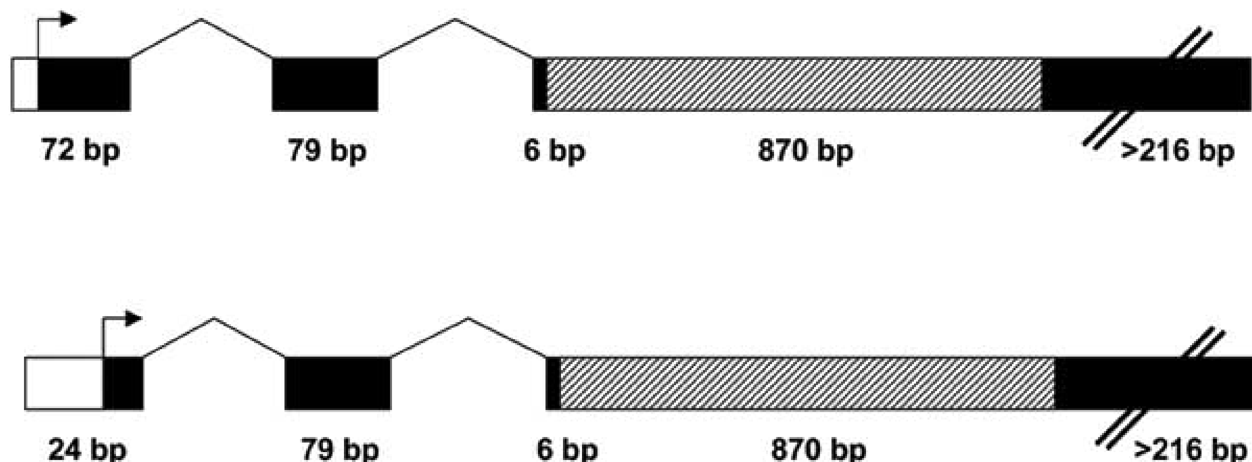

Fig. (2). Major transcripts of human NAT1. Filled boxes represent untranslated regions. Grey box is the coding sequence. The size of each exon is shown. Arrows indicate transcription start sites. For details see [81].

may lead to a better understanding of the relationships between NAT1 mRNA expression and enzymatic activity.

STRUCTURAL DATA AND COVALENT REGULATION

NAT isoforms have been detected in a number of species, from bacteria to mammals [82, 83]. The crystallographic determination of the structure of the NATs from *Salmonella typhimurium* and *Mycobacterium smegmatis* and the subsequent construction of theoretical models of human NAT1 and NAT2 have revealed structural similarities to cysteine proteases [84-87]. These data demonstrate the existence of a conserved cysteine protease-like catalytic triad (Cys, His, Asp) in NATs [84-87], confirming the fundamental role in catalysis of a reactive cysteine residue located at position 68 in the human sequence [82, 88, 89]. Thus, NATs may be prone to covalent modification of the reactive Cys residue, as described below.

The 3D model of human NAT2 has also provided clues to the possible structural and hence functional consequences of the $Arg^{64}\rightarrow Gln$ substitution present in polymorphic variants *NAT2*14A-G* [86]. However, resolution of the crystallographic structure of human NATs would be a key step towards a fuller understanding of the structural basis of the effects of polymorphic amino acid substitutions on NAT activity.

The demonstration of a reactive cysteine residue forming part of a cysteine protease-like catalytic triad in the activity of NAT enzymes has provided insight into the regulation of NAT1 activity. Minchin and co-workers have shown that the hydroxylamine of PABA (N-OH-PABA) irreversibly inactivates NAT1 in cultured cells and cell cytosols. Such inactivation could be due to the fact that N-hydroxylamine could be converted by NAT1 to an unstable intermediate that binds covalently to NAT1 [33]. Other works from Hanna and Wagner's group have shown that 2-nitrosofluorene, generated in the course of bioactivation of N-hydroxy-acetylaminofluorene (N-OH-AAF), is responsible for the irreversible inactivation of hamster NAT1 (Fig. (**3**)). The mechanism of inactivation involves NAT1-catalyzed deacetylation of N-OH-AAF, oxidative conversion to 2-nitrosofluorene and formation of a sulfinamide adduct by

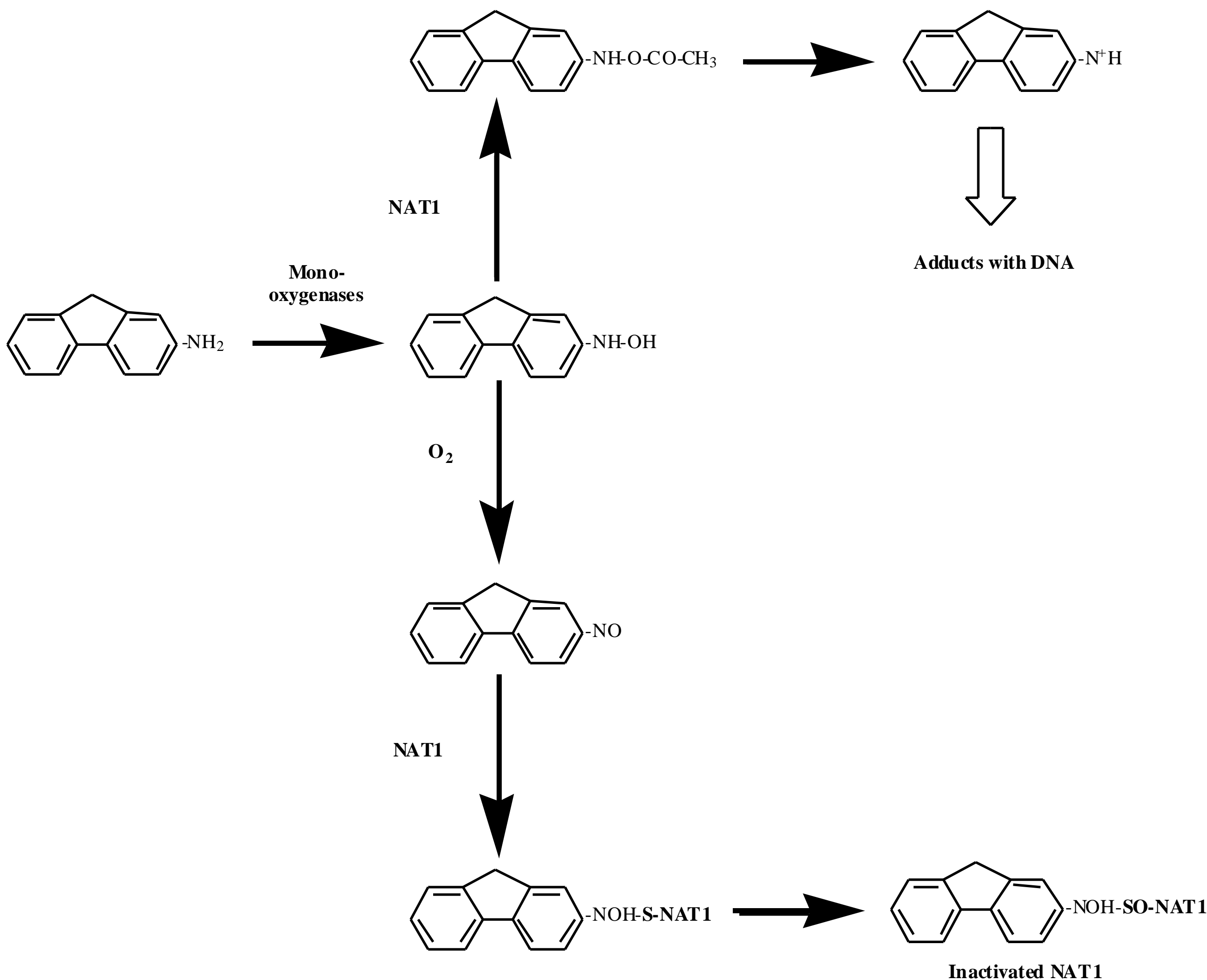

Fig. (3). Bioactivation of 2-aminofluorene and subsequent DNA adduct formation (A) and covalent inhibition of hamster NAT1 (B). Reaction of 2-nitrosofluorene with the reactive catalytic cysteine would proceed through the intermediate semimercaptal and sulfinamide forms. For details see [90].

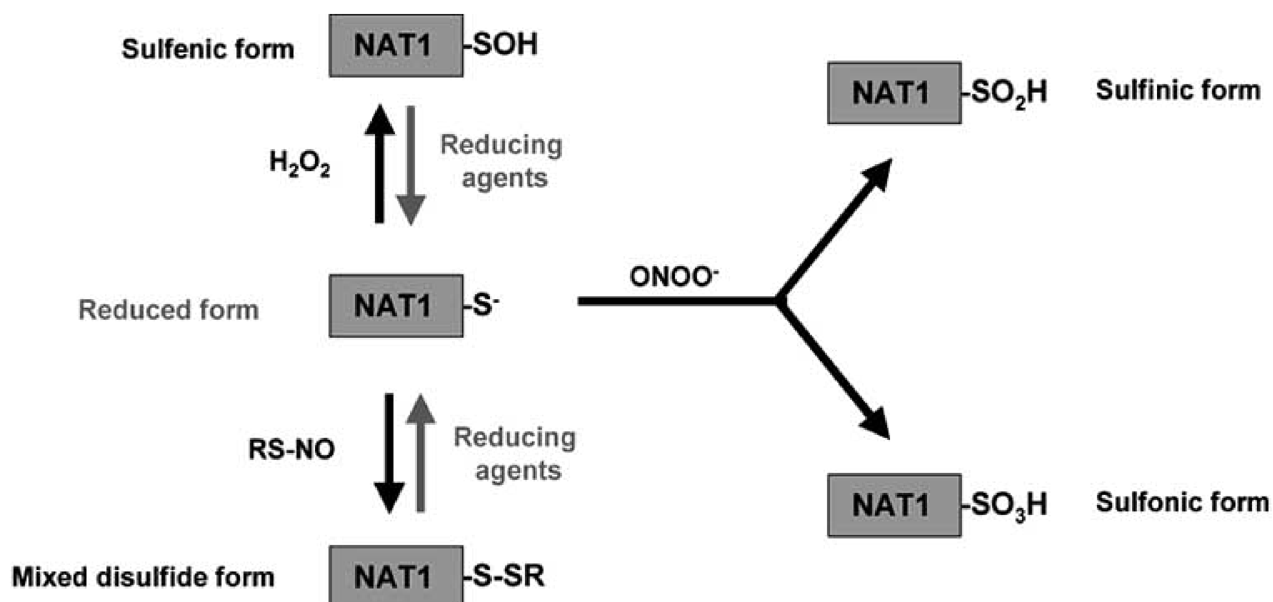

Fig. (4). Oxidative inhibition of human NAT1. The reduced form is active. H_2O_2 (hydrogen peroxide) and RS-NO (S-nitrosothiols) induce reversible inhibition. Peroxynitrite ($ONOO^-$) leads to irreversible inhibition of the enzyme. For details see [99].

reaction between 2-nitrosofluorene and the catalytic cysteine of the NAT enzyme [90]. These findings establish the importance of Cys-68 as the primary site of covalent modification of NAT1 [91]. Although they remain to be confirmed *in vivo*, they may be of significant clinical and toxicological significance.

Several key enzymes with reactive catalytic cysteine residues, such as caspases and phosphatases, have been shown to be regulated *in vitro* and *in vivo* by reactive oxygen or nitrogen species (ROS and RNS, respectively) generated during oxidative stress [92-95]. We recently showed that NAT1 activity is highly sensitive to ROS and RNS and is reversibly inactivated by physiological concentrations of hydrogen peroxide (H_2O_2). This inactivation is due to oxidative modification of the catalytic cysteine residue (Cys-68) to give a sulfenic acid form [96]. We have also shown that RNS such as *S*-nitrosothiols reversibly inactivate NAT1 by targeting the catalytic cysteine residues [97]. *S*-Nitrosothiols are an important class of RNS, believed to be the principal molecules involved in nitrosation reactions under physiological conditions [98]. The effects of hydrogen peroxide and *S*-nitrosothiols (at physiological and pathophysiological concentrations) on NAT1 activity can be abolished by reducing agents such as reduced glutathione. Furthermore, peroxynitrite a highly reactive nitrogen species generated during pathophysiological processes such as inflammation, inactivates NAT1 irreversibly in cultured cells [99]. The redox status of the reactive catalytic cysteine appears to be the key factor governing the activity of NAT1 enzyme in the presence of ROS or RNS (Fig. (**4**)). Depending on the nature of the oxidant, NAT1 inactivation may or may not be reversed by cellular reducing agents such as reduced gluthatione. Fig. (4) shows the putative redox states of the catalytic cysteine residue of NAT1 upon exposure to various oxidants [96, 97, 99]. Other XMEs, such as certain cytochrome P450s and glutathione *S*-transferases, are inactivated by cellular oxidants [100-102]. This suggests that the activity of certain XMEs may be altered or impaired in tissues in which ROS or RNS are generated, with potential effects on drug metabolism in these tissues [103-105]. In addition, high levels of oxidants such as peroxynitrite are generated during inflammation and carcinogenesis, leading to deleterious effects due to the oxidative modification of cellular proteins [106-108]. The pathophysiological significance of our findings requires confirmation in future studies. However, they already provide a possible mechanism by which increases in the formation of nitric oxide and NO-derived oxidants during inflammation and/or some drug adverse reactions can contribute to tissue damage, by decreasing NAT enzyme activity. In addition, by regulating NAT enzymes *in vivo*, nitrosative stress may also affect biotransformation pathways linked to carcinogenesis.

NAT activity is known to be related to genetic polymorphisms. However, recent advances reported in this review show that other mechanisms such as the existence of splice variants, the substrate and oxidative stress-dependent inhibition of NAT1 may also contribute to the overall activity of NAT enzymes. These additional mechanisms could explain why sometimes genotype does not necessarily reflect phenotype.

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