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Arylamine *N*-Acetyltransferase 1: A Novel Drug Target in Cancer Development

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***Abstract*——The human arylamine *N*-acetyltransferases first attracted attention because of their role in drug metabolism. However, much of the current literature has focused on their role in the activation and detoxification of environmental carcinogens and how genetic polymorphisms in the genes create predispositions to increased or decreased cancer risk. There are two closely related genes on chromosome 8 that encode the two human arylamine *N*-acetyltransferases—NAT1 and NAT2. Although NAT2 has restricted tissue expression, NAT1 is found in almost all tissues of the body. There are several single-nucleotide polymorphisms in the protein coding and 3′-untranslated regions of the gene that affect enzyme activity. However, NAT1 is also regulated by post-translational and environmental factors, which may be of greater importance than genotype in determining tissue NAT1 activities. Recent studies have suggested a novel role for this enzyme in cancer cell growth. NAT1 is up-regulated in several cancer types, and overexpression can lead to increased survival and resistance to chemotherapy. Although a link to folate homeostasis has been suggested, many of the effects attributed to NAT1 and cancer cell growth remain to be explained. Nevertheless, the enzyme has emerged as a viable candidate for drug development, which should lead to small molecule inhibitors for preclinical and clinical evaluation.**

I. Introduction: Historical Research into the *N*-Acetyltransferases

Arylamine *N*-acetyltransferases (NATs[1]; EC 2.3.1.5) are a family of highly conserved enzymes that are present in both prokaryotes and eukaryotes (Butcher et al., 2002). In humans, there are two genes that express different isozymes—*NAT1* and *NAT2*. Both enzymes acetylate a range of arylamine, heterocyclic amine, and

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[1]Abbreviations: NAT, arylamine *N*-acetyltransferase; NATb, proximal NAT1 promoter; pABA, *para*-aminobenzoic acid; pABG, *para*-aminobenzoylglutamate; PBMC, peripheral blood mononuclear cell; Rhodo-hp, (*Z*)-5-(2′-hydroxybenzylidene)-2-thioxothiazolidin-4-one; shRNA, short-hairpin RNA; THF, tetrahydrofolate; TSA, trichostatin A.

hydrazine substrates, including many common carcinogens and therapeutic agents (Hein, 2000). Both NATs are predominantly cytosolic proteins of 289 amino acids, giving them a size of approximately 33 kDa.

The NATs were among the first drug-metabolizing enzymes shown to demonstrate genetic variation in humans (Weber and Hein, 1985). Plasma levels of drugs predominantly metabolized by NAT2, such as dapsone, isoniazid, and caffeine (Gardiner and Begg, 2006), often showed a nonunimodal distribution after administration. This was of interest in carcinogenesis because many arylamine carcinogens require metabolic activation, including acetylation, to induce cancer. After the cloning of *NAT1* and *NAT2* in the early 1990s (Ohsako and Deguchi, 1990), the molecular mechanism for human acetylator phenotype was finally understood. Several important single nucleotide polymorphisms in the *NAT2* gene were identified that resulted in a loss in enzyme activity (Deguchi, 1992). On the basis of this early work, numerous studies have been published associating NAT2 genotype, carcinogen exposure, and cancer risk. These studies have been widely reviewed elsewhere (Hein, 2000, 2006).

Compared with NAT2, NAT1 and its potential role in cancer have been largely ignored. However, in recent years, it has become increasingly evident that NAT1 is aberrantly expressed in malignancies and that it is inhibited by a variety of dietary and chemotherapeutic agents. This review summarizes the current knowledge of NAT1 and human cancer and its regulation and potential as a drug target in cancer treatment.

II. Regulation of *N*-Acetyltransferase 1 Enzyme Activity

Over the last decade, a number of studies have investigated both the genetic and nongenetic regulation of NAT1 expression. First, it was established that NAT1, like NAT2, is genetically polymorphic, with some genotypes showing an increased susceptibility to certain types of cancers (see section IV.A). However, the transcriptional and post-transcriptional regulation of NAT1 seems to have a greater effect on NAT1 activity than genotype. In addition, important post-transcription regulatory mechanisms have also been reported both in vitro and in vivo. Because the pharmacogenetics of both NAT1 and NAT2 have been extensively reviewed (Walraven et al., 2008a,b), this section focuses on the dynamic regulation of NAT1 by epigenetic and post-translational mechanisms and how this could affect human malignancy.

A. Structure and Catalytic Function

Early studies recognized that the acetylation of substrates by the NATs involved sulfhydryl-containing amino acids (Tabor et al., 1953; Andres et al., 1988; Cheon et al., 1992), and subsequent site-directed mutagenesis studies identified Cys^{68} as the critical sulfhydryl involved in acetyl transfer (Dupret and Grant, 1992). The acetylation reaction occurs in two sequential steps according to a "ping-pong bi bi" reaction mechanism, where first the acetyl group is transferred from the donor acetyl-CoA to Cys^{68} of the enzyme and then to the primary amine of the acceptor substrate (Minchin et al., 2007). Further mutagenesis studies identified the amino acids and regions of the proteins that are important for determining substrate specificity and intrinsic stability (Dupret et al., 1994; Deloménie et al., 1997; Goodfellow et al., 2000). A major breakthrough in our understanding of the structure and catalytic mechanism of NATs came about when Sinclair et al. (2000) successfully crystallized the enzyme from *Salmonella typhimurium*. Unexpectedly, the NAT active site consisted of a Cys-His-Asp catalytic triad that is structurally similar to that found in cysteine proteases and transglutaminases. This catalytic triad is strictly conserved in all known functional NATs from bacteria to humans. Computational modeling of human NAT1, using the bacterial crystal data, identified the catalytic triad Cys^{68}-His^{107}-Asp^{122} and suggested that the active site of the enzyme resides in a cleft that runs across the molecule (Rodrigues-Lima et al., 2001). This was later confirmed by the successful crystallization of the human NATs, which revealed that the active site is buried deep within the core of the proteins, possibly to exclude water and ensure that the rate of hydrolysis of the acetyl-sulfhydryl intermediate is not too rapid (Wu et al., 2007). The molecular mechanism of acetylation by NAT1 has been characterized by Wang et al. (2004, 2005a) using hamster purified NAT2 (orthologous to human NAT1). Their model proposes that a thiolate-imidazolium ion pair is formed between Cys^{68} and His^{107} with a pK_a of 5.2 and that Asp^{122} is required for optimal catalysis and structural stability. Upon acetylation of the thiolate, the ion pair is lost, and the pK_a of His^{107} shifts to 5.5. The process of deacetylation of the thiolate is dependent on the nucleophilic strength of the arylamine substrate. Those substrates with pK_a values <5.5 (weak nucleophiles) cause deacetylation by nucleophilic attack of the thiol ester, whereas those having pK_a values ≥ 5.5 (strong nucleophiles) do so by deprotonation of a tetrahedral intermediate (Fig. 1).

B. Susceptibility to Oxidation and Active Site Modification

Oxidative stress is a common mediator of cancer; reactive species are now known to directly damage DNA, causing tumor initiation and progression, altering gene expression and signaling pathways important in tumor promotion, and inactivating enzymes involved in the metabolic activation and detoxification of chemical carcinogens (Halliwell, 2007). During oxidative stress, a number of different reactive oxygen and nitrogen species are generated that can covalently bind to and inactivate

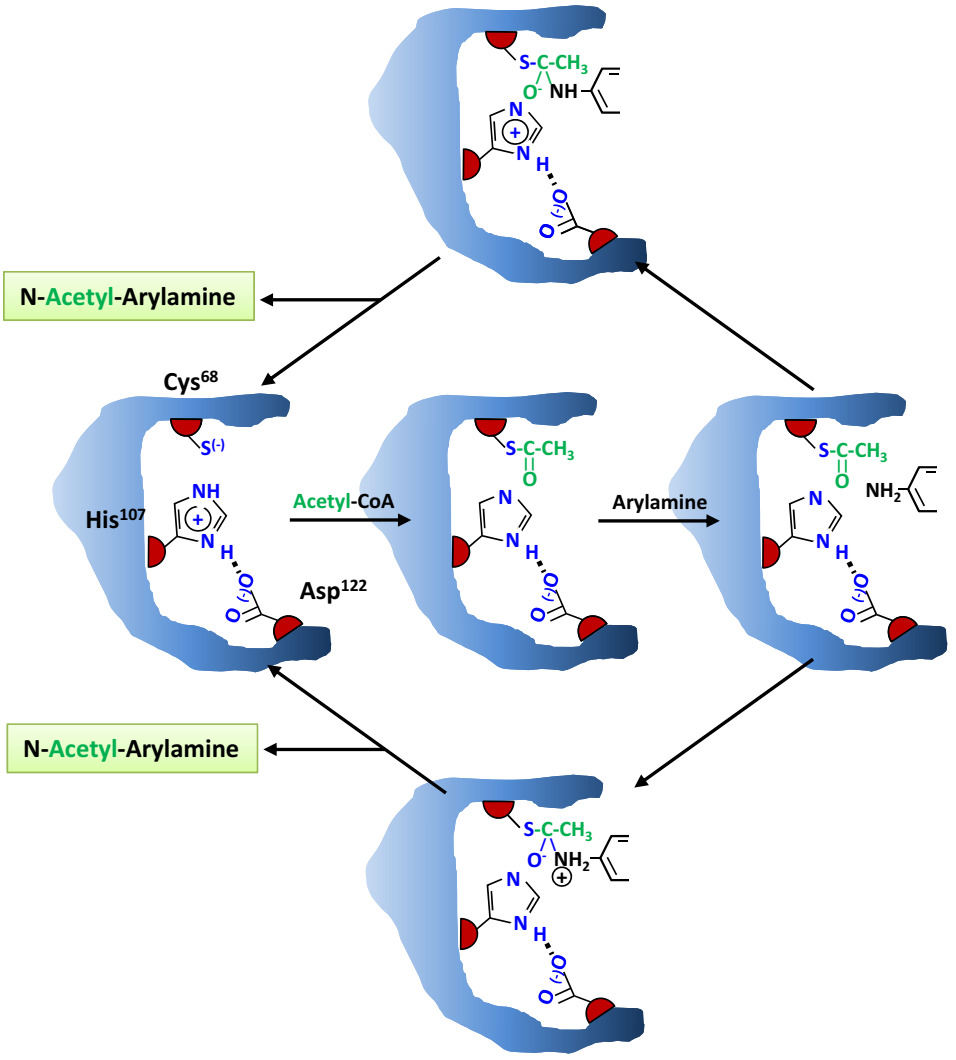

FIG. 1. Transfer of acetyl group from acetyl-CoA to substrate by the catalytic triad of NAT1. The ion pair between His^{107} and Cys^{68} allows for the efficient formation of an acetylated thiolate (center of figure). This results in a shift in the pK_a of His^{107} from 5.2 to 5.5. Deacetylation of Cys^{68} is dependent on the nucleophilic strength of the substrate. Weak nucleophiles directly attach the thiol ester (upper pathway), whereas strong nucleophiles deprotonate the tetrahedral intermediate (lower pathway). Data from Wang et al. (2004, 2005a).

redox-sensitive enzymes. Several xenobiotic metabolizing enzymes that have cysteine-containing active sites, such as glutathione transferases and sulfotransferases, are susceptible to oxidation and loss of catalytic function (Wong et al., 2001; Maiti et al., 2007).

It has been demonstrated that the enzymatic activity of NAT1 can be modulated by direct chemical modification of the active-site cysteine (Cys^{68}) by reactive chemical species (Dupret et al., 2005; Rodrigues-Lima et al., 2008). Using purified recombinant human enzyme, Atmane et al. (2003) showed that NAT1 is rapidly inactivated by physiological concentrations of hydrogen peroxide (H_2O_2). The inactivation was reversible upon treatment with thiols such as GSH or dithiothreitol, and acetyl-CoA could protect against inactivation, implicating the active-site cysteine as the site of oxidation. Dairou et al. (2003) investigated the effect of nitric oxide-derived oxidants on NAT1 activity, because its catalytic triad is structurally similar to that of factor XIII transglutaminase, which is regulated by *S*-nitrosothiols. They showed that human NAT1 was reversibly inactivated by *S*-nitrosothiols, such as *S*-nitroso-*N*-acetyl-DL-penicillamine and *S*-nitrosoglutathione, via the formation of mixed disulfides involving Cys^{68} (Dairou et al., 2003). Again, inactivation was reversed by reducing agents such as GSH or dithiothreitol. The powerful sulfhydryl oxidant peroxynitrite rapidly inactivated purified recombinant human NAT1 by irreversible modification of the active-site cysteine. High concentrations of the reducing agents could partially protect against inactivation but could not reverse it. GSH is the major determinant of cellular redox potential and is generally present in cells at concentrations of at least 1 to 2 mM, which is sufficient to partially protect against and/or reverse the effects of cellular oxidants on NAT1 activity. As a result, factors that induce oxidative stress and alter GSH levels, such as UV light, inflammation, and various disease states, could potentially modulate the susceptibility of

NAT1 to oxidation and subsequent inactivation. It has been reported that cytokines can cause a decrease in NAT1 activity, possibly by oxidative stress-induced inactivation of the enzyme (Buranrat et al., 2007). However, this study also showed that NAT1 mRNA levels were considerably lower in cytokine-treated cells, suggesting that additional mechanisms may contribute to the loss of NAT1 activity, particularly for longer exposure times.

Inactivation of NAT1 by cellular oxidants has also been demonstrated in cultured cells. Physiologically relevant levels of peroxynitrite irreversibly inactivated NAT1 in both human breast cancer MCF7 cells (Dairou et al., 2004) and human lens epithelial HLEB3 cells (Dairou et al., 2005). By contrast, H_2O_2 caused inactivation that was reversible by GSH (Dairou et al., 2005). In addition, UVB irradiation caused a dose-dependent inactivation of endogenous NAT1 that was fully reversible by GSH, suggesting the involvement of oxidants such as superoxide or H_2O_2. Similar results were observed when human bronchial epithelial cells were exposed to H_2O_2 or peroxynitrite (Dairou et al., 2009). It is noteworthy that exposure to pathophysiologically relevant amounts of these oxidants impaired the NAT1-dependent biotransformation of the carcinogens 2-aminofluorene and 4-aminobiphenyl by these cells, potentially affecting their carcinogenic activity (Dairou et al., 2009). Taken together, these studies show that NAT1 is a redox-sensitive enzyme. Moreover, the redox state of the cell may determine the ability of the enzyme to catalyze the acetylation of important human carcinogens.

NAT1 activity can be modulated by substrates after acetylation to reactive metabolites. NAT1 is able to catalyze both N- and O-acetylation reactions. N-acetylation is generally a detoxification step, whereas O-acetylation of *N*-hydroxylated arylamines or intramolecular N,O-acetyltransfer of *N*-arylhydroxamic acids results in bioactivation (Hanna et al., 1982; Hanna, 1994). The resulting acetoxy esters are unstable and spontaneously decompose to arylnitrenium ions, which are highly electrophilic and able to form covalent adducts with DNA to initiate cancer. In addition to O-acetylation, *N*-hydroxyarylamine metabolites can also undergo oxidation to nitrosoarenes, which react readily with nucleophilic thiols present in proteins (Shear and Spielberg, 1985; Cribb and Spielberg, 1990). Several studies have demonstrated that reactive arylamine metabolites can act as irreversible "suicide" inhibitors of NAT1 (Hanna, 1994). Early studies demonstrated that the *N*-arylhydroxamic acid *N*-hydroxy-2-acetylaminofluorene, as well as a variety of structurally similar *N*-arylhydroxamic acids, function as irreversible mechanism-based inhibitors of both hamster and rat hepatic NAT (Smith and Hanna, 1988; Wick et al., 1988; Hanna et al., 1990; Sticha et al., 1998). The mechanism of inactivation of hamster NAT involved NAT-catalyzed deacetylation of *N*-arylhydroxamic acid *N*-hydroxy-2-acetylaminofluorene to the hydroxylamine *N*-hydroxy-2-aminofluorene, which undergoes oxidation to the nitrosoarene 2-nitrosofluorene, followed by reaction with the active-site cysteine of the NAT enzyme.

More recently, Liu et al. (2008) showed that the nitrosoarene metabolites of several important arylamine carcinogens could inactivate human NAT1 both in vitro and in cultured cells. Nitrosoarenes derived from arylamines that were efficiently acetylated by NAT1 were the most potent inactivators of the enzyme. Mass spectrometric analysis of FLAG-tagged NAT1 protein purified from HeLa cells treated with the nitrosoarene 4-nitrosobiphenyl confirmed that intracellular inactivation of NAT1 was due to the formation of a sulfinamide adduct between 4-nitrosobiphenyl and the active-site cysteine (Cys^{68}) (Liu et al., 2008), confirming earlier in vitro studies using hamster NAT (Guo et al., 2004; Wang et al., 2005b).

In addition to the carcinogenic arylamines, the hydroxylamine metabolites of the prototype substrate *p*-aminobenzoic acid (pABA) and the sulfonamide drug sulfamethoxazole also inactivate human NAT1 (Butcher et al., 2000a). Treatment of human peripheral blood mononuclear cell (PBMC) cytosols with low micromolar concentrations of the hydroxylamines caused irreversible inhibition of the enzyme. Addition of acetyl-CoA completely protected against inactivation, suggesting that the mechanism involved covalent modification of the active-site cysteine. Hydroxylamine metabolites do not readily react with proteins or thiols themselves, so it is likely that the inactivation of NAT1 is mediated by the more reactive nitroso metabolite formed by spontaneous oxidation, as found to be the case for the carcinogenic arylamines (Liu et al., 2000). Similar results were observed using cultured PBMCs, substantial inactivation of NAT1 occurring within 30 min of treatment and remaining in excess of 24 h.

NAT1 activity can also be inhibited by a range of chemically diverse compounds that are not known substrates for the enzyme, including novel small-molecule inhibitors, heavy metals, plant extracts, nanoparticles, and therapeutic agents. Ragunathan et al. (2010a,b) investigated the effects of mercury and cadmium on NAT-dependent acetylation. These heavy metals have high affinities for reactive thiol groups and are capable of inactivating thiol-containing enzymes (Jacoby et al., 1999; Bridges and Zalups, 2005). Both inorganic (Hg^{2+}) and organic (CH_3Hg^+) mercury inactivated purified recombinant human NAT1 at biologically relevant concentrations, with IC_{50} values of 0.25 and 1.4 μM, respectively (Ragunathan et al., 2010a). Cadmium also inactivated the enzyme (IC_{50}, 0.055 μM); total inhibition was observed at concentrations as low as 0.3 μM (Ragunathan et al., 2010b).

Exposure to mercury and cadmium commonly occurs via cigarette smoke (Bridges and Zalups, 2005; Martelli et al., 2006), so Ragunathan et al. (2010a,b) used lung

epithelial cells to assess the effect of these heavy metals on acetylation of the arylamine carcinogens 2-aminofluorene and 4-aminobiphenyl, which are also found in cigarette smoke (Hein, 1988). Human A549 cells exposed to either inorganic or organic mercury showed a dose-dependent inhibition of NAT1 activity, with IC_{50} values of 3 and 20 μM, respectively (Ragunathan et al., 2010a). Murine Clara cells exposed to cadmium had a decreased capacity to acetylate the carcinogenic arylamine substrates (Ragunathan et al., 2010b). Furthermore, several tissues from C57BL/6J mice treated with cadmium showed impaired endogenous acetylation capacity compared with nontreated control mice. Local inactivation of NAT1 in lung tissue by heavy metals in cigarette smoke could therefore result in increased toxicity to arylamine carcinogens as a result of impaired detoxification (N-acetylation) and subsequent increased bioactivation by other enzymes such as the cytochromes P450 and sulfotransferases. Inactivation of NAT1 by oxidants in the lung could result in a similar scenario. Further study is required to elucidate the overall role of these inactivating compounds on in vivo drug and carcinogen acetylation capacity.

Recent studies have shown that various nanoparticles can interact with proteins and cause unfolding and loss of function (Cedervall et al., 2007; Zhang et al., 2009; Deng et al., 2011). In particular, the activities of some cytochrome P450 isozymes are impaired by interaction with silver nanoparticles (Lamb et al., 2010). Sanfins et al. (2011) assessed whether the acetylating capacity of pulmonary epithelial Clara cells was affected by carbon black nanoparticles, which are widely used in industry (Lin et al., 2002). They showed that the nanoparticles impaired acetylation of the carcinogen 2-aminofluorene in both cell lysates and intact cells. In addition, using purified recombinant human NAT1, they showed that the molecular mechanism involved a direct interaction of the nanoparticle with the NAT1 enzyme and that nanoparticle-bound enzyme was devoid of activity. Circular dichroism spectroscopy showed conformational changes in NAT1 structure upon interaction with the nanoparticles, resulting in enzyme inactivation. Because of the widespread exposure to carbon black nanoparticles, modification of aromatic amine metabolism by these particles could contribute to individual susceptibility to some forms of cancers.

A number of studies by Chung and colleagues (Chung et al., 2003; Lin et al., 2005b; Yu et al., 2005) have shown that several plant extracts that exhibit anticancer activity and are used as Chinese herbal remedies can decrease the acetylation capacity of mammalian cancer cell lines. Their studies suggest that the effect of these compounds on NAT1 activity is due to both decrease in gene expression and direct inactivation of the enzyme itself. Berberine, wogonin, and aloe-emodin all inhibited the mouse homolog of human NAT1 in mouse leukemia L1210 cell cytosols at concentrations of less than 50 μM (Chung et al., 2003; Lin et al., 2005b; Yu et al., 2005). Kinetic studies suggested that the inhibition was uncompetitive. In addition, at higher concentrations in intact cells, these compounds caused a reduction in NAT protein and mRNA expression. Similar results were obtained using various human cancer cell lines, where a decrease in the acetylation of the carcinogen 2-aminofluorene as well as reduced DNA adduct formation was observed (Chiu et al., 2004; Lin et al., 2005a,c). The possible involvement of NAT1 inhibition in the action of these compounds as anticancer agents remains to be fully elucidated. The effects of small-molecule inhibitors and therapeutic agents on NAT1 activity are discussed in section IV.

C. Substrate-Dependent Down-Regulation

There are many examples of drug-metabolizing enzymes that are regulated by their substrates, particularly the cytochromes P450 (Xu et al., 2005). A similar effect has been reported for NAT1. When cultured in RPMI 1640 medium, human PBMCs lost their ability to acetylate aromatic substrates (Butcher et al., 2000b). The loss of NAT1 activity was caused by the presence of 6 μM pABA in the culture medium. NAT1 activity also was down-regulated by other substrates for NAT1 but not by substrates for the closely related enzyme NAT2. The loss of activity was the result of a concomitant loss of NAT1 protein. In addition to PBMCs, pABA down-regulated NAT1 in several cancer cell lines of differing origin, but only when cells were grown at confluence and growth arrest was evident. It is noteworthy that no loss in NAT1 activity was observed when cells were in exponential growth. Furthermore, the NAT1 activity of pABA-treated PBMCs could be restored to original levels by treatment with phorbol ester, which induces cell proliferation of normally quiescent PBMCs. A more recent study showed that the hair dye ingredient *p*-phenylenediamine, which is acetylated by NAT1, was able to down-regulate NAT1 activity and protein in human primary epidermal keratinocytes as well as in the immortalized keratinocyte cell line HaCaT (Bonifas et al., 2010). In addition, NAT1 activity varied with cell-cycle phase and the HaCaT cells with high NAT1 activity proliferated more rapidly that those with low NAT1 activity. Together, these findings suggest that NAT1 may be regulated differently depending on the proliferative status of the cell or that NAT1 may be linked to proliferation in cancer cells.

Although the molecular mechanism linking NAT1 to cell proliferation has yet to be elucidated, a hypothesis explaining the mechanism of substrate-dependent down-regulation has been presented (Butcher et al., 2004). In the absence of substrate, the NAT1 protein is very stable, having a half-life in excess of 24 h. Because the active-site cysteine can be acetylated by cofactor (acetyl-CoA) in the absence of substrate, NAT1 may exist inside the cell in nonacetylated or acetylated form,

the latter being resistant to degradation. In the presence of substrate, this equilibrium is shifted to the nonacetylated form, which is then rapidly degraded. Immunoprecipitation studies using cells transfected with NAT1 and a hemagglutinin-tagged ubiquitin vector showed that the presence of pABA markedly increased the polyubiquitination of NAT1 (Butcher et al., 2004). This demonstrated that NAT1 turnover involved targeting to the proteasomes and that substrate binding enhanced this targeting. Mutagenesis studies, where the active-site cysteine was changed to a tyrosine, showed that the resulting protein was unable to be acetylated and was rapidly polyubiquitinated and degraded by the proteasomes, supporting the model for the regulation of NAT1 protein stability.

Acetylation of proteins has emerged as an important determinant of protein stability, and an increasing number of important proteins are reportedly acetylated (Caron et al., 2005). Most often, a lysine residue is modified, which blocks ubiquitination and subsequently leads to protein stabilization. However, there are several examples in which lysine acetylation leads to enhanced protein degradation or N-terminal acetylation provides protection against polyubiquitination and subsequent protein degradation, suggesting that acetylation-dependent regulation of protein stability is more complex than initially thought (Caron et al., 2005). The regulation of NAT1 stability by reversible cysteine acetylation is novel and may represent a post-translational modification applicable to other proteins, in particular some caspases and transglutaminases that have similar catalytic triads to NAT1.

D. Epigenetic Regulation

Epigenetic regulation is an important determinant of gene expression (Razin, 1998). Genes can be silenced by DNA methylation at CpG islands in their promoter regions and/or by histone deacetylation, which closes the chromatin structure and prevents transcription. Both of these processes are reversible, resulting in a dynamic control of gene expression. Epigenetic regulation affects the expression of many drug metabolizing enzymes (Hirota et al., 2008; Gomez and Ingelman-Sundberg, 2009; Glubb and Innocenti, 2011), and recent studies suggest that similar factors contribute to the regulation of NAT1.

The first study reporting epigenetic control of the *NAT1* gene investigated the methylation status of the 5′-untranslated region proximal to the start codon in normal, benign, and malignant breast tissues (Kim et al., 2008). Using bisulfite sequencing, these authors showed that the CpG islands in this region of the gene were significantly less methylated in malignant cancer tissue compared with normal and benign tissues. Furthermore, they showed that NAT1 mRNA expression in malignant breast tissue was approximately 1000-fold higher than that for benign and normal breast tissues. The 5′-untranslated region of the *NAT1* gene investigated in this study is located in an intron approximately 11 kilobases downstream of the constitutive promoter for NAT1 (NATb) (Butcher et al., 2003), so methylation in this region is unlikely to directly silence the *NAT1* gene. However, methylation at this distant region may closely correlate with methylation of CpG islands located in the promoter region of the gene. In a follow-up study, Kim et al. (2010) showed that methylation of the *NAT1* gene was significantly lower in control compared with tamoxifen-resistant breast cancer tissue (Kim et al., 2010), suggesting that DNA methylation of the *NAT1* gene may serve as a marker for tamoxifen resistance. These studies show that the *NAT1* gene is normally partially silenced by DNA methylation, at least in breast tissue.

Wakefield et al. (2010) reported tissue-specific CpG methylation of the mouse *Nat2* gene (functional equivalent of human NAT1). They assessed the methylation status of several CpG islands located within or near the core promoter and found that, in this region, the gene was predominantly unmethylated (<20%) in all tissues examined. However, the levels of methylation did seem to vary in a tissue-specific manner.

The histone deacetylase inhibitors sodium butyrate and trichostatin A (TSA) increased NAT1 activity in human cancer cells (Paterson et al., 2011). Acetylation and deacetylation of histones regulates their association with DNA and influences chromatin structure and gene transcription. TSA treatment resulted in a greater than 25-fold increase in NAT1 mRNA expression driven by NAT1 promoter NATb. Using NATb luciferase reporter constructs, the Sp1 binding motif, previously shown to be essential for constitutive expression of NAT1 (Boukouvala and Sim, 2005; Husain et al., 2007), was identified as essential for TSA responsiveness.

In summary, it is now evident that epigenetic regulation, substrate-dependent down-regulation, and cellular redox potential can affect NAT1 activity in normal and transformed cells. These nongenetic mechanisms provide an additional level of complexity to our understanding of the role of NAT1 in health and disease.

III. Role of *N*-Acetyltransferase 1 In Vivo

A. Role of N*-Acetyltransferase 1 in Folate Metabolism*

The NATs have a relatively restricted substrate specificity that almost exclusively comprises primary aromatic and heterocyclic amines and hydrazines. These compounds are rarely found naturally in cells. Unlike many of the aliphatic *N*-acetyltransferases that have been described, the NATs are not known to acetylate proteins or other biological macromolecules. An exception is *p*-aminobenzoylglutamate (pABG), a catabolite produced when folates are cleaved at the C_9-N_{10} bond. pABG was first identified as a selective NAT1 substrate in 1995 (Minchin, 1995), which has been sub-

sequently confirmed both in vitro (Ward et al., 1995) and in vivo (Wakefield et al., 2007a). It was originally suggested that intracellular acetylation of pABG might enhance its excretion because *N*-acetyl-pABG is a major folate metabolite in human urine (McPartlin et al., 1993).

pABG can be formed after cleavage of the reduced folates [dihydrofolate, tetrahydrofolate (THF), and their derivatives] in an oxidative environment (Ha et al., 1990). However, its formation can be accelerated by other factors. For example, overexpression of methylene-THF synthetase, which converts the inactive 5-formyl-THF (leucovorin) to 5,10-methylene-THF, significantly enhances the intracellular production of pABG (Anguera et al., 2003). Moreover, Suh et al. (2000) have shown that the iron binding protein H-ferritin can catalyze the cleavage of folates to pABG.

There have been very few studies into the biological effects of pABG. It is a relatively weak inhibitor of dihydrofolate reductase (Williams et al., 1980) as well as of the proton-coupled folate transporter (Nakai et al., 2007). Both of these studies used monoglutamated pABG, and it is well known that polyglutamation enhances binding affinity of most folates for their respective target proteins (Suh et al., 2001). This may also be the case for pABG. For example, pABG inhibition of glutamate dehydrogenase is significantly influenced by the degree of polyglutamation (White et al., 1976). It is therefore possible that the intracellular accumulation of pABG, or its polyglutamated forms, after NAT1 inhibition leads to inhibition of folate-dependent pathways.

In mammalian cells, reduced folates are essential for the formation of *S*-adenosylmethionine, the cofactor for both protein and DNA methylation reactions. Any perturbation of the folate pathway might therefore alter *S*-adenosylmethionine levels. The therapeutic agents procaine and procainamide are structurally similar to pABG and have been shown to be specific inhibitors of human DNA methyltransferase (Villar-Garea et al., 2003; Lee et al., 2005). Docking studies show that the aromatic amine moiety of each drug can form hydrogen bonds with Pro^{86} and Glu^{128} within the active site of the enzyme. In addition, the modeling identified potential ionic interactions between the carbonyl group of each drug and Arg^{174} of the enzyme (Singh et al., 2009). Both the aromatic amine group and the carbonyl group are conserved in pABG (Fig. 2), suggesting it too may inhibit methyltransferases. In a study using siRNA-mediated NAT1 depletion in HT-29 cells, up-regulation of the tumor suppressor gene E-cadherin was reported (Tiang et al., 2011). It is noteworthy that this was not due to changes in Snail, Slug, or Twist, which have a major repressive role in E-cadherin expression (Wu and Bonavida, 2009). Instead, it was proposed that NAT1 down-regulation altered E-cadherin methylation status, possibly as a result of intracellular pABG accumulation. Further investigation is needed to establish whether loss of NAT1 activity alters intracellular pABG and whether methyltransferase activity is affected.

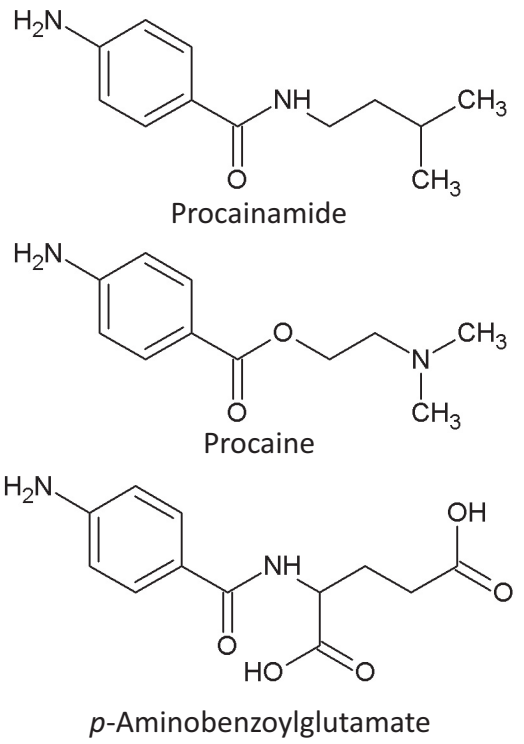

FIG. 2. Structural similarities of *p*-aminobenzoylglutamate and the therapeutic agents procainamide and procaine. Both drugs are inhibitors of human DNA methyltransferases. It has been proposed that the primary aromatic amine forms hydrogen bonds with Pro^{86} and Glu^{128} in the active site of DNMT1, whereas the carbonyl group interacts ionically with Arg^{174} (Singh et al., 2009).

Jensen et al. (2006) have reported that the risk of spina bifida, a congenital abnormality closely linked to folate intake before and during pregnancy, was related to the relatively rare *NAT1* alleles known to result in loss of enzyme function. Reduced maternal or offspring NAT1 activity decreased the incidence of spina bifida, suggesting that higher folate levels may be present in these subjects, although this was not quantified. Alternatively, the authors suggested NAT1 might have a role in activating endogenous/exogenous teratogens that enhance risk of spina bifida. In support of this, they independently reported that the *NAT1*10* allele was associated with a greater risk of spina bifida but only in offspring of mothers who smoked (Jensen et al., 2005). It is noteworthy that persons with the *NAT1*10* allele who smoked also had significantly higher levels of circulating homocysteine (Stanisławska-Sachadyn et al., 2006), which is formed during *S*-adenosylmethionine turnover.

Lammer et al. (2004) reported an increased risk of orofacial cleft in persons homozygous for the 1095A allele in the *NAT1* gene compared with those homozygous for the 1095C allele, although the data did not reach statistical significance. There was no change in risk for cleft palate in any of the NAT1 genotypes examined. Both of these abnormalities have been linked to folate insufficiency.

B. Mouse Knockout Models

The mouse homolog (*Nat2*) of the human *NAT1* gene was first deleted in a murine model in 2003 by two independent laboratories (Cornish et al., 2003; Sugamori et al., 2003). Cornish et al. (2003) replaced the *Nat2* gene with LacZ and backcrossed progeny onto an A/J and C57BL/6 background. There was no observable effect on allelic inheritance or fertility, although a statistically significant sex bias for female offspring in *Nat2*(−/−) mice was noted. Sugamori et al. (2003) generated a double knockout of both the *Nat1* and *Nat2* genes. These were also bred onto a C57BL/6 background, and no overt phenotype attributable to the gene knockout was reported, indicating that *Nat2* is not required for normal growth and development. Both knockout mouse lines demonstrated loss of enzyme activity in all tissues examined. Although originally generated as models to examine the role of arylamine *N*-acetyltransferases in the bioactivation and detoxification of carcinogens and mutagens, they have primarily been used to examine the possible endogenous role of the Nat2 enzyme. In Nat2-null animals, there was a complete loss of acetylated pABG in urine, confirming that Nat2 is homologous to human NAT1 and that no other enzyme seems to catalyze pABG acetylation, at least in mice (Wakefield et al., 2007a).

Continual breeding of the *Nat2* deficient A/J strain revealed ocular defects after approximately seven generations, at which time the A/J sequence homozygosity was almost 100% (Wakefield et al., 2007b). The incidence of the defects (cataract, microphthalmia, and anophthalmia) rose from less than 1% to greater than 10% by generation 12 and was more common in the right eye. In the *Nat2*-null mice bred on the C57BL/6 background, this increase in ocular defect was not observed. It is noteworthy that the phenotype in the A/J mice was more prevalent in offspring from at least one heterozygote parent than from parents homozygous for either wild type [*Nat2*(+/+)] or null [*Nat2*(−/−)[. A similar observation was reported in a much smaller study of neural tube defects in the C57BL/6 *Nat2*-null strain (Wakefield et al., 2007a). Although the incidence of the defect was seen in only 1 of 64 embryos (<2%) from *Nat2*(−/−) mice, it was as high as 14% in heterozygotes. Taken together, these results suggest the homozygous null mice may have undergone some form of adaptation to compensate for the loss of Nat2 activity. This may not have been necessary when only one *Nat2* allele was deleted, so the lack of compensation was then evident in F1 offspring.

IV. *N*-Acetyltransferase 1 Expression in Cancers

A. Epidemiological Association of N*-Acetyltransferase 1 with Cancer Risk*

The first evidence that the NAT1 locus might be genetically variant was published in 1993, when a single-nucleotide polymorphism in a consensus polyadenylation signal (T1088A) in the 3′-untranslated region of the gene was reported (Vatsis and Weber, 1993). Other polymorphisms began to emerge in the literature, but it was not until 1998 that single nucleotide polymorphisms in the coding region of *NAT1* were shown to affect enzyme activity (Butcher et al., 1998; Hughes et al., 1998; Lin et al., 1998). However, each of these polymorphisms is relatively rare compared with many in the *NAT2* gene. Consequently, thorough studies of their epidemiology and association with disease are lacking because of the many thousands of case and control subjects required for meaningful results. One allele (*NAT1*10*) has received considerable attention, first because it is more prevalent in the population and second because it has been associated with an increase in NAT1 activity. The *NAT1*10* allele is characterized by two single-nucleotide polymorphisms (T1088A, C1095A), both in the 3′ untranslated region of the gene. Consequently, the polymorphisms do not affect protein sequence. However, Bell et al. (1995a) reported that this allele led to higher NAT1 activity in the bladder, which they speculated was due to increased mRNA stability . Moreover, patients with bladder cancer and at least one *NAT1*10* allele showed a significantly higher level of DNA adducts compared with those having the wide-type *NAT1*4* allele (Badawi et al., 1995). A similar increase in acetylation toward benzidine was reported in liver slices from subjects with at least one *NAT1*10* allele (Zenser et al., 1996). However, NAT1 activity in peripheral blood did not seem to differ among subjects with the *NAT1*10* or *NAT1*4* allele in two independent studies (Bruhn et al., 1999; Kukongviriyapan et al., 2003). By contrast, both *NAT1*10* heterozygotes and homozygotes exhibited higher acetylation of pABA in peripheral blood leukocytes compared with *NAT1*4* control subjects, which was due primarily to a higher V_{max} (Zhangwei et al., 2006). To add further complexity, a recent study by Zhu et al. (2011) showed that each nucleotide polymorphism in the *NAT1*10* allele results in lower mRNA and lower protein levels when transfected into COS-1 cells, suggesting that mRNA stability does not account for higher NAT1 activity. Some of these conflicting results may be related to tissue-specific increases in NAT1 mRNA stability. However, a recent study that investigated the translational efficiency of mRNA derived from different NAT1 haplotypes may provide some insight into the molecular mechanism that leads to an apparent rapid phenotype with the *NAT1*10* and *NAT1*11* alleles (Wang et al., 2011). These investigators showed, using human liver and B-cell preparations, that total mRNA did not change between phenotypes, but translational efficiency of the mRNA was greater for the **10* and **11* alleles. This resulted in significantly higher protein expression and enzyme activity. The increase in activity was relatively minor (<2 fold) when considered in light of the many other transcriptional and post-translational mecha-

nisms that regulate NAT1 expression. Nevertheless, the same investigators demonstrated that hypersensitivity to sulfamethoxazole was much less in *NAT1*10* homozygotes or *NAT1*11* heterozygotes if they were also NAT2 slow acetylators, suggesting that the NAT1 rapid acetylator phenotype is physiologically relevant. Further studies into the specific effects of the *NAT1*10* and *NAT1*11* alleles on enzymatic activity in vivo are warranted.

Despite the controversy surrounding the *NAT1*10* allele, numerous studies have investigated its association with cancer on the assumption that it represents a rapid allele and therefore may increase risk in those persons exposed to carcinogens activated by NAT1. Many of these studies are summarized in Table 1. Of the 17 studies in colorectal cancer, 11 (65%) failed to demonstrate any significant association with the *NAT1*10* allele. For breast cancer, 70% of studies showed no change in risk with the *NAT1*10* allele. However, for pancreatic cancer, all three studies to date reported a significant increase in the odds ratio in subjects with one or more *NAT1*10* alleles, ranging from 2.23 to 4.15 (Table 1).

Two studies showed a significant increase in risk of colorectal cancer when the *NAT1*10* allele was combined with intake of well done meat (Chen et al., 1998; Lilla et al., 2006), a known source of heterocyclic amine carcinogens. A similar significant increase in risk was reported for both breast (Krajinovic et al., 2001) and pancreatic cancer (Suzuki et al., 2008). However, NAT1 is a poor metabolic activator of the major food-derived mutagens compared with NAT2 (Minchin et al., 1992), although 2-amino-α-carboline seems to be an exception (King et al., 2000). In some studies, *NAT1*10* was associated with a decrease in cancer prevalence, providing further complexity to understanding the role that this enzyme may play in cancer risk (Cascorbi et al., 2001; Moslehi et al., 2006).

Overall, there is little consistency among the various independent studies that have linked cancer risk with the *NAT1*10* allele. Some of these studies suffer from a lack of power with small sample sizes. However, the evidence that *NAT1*10* is a rapid allele in vivo and that NAT1 is responsible for bioactivating important human carcinogens is still inconclusive. The epidemiological studies to date suggest that NAT1 genotypes are linked only weakly, if at all, to cancer risk.

B. N-*Acetyltransferase 1 Expression in Human Cancers—Microarray Data*

NAT1 is represented on most microarray chips, so interrogation of public databases has revealed changes in NAT1 mRNA levels associated with different cancers and cancer subtypes. Although many of these array studies have not been validated, consistent trends seen among independent studies provide a guide for identifying potential changes worthy of further investigation. Perhaps the best example of microarray data that have stimulated NAT1 research emerged from an original study by Perou et al. (2000), who analyzed gene expression in 39 breast cancer samples and 3 control samples. Although this study did not specifically report NAT1 mRNA levels, subsequent analysis of the same data showed NAT1 expression clustered with expression of the estrogen receptor (Sørlie et al., 2001). This has been confirmed in several independent studies (van 't Veer et al., 2002; Weigelt et al., 2003; Bertucci et al., 2004; Abba et al., 2005). The positive association of NAT1 and estrogen receptor was strengthened by Adam et al. (2003)), who showed immunohistochemically that NAT1 protein was higher in estrogen receptor-positive breast cancers compared with estrogen receptor-negative tissue. This has led to the suggestion that NAT1 may be a useful additional biomarker for categorizing breast cancer subtypes (Wakefield et al., 2008).

Microarray analysis of breast cancers (Sørlie et al., 2001; Farmer et al., 2005; Alimonti et al., 2010) has also revealed that NAT1 expression is higher in luminal carcinomas compared with basal-like carcinomas, which are generally more aggressive and have a poorer prognosis (Fadare and Tavassoli, 2008). This is consistent with higher estrogen receptor mRNA in luminal cancers. Luminal breast cancers metastasize primarily to the bone, whereas basal-like carcinomas metastasize to the brain and lungs (Kennecke et al., 2010). In a study of 107 breast cancer samples, high NAT1 levels in primary tumors was significantly associated with increased metastasis to the bone (Smid et al., 2006). Moreover, invasiveness of breast carcinomas has been positively correlated with increased NAT1 mRNA levels (Casey et al., 2009).

It has been suggested that metastatic cancers retain the molecular programming of the primary cancers from which they derive (Perou et al., 2000). In Fig. 3, the change in *NAT1* and estrogen receptor gene profiles between primary and secondary breast cancers is shown using microarray data from Weigelt et al. (2003). NAT1 and estrogen receptor expression were significantly correlated in primary cancers (Fig. 3A), which is in agreement with other studies (Table 2). However, this correlation was lost in metastatic tumors from the same patients (Fig. 3B). Although NAT1 mRNA levels in metastases reflected that in the matched primary cancers (Fig. 3C), this was not the case for estrogen receptor status (Fig. 3D). By contrast, in their study of NAT1 protein levels in human breast carcinomas, Adam et al. (2003) found no an association between the NAT1 in primary tumors and their metastatic potential. These results suggest that estrogen receptor is not responsible for the level of NAT1 expression in breast carcinomas.

Microarray data of other human cancers have not been interrogated for NAT1 expression to the same extent as for breast cancer. Table 2 shows the findings of several studies from which data are available in public databases. For prostate cancer, two reports showed no

TABLE 1
Summary of studies of NAT1 genotypes and cancer risk

Cancer Type	Allele(s)	Sample Size		Reported Significance (95% Confidence Limits)	Modifiers	Reference
		Controls	Cases			
Colorectal	*1*10*	112	202	OR = 2.5 (1.3–4.7)		Bell et al., 1995b
Colorectal	*1*10*	484	441	N.S.		Probst-Hensch et al., 1996
Colorectal	*1*10*	221	212	OR = 5.82 (1.11–30.6)	Well-done meat intake and rapid NAT2 phenotype	Chen et al., 1998
Colorectal	*1*10*	122	103	N.S.		Katoh et al., 2000
Colorectal	*1*10*	228	146	N.S.		Ishibe et al., 2002
Colorectal	*1*10*	537	102	N.S.		Tiemersma et al., 2002
Colorectal	*1*10*	467	349	N.S.		Le Marchand et al., 2001
Colorectal	*1*10*	804	218	N.S.		van der Hel et al., 2003
Colorectal	*1*10/11*	500	500	N.S.		Kiss et al., 2004
Colorectal	*1*10*	433	520	N.S.		Chen et al., 2005
Colorectal	*1*10*	604	505	OR = 2.6 (1.1–6.1)	Well-done meat intake	Lilla et al., 2006
Colorectal	*1*4/1*10*	777	772	OR = 0.5 (0.3–0.9)	Smoking	Moslehi et al., 2006
Colorectal	*1*10*	315	217	N.S.		Butler et al., 2008
Colorectal	*Various*	223	123	N.S.		Mahid et al., 2007
Colorectal	*1*10*	769	379	OR = 1.3 (1.01–1.71)		Sørensen et al., 2008
Colorectal	*1*10*	1522	1009	N.S.		Nöthlings et al., 2009
Prostate	*1*10*	97	101	OR = 2.4 (1.0–5.6)		Fukutome et al., 1999
Prostate	*1*10*	121	47	OR = 2.17 (1.08–4.33)		Hein et al., 2002
Prostate	*1*10*	121	47	OR = 5.08 (1.56–16.5)	Slow NAT2 phenotype	Hein et al., 2002
Prostate	*1*10*	161	152	N.S.		Rovito et al., 2005
Prostate	*1*10*	175	187	N.S.		Iguchi et al., 2009
Prostate	*1*10*	2063	2106	N.S.		Sharma et al., 2010
Prostate	*1*10*	196	206	N.S.		Kidd et al., 2011
Breast	*1*10*	473	498	N.S.		Millikan et al., 1998
Breast	*1*10*	330	154	N.S.		Zheng et al., 1999
Breast	*1*11*	330	154	OR = 3.8 (1.4–10.2)		Zheng et al., 1999
Breast	*1*11*	273	290	N.S.		Millikan, 2000
Breast	*1*10*	273	290	N.S.		Millikan, 2000
Breast	*1*10*	207	149	OR = 4.4 (1.0–18.9)	Well-done meat intake	Krajinovic et al., 2001
Breast	*1*10*	301	254	N.S.		Lee et al., 2003
Bladder	*1*10/11*	242	254	N.S.		Okkels et al., 1997
Bladder	*1*10*	72	110	OR = 5.7 (1.9–17.7)	Smoking and rapid NAT2	Taylor et al., 1998
Bladder	*1*10*	343	425	OR = 0.39 (0.22–0.68)	Rapid NAT2	Cascorbi et al., 2001
Bladder	*1*10*	320	56	N.S.		Jaskuła-Sztul et al., 2001
Bladder	*1*10*	513	507	N.S.		Gu et al., 2005
Bladder	*1*10*	234	78	N.S.		McGrath et al., 2006
Bladder/Prostate	*1*10*	34	17	N.S.		Wang et al., 2002
Pancreatic	*1*10*	379	365	OR = 3.0 (1.6–5.4)	Smoking	Li et al., 2006
Pancreatic	*1*10/11*	581	32	OR = 4.15 (1.15–15)		Jiao et al., 2007
Pancreatic	*1*10*	636	755	OR = 2.23 (1.33–3.72)	Heterocyclic amine intake	Suzuki et al., 2008
Lung	*1*10*	47	45	RR = 3.7 (1.2–16)		Abdel-Rahman et al., 1998
Lung	*1*10*	172	150	OR = 6.4 (1.4–30.5)		Bouchardy et al., 1998
Lung	*1*10*	811	2250	OR = 0.81 (0.7–0.93)	Heterozygotes only	McKay et al., 2008
Lung (adenocarcinoma)	*1*10/11*	392	152	OR = 1.92 (1.16–3.16)		Wikman et al., 2001
Lung (squamous cell)	*1*10/11*	392	173	N.S.		Wikman et al., 2001
Laryngeal	*1*10/11*	510	255	N.S.		Henning et al., 1999
Laryngeal	*1*10/11*	172	129	N.S.		Jourenkova-Mironova et al., 1999
Laryngeal	*1*10/11*	172	8	$P < 0.038$		Varzim et al., 2002
Gastric	*1*10*	122	103	2.97 (1.23–7.14)	Smoking	Katoh et al., 2000
Gastric	*1*10*	112	94	OR = 2.2 (1.2–3.9)		Boissy et al., 2000
Gastric	*1*10*	356	257	N.S.		Lan et al., 2003
Gastric	*1*10*	593	149	N.S.		Agudo et al., 2006
Gastric	*1*10/11*	209	183	N.S.		Wideroff et al., 2007
Non-Hodgkin's lymphoma	*1*10*	922	1136	OR = 1.6 (1.04–2.46)		Morton et al., 2006
Non-Hodgkin's lymphoma	*1*10*	413	509	N.S.		Morton et al., 2007
Non-Hodgkin's lymphoma	*1*10*	535	461	OR = 2.0 (1.0–2.4)		Kilfoy et al., 2010
Oral	*1*10*	122	62	OR = 3.72 (1.56–8.9)		Katoh et al., 1998
Urothelial	*1*10*	122	116	OR = 2.09 (1.02–4.35)		Katoh et al., 1999
Head and neck	*1*10/11*	202	182	N.S.		Olshan et al., 2000
Head and neck	*1*10/11*	300	291	N.S.		Fronhoffs et al., 2001
Head and neck	*1*10*	93	96	N.S.		Demokan et al., 2010
Multiple myeloma	*1*10*	205	90	N.S.		Lincz et al., 2004
Cholangiocarcinoma	*1*10*	233	216	N.S.		Prawan et al., 2005
Neuroblastoma	*1*11*		209	$P < 0.05$	Time to relapse	Ashton et al., 2007
Liver	*1*10*	173	96	OR = 3.4 (1.03–11.22)		Zhang et al., 2005

OR, odds ratio; HR, hazard ratio; RR, relative risk; N.S., not significant.

association between NAT1 mRNA levels and disease (Varambally et al., 2005; Chandran et al., 2007). An exception was the study of Lapointe et al. (2004), who investigated 112 prostate tissue samples and categorized tumors based on gene expression. Figure 4 illustrates the results of that study. NAT1 was significantly

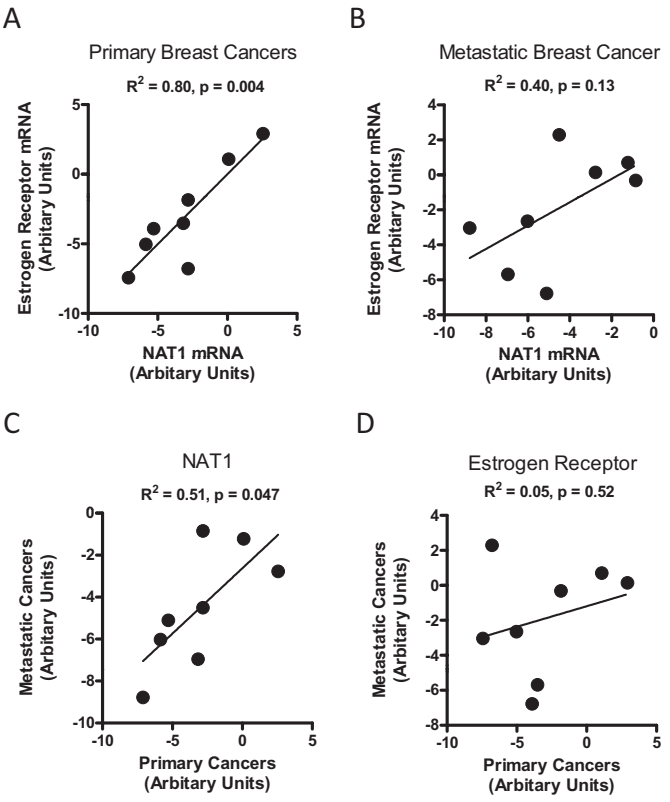

FIG. 3. Correlation between NAT1 and estrogen receptor mRNA measured in eight matched primary and secondary breast carcinomas. A, correlation of expression of the two genes in primary tumors. B, correlation of expression of the two genes in metastatic tumors. C, NAT1 expression in primary and matched secondary breast cancers. D, estrogen receptor expression in primary and matched secondary breast cancers. Data from Weigelt et al. (2003).

higher in normal tissue than in group I tumors, which were mostly lower grade carcinomas (Gleason score <3). In brain cancers, NAT1 was consistently more highly expressed in cancers than in normal tissue (Bredel et al., 2005; Sun et al., 2006). By contrast, three studies of lung cancers and four studies of leukemia reported no differences (Table 2). Finally, several studies have shown that NAT1 expression increases with increasing grade of glioma (Freije et al., 2004; Phillips et al., 2006).

The reason for the altered expression of NAT1 in cancers is currently unknown. However, there are several plausible explanations worthy of further investigation. For breast cancer, NAT1 mRNA levels segregate with a group of genes that included the estrogen receptor. Although there is no evidence to date that NAT1 is estrogen-responsive, expression of the estrogen receptor correlates closely with that of the androgen receptor, especially in breast cancers of luminal origin (Niemeier et al., 2010). NAT1 has been previously shown to be regulated transcriptionally by androgens (Butcher et al., 2007) and this may explain, at least in part, why NAT1 mRNA is more highly expressed in luminal-type breast cancers. NAT1 is also more highly expressed in normal epithelial cells than adjacent mesenchymal cells in the GI tract (Hickman et al., 1998; Windmill et al., 2000), bladder (Windmill et al., 2000), breast (Adam et al., 2003), and prostate (Butcher et al., 2007) tissue. It is noteworthy that basal-like breast carcinomas are characteristically similar to myoepithelial cells in normal breast tissue, which stain negative for NAT1 (Adam et al., 2003). It is possible that expression of NAT1 in different tumors simply reflects their cell of origin.

Many genes are regulated epigenetically by methylation of their promoter regions or by modification of histones and other proteins that are involved in heterochromatin formation. A change in global methylation is common in malignant cells, mostly as a result of a shift to a hypomethylated state. In a study of 119 breast cancer samples, Kamalakaran et al. (2011) demonstrated gene-specific alterations in methylation status in luminal compared with nonluminal breast subtypes. They also showed that many of the genes previously reported to be differentially expressed in breast carcinoma subtypes undergo a change in their methylation patterns that is predictive of their level of expression. Estrogen receptor is hypomethylated in luminal cancers compared with basal-like cancers, which may account for the higher level of expression (Bediaga et al., 2010). NAT1 is also hypomethylated in breast cancer compared with normal and benign tissue, and tumors with low NAT1 methylation exhibited a higher incidence of estrogen receptor expression (Kim et al., 2008). These results suggest that the two genes are linked by methylation status in breast cancer.

Another possible cause for up-regulation of NAT1 in various cancers is gene amplification. In a study of 89 breast cancer samples originally published by Chin et al. (2006), amplification of the *NAT1* gene was common. Moreover, the estrogen receptor was also found to be amplified in the same samples (Yuan et al., 2010).

Finally, the *NAT1* gene is located at 8p23.1, a region commonly deleted in cancers (Knuutila et al., 1999). In cells that carry an 8p23 deletion, NAT1 protein levels and enzyme activity should be significantly less than that in cells with two functional alleles. Thus, deletion of this region of the chromosome may account for lower NAT1 expression in some cancer subtypes.

In summary, microarray data have provided some interesting leads into changes in NAT1 expression in various cancers. However, the causes and consequences of these changes remain to be elucidated.

C. N-Acetyltransferase 1 Expression in Cancer Cell Lines

Cancer cells have been used extensively to investigate the role of NAT1 in xenobiotic metabolism and, more recently, in cell proliferation and survival. NAT1 is endogenously expressed in all immortalized cells reported to date. However, activity is highly variable because of both transcriptional and post-transcriptional regulation. This was illustrated in a study by Wakefield et al. (2008), who reported NAT1 activity in a variety of breast

TABLE 2
NAT1 expression in cancers by microarray analysis

Cancer Type	Year of Study	Sample Size	Reported Findings	Reference
Breast	2001	85	NAT1 clustered with estrogen receptor expression and was more highly expressed in luminal carcinomas compared with basal carcinomas. Both relapse-free survival and overall survival correlated positively with NAT1 mRNA.	Sørlie et al., 2001
Breast	2001	49	NAT1 was ranked 12th in a group of 40 genes that discriminated estrogen receptor status.	West et al., 2001
Breast	2002	78	NAT1 mRNA correlated with estrogen receptor status determined by immunohistochemistry. NAT1 did not correlate with recurrence.	van't Veer et al., 2002
Breast	2003		NAT1 correlated with estrogen receptor expression in eight primary cancers but not in matched metastatic samples.	Weigelt et al., 2003
Breast	2003	14	NAT1 was not altered in bone metastases in a comparison of seven primary and seven metastatic tumors.	Woelfle et al., 2003
Breast	2004	213	NAT1 expression clustered with the estrogen receptor and was negatively correlated with ERBB2 expression, which was determined by immunohistochemistry.	Bertucci et al., 2004
Breast	2005	26	NAT1 was overexpressed in estrogen-positive cancers.	Abba et al., 2005
Breast	2005	49	Study demonstrated basal cancers have lower NAT1 mRNA than luminal cancers.	Farmer et al., 2005
Breast	2006	107	High NAT1 expression in primary breast carcinomas was significantly associated with increased risk of bone metastasis.	Smid et al., 2006
Breast	2006	14	No difference in NAT1 expression in preinvasive ductal carcinoma in situ and invasive ductal carcinoma.	Schuetz et al., 2006
Breast	2007	30	No difference in NAT1 expression in lobular versus ductal carcinomas.	Turashvili et al., 2007
Breast	2007	57	Following treatment with letrozole, NAT1 expression increased (>110%) in 17%, did not change in 20% (90–110%), and decreased in 63% of patients.	Miller et al., 2007
Breast	2008	47	NAT1 mRNA was significantly less in inflamed stroma compared with noninflamed stroma.	Boersma et al., 2008
Breast	2008	60	NAT1 expression was similar in 5-year disease-free and recurrent patient tumor samples.	Loi et al., 2008
Breast	2009	66	NAT1 expression was higher in invasive carcinoma compared with normal tissue.	Casey et al., 2009
Breast	2010	42	No difference in NAT1 expression between control and cancer tissue or between estrogen-positive and estrogen-negative cancers.	Graham et al., 2010
Breast	2010	45	NAT1 mRNA was significantly lower in basal carcinomas compared with nonbasal carcinomas. Nonbasal carcinomas were similar to normal tissue	Alimonti et al., 2010
Breast	2010	89	Using a previously published dataset (Chin et al., 2006), computational approaches were used to demonstrate NAT1 is commonly amplified in breast carcinomas.	Yuan et al., 2010
Prostate	2004	112	NAT1 expression was significantly higher in one subtype of prostate carcinoma compared with normal tissue.	Lapointe et al., 2004
Prostate	2005	19	No differences in NAT1 expression between benign, primary, or metastatic cancers.	Varambally et al., 2005
Prostate	2007	164	No difference in NAT1 mRNA levels in primary or metastatic disease compared with normal tissue.	Chandran et al., 2007
Melanoma	2005	18	NAT1 gene expression increased from benign to vertical growth phase and metastatic phase of the disease.	Smith et al., 2005
Melanoma	2005	70	No difference in NAT1 expression in normal tissue and benign or metastatic cancers.	Talantov et al., 2005
Lung	2005	39	No difference in NAT1 expression in tumor versus adjacent normal tissue.	Stearman et al., 2005
Lung	2006	130	No change in NAT1 expression with stage of small-cell carcinoma.	Raponi et al., 2006
Lung	2009	58	No difference in NAT1 expression in small-cell carcinoma compared with adenocarcinoma.	Kuner et al., 2009
Ovarian	2009	24	No difference in NAT1 expression between normal and adenocarcinomas.	Bowen et al., 2009
Brain	2004	85	NAT1 expression significantly higher in grade IV versus grade II glioma.	Freije et al., 2004
Brain	2005	53	NAT1 expression significantly higher in brain cancers compared with normal tissue.	Bredel et al., 2005
Brain	2006	180	NAT1 mRNA levels significantly higher in astrocytic, glial, and oligodendroglial tumors compared with noncancerous tissue. No difference in expression between tumor types.	Sun et al., 2006
Brain	2006	100	NAT1 expression significantly higher in grade IV versus grade III glioma.	Phillips et al., 2006
Colon	2007	16	No difference in NAT1 expression in tumors from nonrelapsed and relapsed patients with Duke's B cancer.	Bandrés et al., 2007
Colon	2007	64	No difference in NAT1 expression in adjacent normal tissue and adenomas.	Sabates-Bellver et al., 2007
Colon	2007	22	NAT1 expression significantly less in early-onset cancers compared with normal tissue.	Hong et al., 2007
Renal	2005	34	No difference in NAT1 expression between different classes of papillary renal cell carcinoma.	Yang et al., 2005
Renal	2005	35	NAT1 mRNA levels in clear cell renal carcinoma were significantly higher than normal tissue. In Wilm's tumors, no change was seen.	Cutcliffe et al., 2005
Renal	2006	18	NAT1 expression was significantly higher in malignant compared with benign cancers.	Rohan et al., 2006

TABLE 2—*Continued*

Cancer Type	Year of Study	Sample Size	Reported Findings	Reference
Renal	2007	20	NAT1 expression was significantly lower in clear cell carcinoma compared with normal tissues.	Gumz et al., 2007
Mesothelioma	2005	56	No difference in NAT1 expression between normal and malignant mesothelioma tissue.	Gordon et al., 2005
Leukemia	2003	58	No difference in NAT1 expression between complete remission and relapse in patients with pediatric acute myeloid leukemia.	Yagi et al., 2003
Leukemia	2006	29	No difference in NAT1 expression between glucocorticoid-sensitive and -resistant lymphoblastic leukemia.	Wei et al., 2006
Leukemia	2007	14	No difference in NAT1 expression between normal cells and T-cell prolymphocytic leukemia.	Dürig et al., 2007
Leukemia	2008	64	No difference in NAT1 expression in acute myeloid leukemia compared with normal hematopoietic cells.	Stirewalt et al., 2008

cancer cell lines. They showed that enzyme activity, using pABA as substrate, varied from less than 0.3 nmol · min^{-1} · mg protein^{-1} in Cal51 cells to almost 200 nmol · min^{-1} · mg protein^{-1} in ZR-75-1 cells. Most other cells showed activities of 1 to 2 nmol · min^{-1} · mg protein^{-1}. The ZR-75-1 cells were the only cell line that generated transcripts from the more distal NAT1 promoter, suggesting that they may express transcription factor(s) specific for that promoter. A summary of NAT1 activity in different human cell lines determined in the author's laboratory is shown in Fig. 5. The lowest activity was seen in the colon carcinoma HT-29 cells, which have a large deletion at 8p22 (Arbieva et al., 2000). Consistent with the observations from Wakefield et al. (2008), ZR-75-1 showed high activity, as did T-47D. In the prostate cancer cells, NAT1 activity was high in the androgen sensitive lines 22Rv1 and LNCaP and low in the androgen-insensitive line PC-3, which is consistent with an independent microarray study of various prostate cancer cell lines (Zhao et al., 2005). NAT1 is known to be induced by androgens (Butcher et al., 2007). Because NAT1 expression is regulated transcriptionally, post-transcriptionally, and epigenetically (see sections II.B–II.D), it is likely that all of these factors contribute to the highly variable levels of NAT1 activity in cancer cell lines. The important question is whether this variation affects cell biology.

Using nontransformed breast epithelial HB4a cells, Adam et al. (2003) reported that NAT1 overexpression conferred a growth and survival advantage, even in low serum. Moreover, these cells were more resistant to etoposide-induced cell death, prompting the authors to suggest that NAT1 may have indirect oncogenic effects. Reanalysis of several microarray studies revealed an association between increased NAT1 expression and resistance. For example, in HT-29 cells resistant to methotrexate, NAT1 mRNA levels were significantly higher than in sensitive cells (Selga et al., 2008). Likewise, NAT1 expression was higher in gemcitabine-resistant Calu3 cells compared with sensitive cells (Tooker et al., 2007). The underlying mechanism explaining this apparent association is not clear, but it is unlikely to result from drug metabolism because none of these drugs is known to be acetylated in humans.

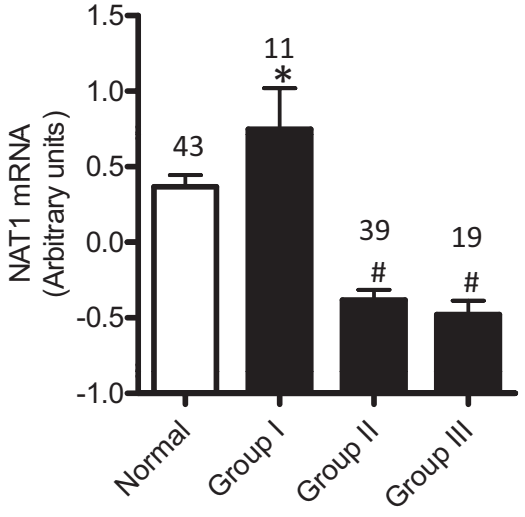

FIG. 4. Expression of NAT1 in normal prostate tissue (open bar) and different subtypes of prostate carcinomas (closed bars). Sample numbers are shown above each bar. *, significantly greater than normal ($p < 0.05$); #, significantly less than normal ($p < 0.05$). Data from Lapointe et al. (2004)).

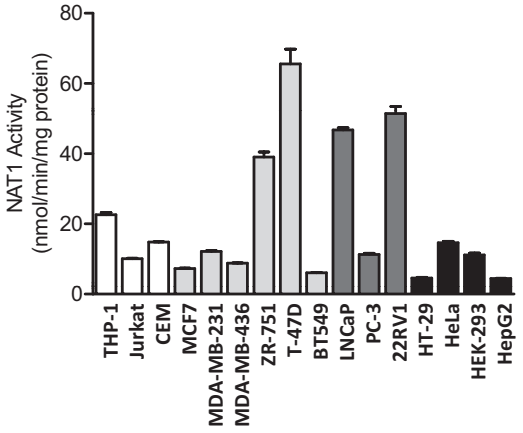

FIG. 5. NAT1 expression in different human cancer cell lines. Cytosolic preparations were used to determine NAT1 activity using *p*-aminobenzoic acid as substrate. Activity was determined at saturating substrate (440 μM) and cofactor (1.1 mM) concentrations and are expressed as mean ± S.E.M., $n = 3$.

V. *N*-Acetyltransferase 1 as a Novel Drug Target

As outlined in the previous section, there is a growing body of evidence suggesting that NAT1 has an important role in cancer cell biology, particularly for breast cancer. This has been a driving force behind recent research to identify small-molecule inhibitors of NAT1 and to use short-hairpin RNA (shRNA) directed against NAT1 to manipulate its activity. Currently, several laboratories are using these tools to examine the endogenous function of NAT1.

A. Small-Molecule Inhibitors of N-*Acetyltransferase 1*

Mechanism-based inhibitors of the NATs were discovered more than 20 years ago and were used to elucidate the catalytic mechanism of the enzymes. However, none of these was a selective inhibitor of NAT1 and none was suitable to be used in the context of the cell as a result of cytotoxic effects. More recently, structure-based inhibitors showing specificity toward NAT1 and having minimal cell toxicity have been identified. High-throughput screening of a library of 5000 drug-like small molecules against several mammalian and nonmammalian purified recombinant NAT proteins allowed Russell et al. (2009) and Westwood et al. (2010, 2011) to identify isozyme-specific NAT inhibitors. One of the compounds, rhodanine, was a selective inhibitor of recombinant human NAT1 (and the mouse homolog Nat2) and also inhibited NAT activity of cell lysates from ZR-75-1 breast cancer cells. However, this compound was overtly cytotoxic. The same group synthesized a panel of related compounds based on the structure of rhodanine and conducted structure-activity investigations (Russell et al., 2009). The rhodanine analog (*Z*)-5-(2′-hydroxybenzylidene)-2-thioxothiazolidin-4-one (Rhod-o-hp) was one of the most potent inhibitors of human NAT1 (IC_{50}, 1.1 μM) and showed minimal cell toxicity. Kinetic studies demonstrated that Rhod-o-hp was a competitive inhibitor of mouse Nat2, and its binding to the active site of the enzyme was confirmed by NMR and in silico docking studies (Russell et al., 2009).

The small-molecule inhibitor Rhod-o-hp was used to investigate the effect of NAT1 inhibition on the proliferation and invasiveness of MDA-MB-231 breast cancer cells (Tiang et al., 2010). Rhod-o-hp inhibited NAT1 activity in intact MDA-MB-231 cells in a concentration-dependent manner, although the IC_{50} was approximately 100 times higher than that observed in vitro using purified recombinant human NAT1. This may have been due to poor uptake of the inhibitor into the cell or degradation inside the cell. Further chemical modification of Rhod-o-hp resulting in enhanced uptake and/or resistance to cellular degradation may produce a more potent inhibitor for in vivo use. Nonetheless, Rhod-o-hp had some profound effects on the breast cancer cells. First, it inhibited cell proliferation by blocking cells in G_2/M, and this was correlated with inhibition of NAT1 activity. Second, it inhibited anchorage-independent growth of cells in soft agar. Finally, it reduced invasiveness of MDA-MB-231 cells in an in vitro invasion assay. There was no evidence of cell toxicity at the concentrations of inhibitor used in these studies. Small-molecule inhibitors are prone to off-target effects, so NAT1 was also inhibited using a lentiviral-based shRNA directed against NAT1 mRNA (Tiang et al., 2010). The invasiveness of MDA-MB-231 cells was reduced by approximately 50% compared with cells expressing a scrambled shRNA. This suggested that the effects of Rhod-o-hp were most likely due to NAT1 inhibition.

Recent studies have also demonstrated that some currently used therapeutic agents inhibit NAT1 activity both in vitro and in vivo. These include tamoxifen and cisplatin, which are chemotherapeutic agents, and disulfiram, which is used to treat alcoholism. Early studies reported that tamoxifen inhibited NAT1 activity in human tissue samples and cancer cell lines (Lu et al., 2001; Lee et al., 2004). More recently, Ragunathan et al. (2008) showed that NAT1 was a target for cisplatin. MCF-7 and MDA-MB-231 breast cancer cells treated with clinically relevant concentrations of cisplatin showed significant NAT1 inhibition, with an IC_{50} of approximately 100 μM for both cell lines. Furthermore, mouse Nat2 activity (human NAT1 homolog) in various tissues from cisplatin-treated mice was reduced compared with nontreated control mice. Using purified recombinant human NAT1, cisplatin was shown to form adducts with the active site cysteine and to be an almost irreversible inhibitor of the enzyme. The same group has also shown that disulfiram inactivates NAT1 in human cultured cells by reacting irreversibly with the active-site cysteine (Malka et al., 2009). Although these drugs are not specific inhibitors of NAT1, their inhibition of NAT1 activity may contribute to their chemotherapeutic effects if NAT1 is shown to play a role in cancer cell biology.

B. Effects of N-*Acetyltransferase 1-Directed shRNA*

In addition to small-molecule inhibitors of NAT1, shRNA strategies to manipulate NAT1 expression in human cancer cell lines have been reported. As discussed above, a lentiviral-based system to knock-down NAT1 in the highly invasive MDA-BD-231 breast cancer cell line resulted in changes in cell proliferation rates and invasiveness (Tiang et al., 2010). We are currently using this strategy to knock down NAT1 in a number of different human cancer cell lines in an attempt to elucidate the role of NAT1 in cancer cell biology. Initial studies used a shRNA plasmid targeting the human *NAT1* gene to create stable NAT1 knockdown cell lines. It is noteworthy that knockdown of NAT1 expression in the noninvasive HT-29 colon cancer cell line resulted in a marked change in cell morphology that was accompanied by an increase in cell-cell contact inhibition of growth and a loss of cell viability at confluence (Tiang et

al., 2011). NAT1 knockdown also led to attenuation in anchorage-independent growth in soft agar. These results suggested that the cells had regained contact inhibition of growth, a property often lost in malignancies (Smalley and Dale, 1999; Pawlak and Helfman, 2001). NAT1 inhibition resulted in up-regulation of E-cadherin that was not associated with a change in the transcription repressors Snail, Twist, or Slug. Instead, hypomethylation of the E-cadherin promoter, or a regulatory gene of E-cadherin expression, seemed to be responsible for the observed changes. Similar results were seen in the malignant prostate cancer line 22Rv1.

The molecular mechanisms that underlie the effects of NAT1 on cell growth and survival require further investigation. Nevertheless, studies using small-molecule inhibitors and shRNA point to NAT1 as a novel target for anticancer drug development.

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Authorship Contributions

Wrote or contributed to the writing of the manuscript: Butcher and Minchin.

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