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REVIEW

Arylamine N-acetyltransferases: from drug metabolism and pharmacogenetics to drug discovery

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Arylamine N-acetyltransferases (NATs) are polymorphic drug-metabolizing enzymes, acetylating arylamine carcinogens and drugs including hydralazine and sulphonamides. The slow NAT phenotype increases susceptibility to hydralazine and isoniazid toxicity and to occupational bladder cancer. The two polymorphic human *NAT* loci show linkage disequilibrium. All mammalian *Nat* genes have an intronless open reading frame and non-coding exons. The human gene products NAT1 and NAT2 have distinct substrate specificities: NAT2 acetylates hydralazine and human NAT1 acetylates p-aminosalicylate (p-AS) and the folate catabolite para-aminobenzoylglutamate (p-abaglu). Human NAT2 is mainly in liver and gut. Human NAT1 and its murine homologue are in many adult tissues and in early embryos. Human *NAT1* is strongly expressed in oestrogen receptor-positive breast cancer and may contribute to folate and acetyl CoA homeostasis. NAT enzymes act through a catalytic triad of Cys, His and Asp with the architecture of the active site-modulating specificity. Polymorphisms may cause unfolded protein. The C-terminus helps bind acetyl CoA and differs among NATs including prokaryotic homologues. NAT in *Salmonella typhimurium* supports carcinogen activation and NAT in mycobacteria metabolizes isoniazid with polymorphism a minor factor in isoniazid resistance. Importantly, *nat* is in a gene cluster essential for *Mycobacterium tuberculosis* survival inside macrophages. NAT inhibitors are a starting point for novel anti-tuberculosis drugs. Human NAT1-specific inhibitors may act in biomarker detection in breast cancer and in cancer therapy. NAT inhibitors for co-administration with 5-aminosalicylate (5-AS) in inflammatory bowel disease has prompted ongoing investigations of azoreductases in gut bacteria which release 5-AS from prodrugs including balsalazide.

Abbreviations

5-AS, 5-aminosalicylate; ES, embryonic stem; IBD, inflammatory bowel disease; MTP, methyl triazolophthalazine; NAT, arylamine N-acetyltransferase; p-aba, *para*-aminobenzoic acid; p-abaglu, *para*-aminobenzoylglutamate; p-AS, *para*-aminosalicylate; SAR, structure activity relationship; SLE, systemic lupus erythematosus; SNP, single nucleotide polymorphism

Introduction

This review describes studies of the drug-metabolizing enzyme arylamine N-aetyltransferase (NAT) over a period of some 30 years up to the present day, which was presented as the JR Vane Lecture in December 2012. The work includes early pharmacogenetic analysis, use of isoenzyme specific antibodies in Western blotting and immunohistochemistry; transgenic mice, which may have generated more questions than answers, large-scale recombinant protein expression for structural studies and development of isoenzyme specific inhibitors. Isoniazid, the anti-tubercular prodrug, is metabolized by NAT (Figure 1) and prompted work on NAT in mycobacteria, stimulating investigations in drug discovery. Recent work on

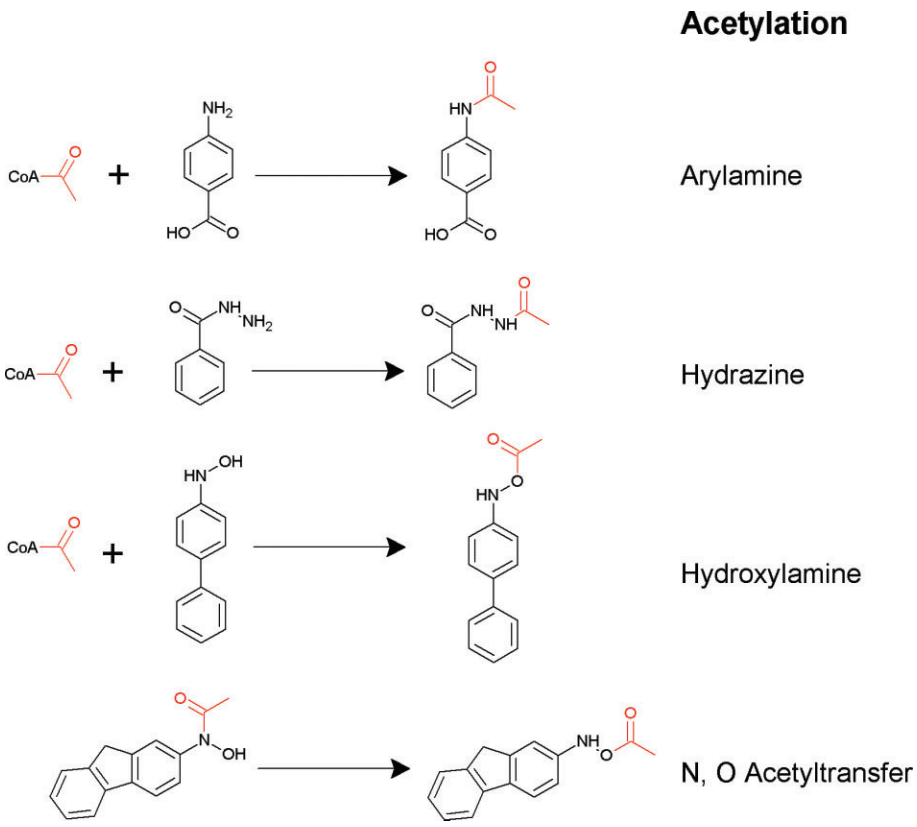

Figure 1

Metabolic reactions catalysed by arylamine N-acetyltransferase. Acetylation reactions from the top show N-acetylation of an arylamine, *p*-aminobenzoic acid; N-acetylation of isoniazid; O-acetylation of an arylhydroxylamine and the bottom reaction shows N,O acetyltransfer in a hydroxamate. The top three reactions use acetyl CoA as a cofactor while the bottom reaction does not. The bottom two reactions are associated with carcinogenesis. From Sim *et al.*, 2010.

azoreductases, which generate arylamines from azo drug precursors, is also covered. These studies have relied on an enthusiastic band of co-investigators and collaborators including project students and graduate research students (Supporting Information Table S1). The present review adds to earlier summaries (Sim *et al.*, 2000; 2007; 2008a–c).

NATs came to prominence in identifying acetyl CoA as a key metabolic intermediate (Lippmann and Kaplan, 1946). Their role in xenobiotic metabolism has been wide-reaching for early insight into interindividual differences in the ability to metabolize drugs (Kalow, 2004; Weber and Hein, 1985 for reviews). Studies on arylamine N-acetyltransferases (NATs) were among the earliest examples of pharmacogenetic variation (Evans *et al.*, 1960) and laid the foundation for understanding that different ethnic populations could differ considerably in drug metabolism (see Jones, 2013) with important implications for clinical trials (Tam *et al.*, 2000). NAT polymorphism also has importance in understanding adverse side effects (see Park *et al.*, 1992).

The corresponding author's journey in drug metabolism was triggered by attempts to understand how an environmental factor, the drug, could lead to an adverse reaction. These studies were carried out firstly in the Pharmacology Department in South Parks Road in Oxford and subsequently in the iconic building in Mansfield Road for which the funds were raised by Prof. David Smith from Squibb. The building was opened by JR Vane in the summer of 1991 (Figure 2). Since 2011, the studies have been continued at Kingston University.

Figure 2

JR Vane at the opening of the Pharmacology Department, Oxford 1991. John Vane is on the right, David Smith, Head of Department, is in the middle and the head of research of Squibb Pharmaceuticals, who gave the endowment before the Bristol-Meyers takeover is on the left.

Adverse drug reactions

Even in the post-genomic era, identification of an environmental trigger of disease can be very difficult, and therefore, adverse drug reactions have the advantage that the drug serves as a readily identifiable environmental factor (McDowell *et al.*, 2013). I chose to study hydralazine-induced systemic lupus erythematosus (SLE; Batchelor *et al.*, 1980). Immune complexes become deposited in capillaries and in kidney glomeruli with inhibition of the complement cascade contributing to SLE susceptibility (Walport, 2002 for review). We showed hydralazine inhibited the solubilization of immune complexes by blocking complement component C4, which is polymorphic (Sim *et al.*, 1984), with hydralazine inhibiting the C4A type more than the C4B type (Sim and Law, 1985). Immune system polymorphisms had a role in predisposition to hydralazine-induced SLE (Mitchell *et al.*, 1987; 1989; Mitchell and Sim, 1989) but the most important polymorphism was the ability to metabolize hydralazine (Drayer and Reidenberg, 1977). Hydralazine is acetylated polymorphically to produce cyclized methyl triazolophthalazine (MTP; Timbrell *et al.*, 1980). When complement component C4 is activated a thiol ester is exposed, which binds covalently to adjacent immune complex (Sim *et al.*, 1981) leading to solubilization. Hydralazine, but not its acetylated metabolite MTP, blocked covalent binding of complement component C4 (Figure 3; Sim *et al.*, 1984). In the process, hydralazine becomes bound to C4 (Sim and Law, 1985).

The metabolism of hydralazine has been investigated extensively and recent structural studies with recombinant NAT protein have demonstrated the mechanisms of the cyclization reaction (Figure 4; Abuhammad *et al.*, 2010).

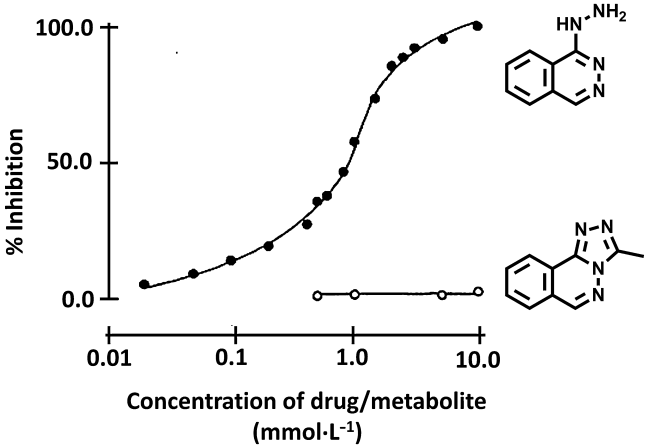

Figure 3
Hydralazine inhibits binding of complement component C4. Radiolabelled C4 was activated by a model for immune complexes (C1s bound to Sepharose) and the inhibition of binding was compared using hydralazine or its acetylated metabolite, methyltriazolophthalazine synthesized by EW Gill. From Sim *et al.* (1984).

NAT and pharmacogenetics

In the 1960's, it had been established that there was polymorphism in isoniazid inactivation by human NAT activity (Evans *et al.*, 1960). This is the classic textbook pattern of distribution into fast and slow acetylators (Figure 5). Fast and slow acetylators were classified with several drugs including isoniazid, hydralazine and sulphamethazine (see Weber and Hein, 1985). It was also identified that ethnic populations varied such that in Orientals, there were approximately 15% slow acetylators while in the Caucasian population, around 50% were slow acetylators (Harris *et al.*, 1958; Sim and Hickman, 1991). The slow acetylator phenotype was, in early studies, found to be more prevalent in isoniazid-induced toxicity as well as in hydralazine-induced lupus (Woosley *et al.*, 1978). More recent studies have demonstrated that isoniazid-induced hepatotoxicity have also been associated with the slow acetylator type (Gupta *et al.*, 2013).

In a landmark study, it was also shown that slow acetylation was a major predisposing factor in industrial bladder cancer (Cartwright *et al.*, 1982) where bladder cancer had been known since the late 19th century to be linked to exposure to aniline dyes (Rehn, 1895; see Dietrich and Dietrich, 2001).

Some drugs were identified, which were metabolized by acetylation but there did not appear to be the same distribution among individuals into fast and slow acetylators. These drugs included *para*-aminosalicylate (p-AS) and the compound *para*-aminobenzoic acid (p-aba). These drugs were referred to as monomorphic as opposed to the drugs like procainamide, isoniazid and hydralazine, which were known as polymorphic (see Weber and Hein, 1985).

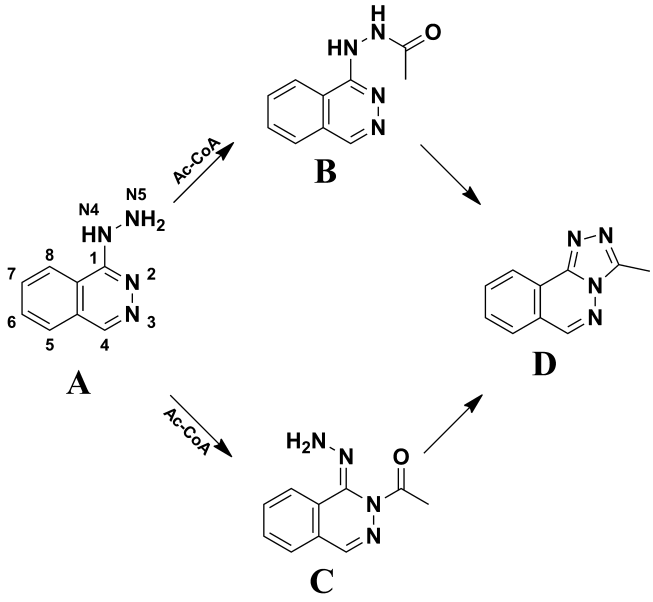

Figure 4
Two possible routes of acetylation of hydralazine. From the crystal structure and the different tautommers and ionization states it is proposed that hydralazine may also be acetylated initially at the N in the heterocyclic ring. In either case, cyclization is proposed to generate methyltriazolophthalazine. From Abuhammad *et al.* (2010).

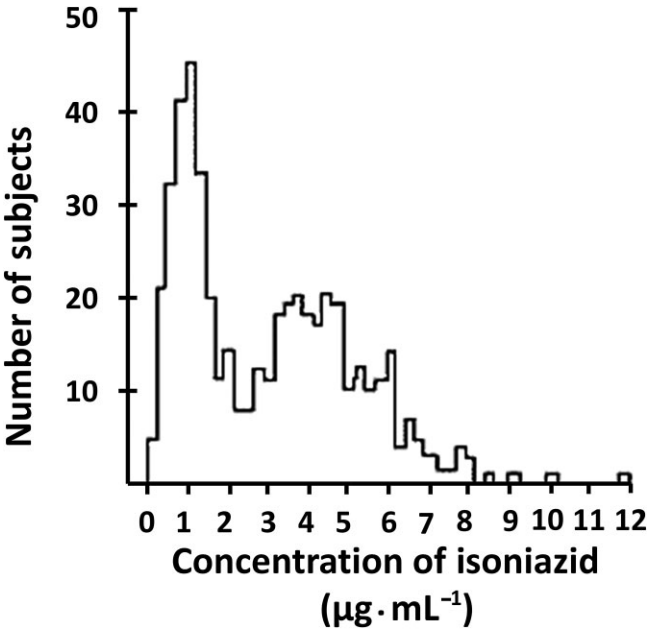

Figure 5
Isoniazid pharmacokinetics in a population. After Evans *et al.* (1960).

An inkling of the cause behind two different classes of substrate came from some outstanding experiments by Jenne (1965). Two enzymic activities in human liver homogenates were separated by ion exchange chromatography and the profile differed when activity was measured with p-AS and with sulphamethazine. Some 10–15 years later using molecular biology, two different human NAT isoenzymes (Blum *et al.*, 1990; Deguchi *et al.*, 1990) were found, now known as human NAT1 and NAT2 each catalysing the acetylation of arylamines using acetyl CoA as the acetyl donor. Only NAT2 catalyses the acetylation of hydralazine, procainamide and isoniazid while p-AS is only acetylated by human NAT1 (Figure 6). As more versatile assay methods have become available (Brooke *et al.*, 2003a), it has been possible to establish an easy method of comparing the substrate specificities of a range of different NAT enzymes (Figure 6) and this demonstrates clearly that the human NAT enzymes have different but overlapping substrate specificity profiles (Figure 6).

Finding two functional human NAT proteins did not solve the basis of polymorphism in metabolism but early cloning experiments provided the clue to the polymorphism in metabolism of drugs by human NAT2. In the pre-PCR, pre-whole genomic age and specific polynucleotide probes to screen DNA libraries were identified through translation of protein sequence. We had previously chosen to purify NAT from mouse liver (Watson *et al.*, 1990) but rabbit was a much better choice (Andres *et al.*, 1987; 1988; Sasaki *et al.*, 1991). Partial protein sequence from pure NAT from rabbit liver NAT was published by Andres, an inventive contributor to the NAT field (Andres *et al.*, 1987). This paper appeared when I was on maternity leave.

Fortuitously, using the sequence information from rabbit, both a Japanese and a European group chose to clone human NAT genes (Blum *et al.*, 1990; Ohsako and Deguchi, 1990). These studies show how the accumulation of data serves to solve apparently intractable problems. The differences, which had been observed in the distribution of fast and slow acetylation in Oriental and Caucasian populations, were reflected in the DNA clones described in these two studies. Both groups found two different genes, *NAT1* and *NAT2*, (Blum *et al.*, 1990; Ohsako and Deguchi, 1990). Point mutations in the human *NAT2* gene leading to poor acetylation (Deguchi *et al.*, 1990; Blum *et al.*, 1991) were found but the point mutations in the Japanese group were different from those in Caucasians (Deguchi *et al.*, 1990; Blum *et al.*, 1991).

Soon afterwards, we developed a restriction fragment length polymorphism PCR genotyping method for *NAT2* (Hickman and Sim, 1991; Hickman *et al.*, 1992). Now microarray-based single nucleotide polymorphism (SNP) analyses (Hein and Doll, 2012) and cheap and comprehensive sequencing across the chromosomal region are used (Patin *et al.*, 2006a,b; Sabbagh *et al.*, 2011; Tilak *et al.*, 2013). These studies have also allowed the establishment of gene frequencies in a wide range of ethnic populations and disease states (e.g. Garte *et al.*, 2001).

In the initial cloning experiments, the Japanese used cDNA (Ohsako and Deguchi, 1990) while the Meyer group used genomic DNA (Blum *et al.*, 1990). The latter studies identified a third pseudogene, which was not transcribed and hence was not found in the cDNA library (Blum *et al.*, 1990). Comparison of the cDNA and genomic DNA clones showed the open reading frames of each of the *NAT1* and *NAT2* genes were intronless. Human *NAT1* (Ebisawa and Deguchi, 1991) has a non-coding exon and we now know there are a series of upstream non-coding exons in the human NAT1 gene and that the splicing of these into the proximal region immediately in front of the coding exon may be tissue specific (Husain *et al.*, 2004; Boukouvala and Sim, 2005; Barker *et al.*, 2006; see Minchin *et al.*, 2007).

Human *NAT2* also has a non-coding exon spliced in frame with its single coding exon (Husain *et al.*, 2007; see Sim *et al.*, 2010). The extended *NAT2* gene is much simpler than the multiple alternative transcripts of human *NAT1*. The wide spread tissue expression of human NAT1 (in most tissues including endocrine tissues, blood cells, neural tissue as well as gut and liver) compared with the more restricted expression of human NAT2 (mainly gut and liver) may underlie the more complex gene organisation (see Minchin *et al.*, 2007). These genes as well as the human pseudogene are in close proximity in the genome in the region 8p22 (Hickman *et al.*, 1994; Franke *et al.*, 1994).

Although in the initial cloning experiments NAT1 was found to be invariant among individuals, we now know that human *NAT1* also has different alleles (Vatsis and Weber, 1993; Grant *et al.*, 1997; Payton and Sim, 1998). Point mutations in the open reading frame along have been described as well as alleles with no mutations in the coding exon but there were insertions and deletions at the 3′ end of the gene (Vatsis and Weber, 1993). Genotypng *NAT1* using PCR-based methods were more complex than for *NAT2* (Smelt *et al.*, 1998; Pirmohamed *et al.*, 2000; Johnson *et al.*, 2004). Multiplex SNP analyses are still used (see Hein, 2009) but genotyping through sequencing is now the method of choice.

We know there is linkage disequilibrium across the 200kb region on chromosome 8 in which all three NAT genes are

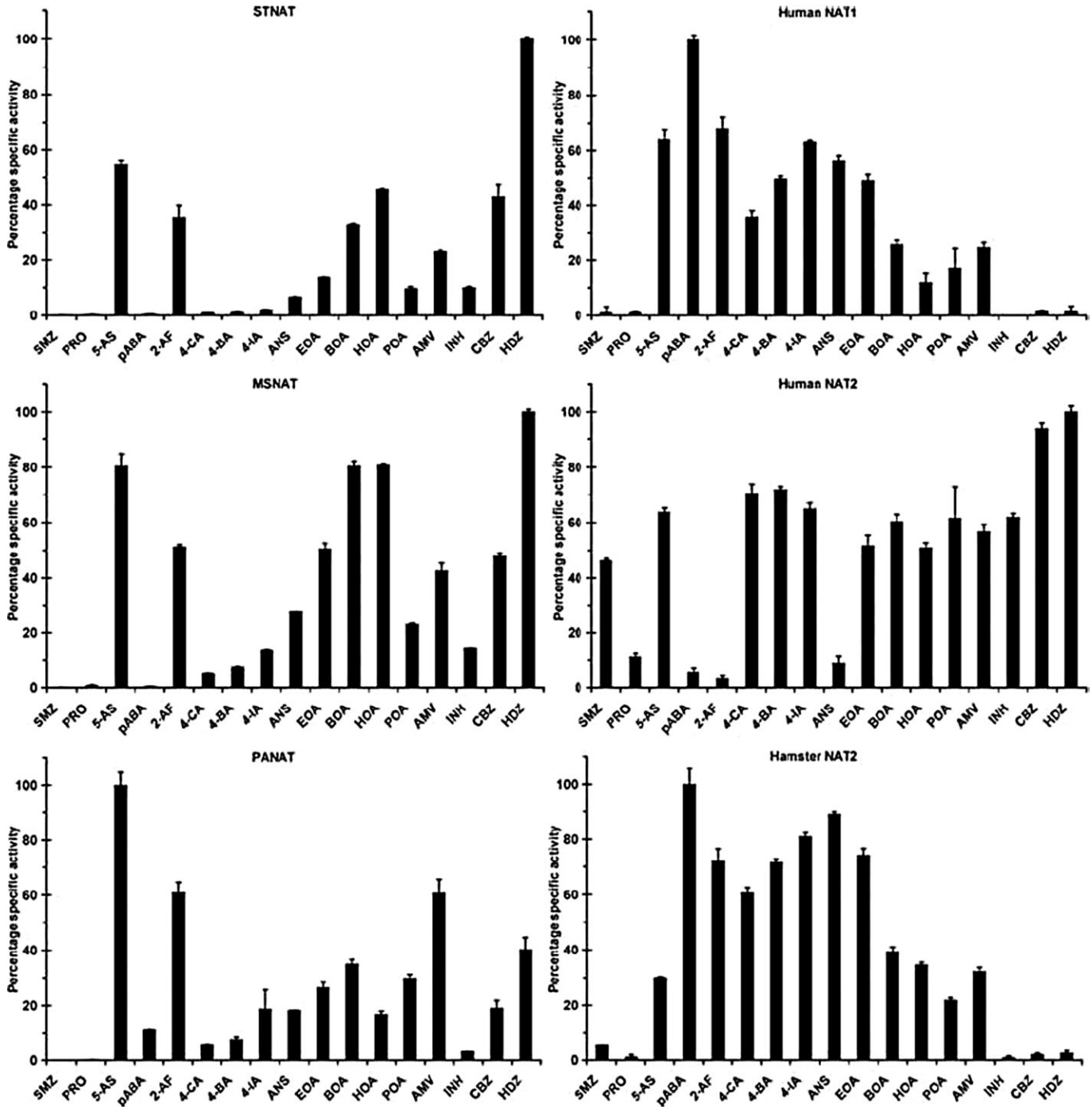

Figure 6

Specificity profile of NATs. A range of different NAT enzymes were prepared and their specificity was determined with the same panel of substrates in each case The specific activity profiles of three eukaryotic NATs and three prokaryotic NATs have been reported. Specific activities are presented as percent compared with the most active substrate for each enzyme. STNAT, NAT from *S. typhimurium*; PANAT, NAT from *P. aeruginosa*; MSNAT, NAT from *M. smegmatis*; SMZ, sulfamethazine; PRO, procainamide; 5-AS, 5-aminosalicylic acid; PABA, 4-aminobenzoic acid; 2-AF, 2-aminofluorene; 4-CA, 4-chloroaniline; 4-BA, 4-bromoaniline; 4-IA, 4-iodoaniline; ANS, 4-anisidine; EOA, 4-ethoxyaniline; BOA, 4-butoxyaniline; HOA, 4-hexyloxyaniline; POA, 4-phenoxyaniline; AMV, 4-aminoveratrole; INH, isoniazid; CBZ, 4-chlorobenzoic hydrazide; HDZ, hydralazine (Westwood *et al.*, 2006).

found (Cascorbi *et al.*, 1995; 2001; Smelt *et al.*, 1998) and this has been exploited in molecular anthropological studies of population migration and microevolution (Patin *et al.*, 2006a,b; Sabbagh *et al.*, 2011). In order to understand differential expression of the two human NATs, we developed antibodies against the intact proteins, which detect both NAT1 and NAT2, as they are over 80% identical in sequence. The C-termini differ sufficiently to use the C-terminal dodecapeptide to generate a human NAT1-specific antibody, which has been widely employed (e.g. Butcher *et al.*, 1998; Adam *et al.*, 2003; Johansson *et al.*, 2012). The anti-NAT2 antibody has not been so successful.

Effects of mutations in human NAT2 and human NAT1

A series of reviews of the effects of each of the mutations in human NAT1 and NAT2 has been carried out through modelling (Rodrigues-Lima and Dupret, 2002; Walraven *et al.*, 2008; Rajasekaran *et al.*, 2011). A full description of all NAT1 and NAT2 alleles is to be found on the web site initially curated by David Hein (http://louisville.edu/medschool/pharmacology/consensus-human-arylamine-n-acetyltransferase-gene-nomenclature/) and now hosted at http://nat.mbg.duth.gr/.

Specific highlights will be covered here.

Most alleles of human *NAT1* and *NAT2* are haplotypes of several point mutations with one being a signature mutation, which alone causes a change such that the resultant protein shows reduced activity. Wide phenotypic spread in the pharmacokinetics of isoniazid (Ellard and Gammon, 1976) had been identified and molecular studies confirmed the earlier twin studies of (Evans *et al.*, 1960), namely, that slow alleles of human *NAT2* are somatically inherited. While one 'slow' allele confers the slow acetylation phenotype, those with two 'slow' alleles show a more extreme phenotype (Hickman and Sim, 1991). Inherent effects of the level of 'slowness' conferred by different *NAT2* alleles have not been fully explored (Ruiz *et al.*, 2012).

However alleles fall into two classes, those that cause unstable protein and those which affect the activity of a folded protein.

A list of the main mutations that affect amino acids in human NAT1 and NAT2 are shown in Table 1. The premature stop codons in human *NAT1* are readily understood (Hubbard *et al.*, 1998; Hughes *et al.*, 1998; Payton and Sim, 1998).

Expressing variant alleles causing amino acid substitutions as recombinant enzymes has identified the effects of point mutations (Hickman *et al.*, 1995a,b; Walraven *et al.*, 2008 for review). One amino acid substitution with tryptophan substituted for aspartate at position 64 (W64D) occurs in both NAT1 and NAT2. Two very different elegant studies (Butcher *et al.*, 2004; Liu *et al.*, 2006) provided the conclusion that Trp (W) at position 64 results in an unfolded variant, which accumulates intracellularly in aggresomes for degradation via ubiquitination through the proteosomal pathway.

An example of a mutation resulting in folded but inactive protein is the change of aspartate 122 to asparagine (D122N). Asp^{122} is an essential part of the charge relay system in the active site (see below) and asparagine is not effective as has been confirmed by site-directed mutagenesis (Wang *et al.*, 2004).

Table 1

Mutations causing amino acid changes in human *NAT* genes

	Mutation	*NAT* allelic family	Amino acid
NAT2	C190T	2*19	Arg64Trp
	G191A	2*14	Arg64Gln
	T341C	2*5	Ile114Thr
	G364A	2*D12	Asp122Asn
	A434C	2*17	Gln145Pro
	G499A	2*10	Glu167Lys
	G590A	2*6	Arg197Gln
	A803G	2*12	Lys268Arg
	G857A	2*7	Gly286Gln
NAT1	C97T	1*19	Arg33Stop
	C190T	1*17	Arg64Trp
	C559T	1*15	Arg187Stop
	G560A	1*14	Arg187Gln
	A752T	1*22	Aso251Val

Apparently, healthy individuals with very little NAT1 activity have each been shown to have two defective alleles (Hughes *et al.*, 1998; Payton and Sim, 1998). It was nevertheless shown there was an inverse correlation between NAT1 activity and folate levels in red blood cells (Ward *et al.*, 1992), which opened up an ongoing debate on the relationship between NAT1 and folate (see Minchin *et al.*, 2007; Butcher and Minchin, 2012).

The allele *NAT1*10* has had more coverage than any other: it has no amino acid substitutions within the coding region – there are deletions and insertions at the 3' end. In colon cancer studies, *1*10* was associated with increased activity (Bell *et al.*, 1995), its effect has been hotly debated for other tissues where the number of copies of the 1*10 allele did not correlate with the level of NAT1 activity (Grant *et al.*, 1997; Payton and Sim, 1998). Effects of NAT1 mutations are being revisited (Millner *et al.*, 2012a,b) and *NAT1*10* remains an interesting allele. A 1*10 homozygous individual was identified in a study of patients with Alzheimer's disease (Johnson *et al.*, 2004) was identified and NAT1 polymorphism, including *1*10*, has been implicated in epidemiological studies of neural tube defects (Lammer *et al.*, 2004; Jensen *et al.*, 2005; 2006).

Epigenetic control of *NAT* gene expression and hence enzyme activity is yet to be fully explored. CpG islands in the vicinity of the *NAT* genes (Matas *et al.*, 1997) and their murine equivalents (Fakis *et al.*, 2000) indicate methylation sites and analyses of the upstream spliced region show this is the case (Kim *et al.*, 2008; Wakefield *et al.*, 2010). Epigenetic control is an important area for the future.

NATs and cancer

NAT has links with cancer at many levels (Table 2).

Carcinogenesis. Early studies identified that many carcinogens were NAT substrates (Bartsch *et al.*, 1973). It was debated

Table 2

Relationships between NAT and cancer

Links between NAT and cancer
Carcinogenesis
• Human NAT1 and human NAT2 metabolize arylamine carcinogens, including N-O transfer of an acetyl group in acetylhydroxylamines to generate N-acetoxyesters
• Strains of *S. typhimurium* with high NAT activity are more sensitive in Ames test
• Cultured human cells transfected with NAT genes exhibit higher level of mutagenesis
Polymorphism
• Slow acetylator polymorphism in human NAT2 has been linked to bladder cancer but depends on level of exposure
• Polymorphism in human NAT1 and colon cancer has been questioned
Gene expression
• Human NAT1 is overexpressed in oestrogen receptor-positive breast cancer in male and female breast cancer
Cytogenetics
• Human NAT genes encoded in a region of human genome harbouring oncogenes and tumour suppressor genes and show cytogenetic differences in tumours

whether the drug-metabolizing N-acetyltransferase (see Weber and Hein, 1985) was the same enzyme as the carcinogen-activating acetylating activity, which catalysed N-acetylation, O-acetylation and N,O acetyl transfer in hydroxamates (Figure 1; King, 1974). The elegant work of Hanna (Hanna, 1994) in this controversy provided balance and has further defined the mechanism of protein adduct formation with human NAT1 (Liu *et al.*, 2009). Recently, N- and O- acetylation activity in preserved human hepatocytes has been carried out (Doll *et al.*, 2010).

Cloning the N-hydroxylamino O-acetyltransferase from *Salmonella typhimurium* was a major step towards showing that this activity and NAT in humans were homologues (Watanabe *et al.*, 1992). Strains of *S. typhimurium* overexpressing its own *nat* gene were more sensitive in the Ames test for carcinogens (Watanabe *et al.*, 1987) and eukaryotic cells showed increased carcinogenesis when human NATs were overexpressed (Watanabe *et al.*, 1994).

Bladder cancer. Bladder cancer is a frequently occurring occupational and smoking-related cancer (Letašiová *et al.*, 2011 overview). In bladder cancer, there are many links with NAT. The link between bladder cancer and exposure to NAT substrates has been known for over a century (Rehn, 1895; see Dietrich and Dietrich, 2001). The phenotypic link of NAT2 slow acetylation to bladder cancer susceptibility (Cartwright *et al.*, 1982) has been confirmed by genotyping studies (Risch *et al.*, 1995) and has been reviewed (Vineis *et al.*, 2001; Hein, 2002). Interestingly, NAT2 is barely expressed in bladder epithelium although NAT1 is. In some bladder tumours, NAT1 is reduced (Stanley *et al.*, 1996) but no systematic study has been carried out.

The location of NAT genes in a hot spot on chromosome 8 (Knowles *et al.*, 1993; Spurr *et al.*, 1995; Hubbard *et al.*, 1997) prompted comparison in bladder tumour and normal DNA of *NAT* genes in this unstable region (Stacey *et al.*, 1996; 1997; 1999; Watters *et al.*, 1998). Loss of heterozygosity (Thygesen *et al.*, 1999) and gene duplication were detected but whether these changes are causative remains to be established. This region was not highlighted in a whole genome analysis (Kiemenev *et al.*, 2008).

Colorectal cancer. The link between NAT and colorectal cancer has had a chequered history. The *NAT 1*10* allele association with increased activity stimulated many studies (Bell *et al.*, 1995). A meta-analysis of 59 studies (Liu *et al.*, 2012) appears to confirm that there is no link between *NAT1* or *NAT2* polymorphism and colorectal cancer. Each of these NAT proteins is detected in the colon and the availability of specific NAT1 antibodies (Hickman *et al.*, 1998) has clearly demonstrated staining in the crypts of the colon. This study showed the ratio of NAT1 to NAT2 among individuals varies up to 70-fold. The activity of human NAT1 in the gut has implications also for the treatment of inflammatory bowel disease (IBD) which is a prediposing factor in colon cancer. IBD is treated with 5-aminosalicylate (5-AS), which is a substrate for human NAT1 and human NAT2 and this will be considered below in relation to NAT as a target for novel drug development.

NAT and breast cancer. From proteomic and genomic studies, human NAT1 but not NAT2 was found to be overexpressed in oestrogen receptor-positive female breast cancer (Adam *et al.*, 2003; see Sim *et al.*, 2008a) and more recently in male breast cancer (Johansson *et al.*, 2012). NAT2 is only marginally expressed in any breast tissue.

Understanding NAT1 overexpression in breast cancer and studies in other endocrine tumours (Butcher *et al.*, 2007; Butcher and Minchin, 2010) has made use of cell lines (Wakefield *et al.*, 2008a). The human breast cancer cell line ZR75 has an extremely high level of human NAT1, which is neither due to gene duplication nor to a mutant version of human NAT1.

Studies with siRNA indicate a reduction in contact-inhibited growth when NAT1 is suppressed (Tiang *et al.*, 2011; see Butcher and Minchin, 2012). Up-regulation of

NAT1 in breast cancer has been linked with improved response to tamoxifen (Bièche *et al.*, 2004) and it has interestingly been observed that human NAT1 (Lu *et al.*, 2001; Lee *et al.*, 2004) and its mouse equivalent (Kawamura *et al.*, 2008) is inhibited by tamoxifen and also bisphenol A.

There is no difference in the proportion of fast acetylating individuals for human NAT2 between a breast cancer and control group (Webster *et al.*, 1989) and this finding illustrates further that the relationship between NAT and breast cancer involves human NAT1 only.

Human NAT1 and NAT2 in development

In adults, human NAT1 has a much wider tissue distribution than human NAT2. During development, the difference is even more marked. In placentae, human NAT1 is found throughout pregnancy (Smelt *et al.*, 1998; 2000; Upton *et al.*, 2000) up to term (Derewlany *et al.*, 1994a,b). Foetal tissues have human NAT1 activity (Pacifici *et al.*, 1986) and NAT1 transcripts were detected at the four-cell stage and in blastocysts (Smelt *et al.*, 2000). Human NAT2 could not be detected in these studies.

Folate is essential for normal human development and a link between human NAT1 and folate was hinted at by the inverse correlation between red blood cell folate and human NAT1 activity in red cells (Ward *et al.*, 1992). Human NAT1 was shown to acetylate the folate catabolite *para*-aminobenzoylglutamate (p-abaglu) in lymphocytic cell extracts (Minchin, 1995), in placenta (Upton *et al.*, 2000) and using recombinant human NAT1 (Ward *et al.*, 1995) but not human NAT2 (Kawamura *et al.*, 2004).

The physiological role of human NAT1 has raised many questions (see Minchin *et al.*, 2007) as apparently healthy adult individuals with two defective human NAT1 alleles exist (Ward *et al.*, 1992; Hughes *et al.*, 1998; Payton and Sim, 1998). It was necessary to establish a model system in order to study further the possible relationship between folate and NAT and to decipher the role of human NAT1 in development.

Mouse NATs

Although mouse liver had proved to be a poor choice for purification of Nat enzymes (Watson *et al.*, 1990), mouse Nats were most suitable for recombinant protein studies and excellent for investigation of Nat in development. Two mouse genes, *Nat1* and *Nat2*, which are over 80% identical, were initially cloned from fast and slow acetylating mouse strains with each having an intronless open reading frame (Martell *et al.*, 1991; 1992). An upstream non-coding exon in mouse *Nat2* has also been characterized (Boukouvala *et al.*, 2003; see Sim *et al.*, 2010).

Mouse *Nat1* was identical in the fast and slow strains and showed specificity for isoniazid while mouse *Nat2* catalysed p-aba acetylation and had a point mutation resulting in asparagine 99 in the fast strain and isoleucine in the slow strain resulting in a less stable enzyme (Martell *et al.*, 1992; De Leon *et al.*, 1995). A third functional mouse gene, *Nat3* (Kelly and Sim, 1994), is highly polymorphic and less similar to the other genes (around 70% identity) but with very low activity (Fretland *et al.*, 1997; Boukouvala *et al.*, 2002). All three mouse genes are found in close proximity in the syngenic region to chromosome 8p22 where the human NAT genes are found (Hickman *et al.*, 1994; Fakis *et al.*, 2000).

The naming of mouse *Nat2* is historical (Martell *et al.*, 1991) but perversely has been retained (Hein *et al.*, 2008). It is the functional equivalent of human NAT1; the substrate specificity profile of mouse Nat2 is like that of human NAT1 (Kawamura *et al.*, 2008): the C-terminal sequence is the same as human NAT1 and mouse NAT2 is also recognized by human NAT1-specific antibodies raised against the C-terminus. This has allowed mouse NAT2 to be detected immunohistochemically in adult mouse tissues (Stanley *et al.*, 1997) and traced during development (Stanley *et al.*, 1998). Like its human counterpart, mouse NAT2 is found in early embryos in embryonic stem (ES) cells (Payton *et al.*, 1999a). The *Nat2* gene is expressed in the neonatal period unlike the other mouse *Nat* genes (Mitchell *et al.*, 1999), again mirroring human NAT1. Studies of congenic mouse strains had identified the region around the *Nat* loci in mice as implicated in susceptibility to neural tube defects including cleft lip and palate (Karolyi *et al.*, 1990) and intriguingly, mouse *Nat2* is expressed in the developing neural tube (Stanley *et al.*, 1998; Cornish *et al.*, 2003; Figure 7).

Knowledge of the genetic region around the mouse *Nat* genes (Fakis *et al.*, 2000) allowed creation of transgenic mice to study development. Many attempts were made to overexpress human NAT1 in mouse ES cells (Johnson, 2002; Sim *et al.*, 2003). No normal mice were produced overexpressing human *NAT1* following targeted insertion using a strong promoter, although two mice with neural tube defects were found as well as deformed embryos and evidence of resorption early in gestation (Sim *et al.*, 2003). Random insertion of human *NAT1* in mouse ES cells under the same strong promoter generated several mice overexpressing human *NAT1* (Cao *et al.*, 2005) but at a much lower level than expected. Overexpression of human *NAT1* appears detrimental during development confirming a role for mouse *Nat2* in teratogen-induced cleft lip and palate (Erickson *et al.*, 2008; Erickson, 2010 for review). These studies mirror the link of human *NAT1 *10* genotype to neural tube defects (Lammer *et al.*, 2004; Jensen *et al.*, 2005; 2006) but as discussed, the link between this allele and increased NAT1 activity, although attractive, is controversial.

It has been reasoned that excess human NAT1/mouse *NAT2* is detrimental in mice during development due to increased folate catabolism driven by metabolism of the folate breakdown product p-abaglu (Wakefield *et al.*, 2007a; Cao *et al.*, 2010). These studies have been challenged recently but additional information linking human NAT1 and mouse NAT2 with folate has been described. These enzymes, but no other NATs tested, are specifically able to catalyse hydrolysis of acetyl CoA in the presence of folate (Rodrigues-Lima *et al.*, 2011). Hydrolysis of acetyl CoA in all NATs tested is accelerated in the presence of an arylamine or hydrazine substrate – the basis of NAT assays (Brooke *et al.*, 2003a). Folate itself is not a substrate for human NAT1 acetylation and these studies open up the possibility that human NAT1 and its homologue in mouse, Nat2, regulate homeostasis between acetyl CoA and folate pools. A direct link remains to be established but the observation explains why folate has been described as an inhibitor of human NAT1 (Ward *et al.*, 1995) since it serves to deplete the cofactor acetyl CoA.

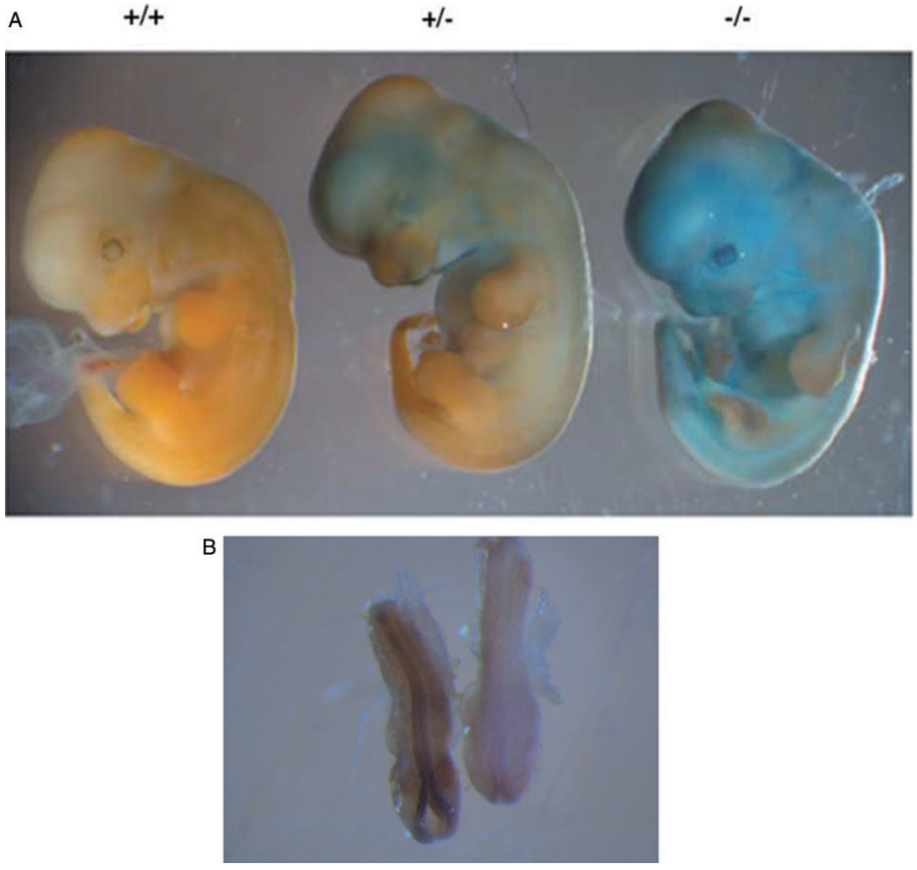

Figure 7

Generation of knockout mice. The Nat2 gene has been interrupted by a cassette in frame, which includes the β-galactosidase gene. Embryos can be stained with X-gal, which is converted to a blue colour in the knockout mice. Ashows 12.5 day embryos with wild type, heterozygote (Nat2+/–), a homozygote null (Nat2–/–). B shows 8.5 day whole mount embryo with wild type on the right and homozygote Nat2–/– on the left. Staining in the developing neural tube is marked. Panel A is from Cornish *et al.*, 2003. Panel B was prepared by L. Wakefield.

Knockout mice

Transgenic mice with mouse *Nat2* deleted (Sugamori *et al.*, 2003) or interrupted by insertion of *lacZ* in frame with the gene have been made (Cornish *et al.*, 2003). The latter strain of mice allows *lacZ* expression under control of the mouse *Nat* promoter. Expression of mouse *Nat2* can therefore be studied by staining whole embryos with the β-galactosidase substrate X-gal. This is illustrated dramatically in the skin of mice (Figure 7) and adds further support to the similarity with human NAT1, which is also found in skin. Mouse *Nat2* is expressed in embryos including in the developing heart in scattered clusters of cells including at the sino-atrial node (Wakefield *et al.*, 2005; 2008b). The detection of *Nat2* expression in ES cells (Payton *et al.*, 1999a) raises the question of whether it is cardiac stem cells, which are expressing *Nat2*. The expression of mouse *Nat1* appears to be mainly restricted to liver and gut and is unmodified in the *Nat2* –/– mice (Loehele *et al.*, 2006).

Mice lacking *Nat3* have been generated (Sugamori *et al.*, 2007) but show no differences in drug metabolism. Mice lacking *Nat1* and /or *Nat2* appear healthy but show differences in drug metabolism (Sugamori *et al.*, 2003) although there appears to be defects that emerge in long-term breeding programmes (Wakefield *et al.*, 2007b). There appears to be a gender imbalance in the offspring on breeding heterozygotes (Cornish *et al.*, 2003; Wakefield *et al.*, 2008b). Gender differences in carcinogen activation have also been identified in mice with the *Nat2* gene deleted (Sugamori *et al.*, 2012).

Mouse NAT2 is found in adult mice in a similar distribution to the human NAT1 (Stanley *et al.*, 1997) including in breast tissue (Williams *et al.*, 2001; Wakefield *et al.*, 2008b). The relationship between human *NAT1* expression and hormonal control (Adam *et al.*, 2003; Butcher *et al.*, 2007) and the earlier studies on hormonal control of murine *Nat* (Estrada-Rodgers *et al.*, 1998) may well be a key to the gender-linked asymmetry observed in transgenic mice and the understanding of epigenetic control of gene expression are areas of relative ignorance at present.

Structural studies

Early enzymology studies of Riddle and Jencks (1971) identified NAT activity involved formation of an acetylated enzyme intermediate in a mechanism known as ping-pong bi-bi. Cysteine was implicated in accepting the acetyl group from acetyl CoA as NAT was inhibited by classic cysteine reactive

agents and by oxidative damage. One of us (ES) was particularly interested to study the NAT enzymes from experience gained with iron sulphur proteins (Sim and Vignais, 1979) although NAT turned out not to be an iron sulphur protein. Recent work has extended the oxidative sensitivity of the NAT enzymes to reactive oxygen species (see Dupret *et al.*, 2005).

The sulphydryl reactive agents N-ethyl maleimide and bromacetanilide inhibited NAT (Andres *et al.*, 1988; Watson *et al.*, 1990) and it was clear from accumulated evidence (see Weber and Hein, 1985) that NAT had an active site cysteine residue. Identifying Cys^{69} as the active site in NAT from *S. typhimurium* (Watanabe *et al.*, 1992) was followed by confirmation through site-directed mutagenesis that the equivalent residue in human NAT2 was Cys^{68} (Dupret and Grant, 1992).

The 3D structure of the NAT enzymes needed sufficient pure protein for crystallization and recombinant protein was the way forward. Following a well trodden path for drug-metabolizing enzymes, sufficient pure stable recombinant protein was obtained by cloning *nat* from *S. typhimurium* (Sinclair *et al.*, 1998) after many attempts to generate sufficient human NAT as a recombinant enzyme for crystallization (Ward *et al.*, 1995; Sinclair and Sim, 1997). While the yield of the *S. typhimurium* NAT was improved (Sinclair *et al.*, 1998), its substrate specificity and characteristics were established (Figure 6). Labelling the active site cysteine with a bromacetanilide conferring selectivity included a ^{13}C label and also ^{19}F. The bromacetanilide binds covalently to the active site Cys (determined by mass spectrometry) and we hedged our bets with the possibility of probing the molecular environment of the active site by ^{13}C and ^{19}F NMR should it prove impossible to obtain crystals. It appeared we might have needed the NMR approach as on one particular rare occasion when there were diffractable crystals, the data collected at the Daresbury Synchrotron was destroyed by an electrical storm in transit to our Oxford computer. This was a particularly low point but we eventually obtained diffraction data with active *S. typhimurium* NAT labelled with selenomethionine to allow the structure of the unique protein fold to be solved (Sinclair *et al.*, 2000).

When the structure was obtained, it was worth waiting for (Figure 8). The active site Cys with its specificity label was visible juxtaposed to a histidine residue and an aspartate residue – a clearly identifiable catalytic triad, which activates the Cys in order to allow the first step of catalysis in which the Cys residue becomes acetylated before being transferred to the incoming arylamine. The catalytic triad was obvious in all NATs and appears essential for activity (Sandy *et al.*, 2005a): indeed in one of the NAT2 slow variants, the Asp of the catalytic triad has been mutated to Asn and is inactive. In all subsequent structures of NATs obtained, the catalytic triad residues are superimposable.

Solving the protein fold allowed excellent work to be done in solving the complete structure of mammalian NAT by NMR (Zhang *et al.*, 2006) and then the same bromacetanilide approach was used (but without the ^{19}F and ^{13}C labels) in studies from the Structural Genomics Consortium along with site-directed mutagenesis to improve the stability of the human NAT1 protein (Wu *et al.*, 2007). The improvement in stability of human NAT1 was based on comparing the substrate specificity and stability of a series of chimeras of human

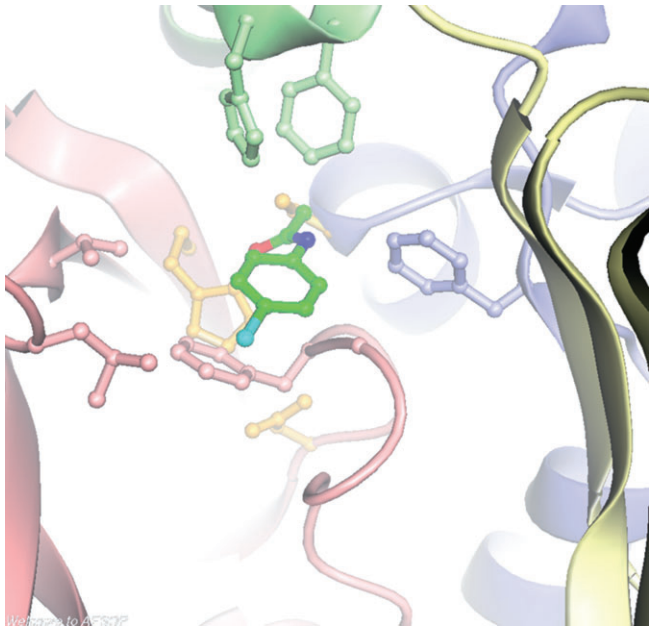

Figure 8

Active site of *S. typhimurium* NAT. The residues forming the catalytic triad are shown in pale yellow Cys^{69}, His^{107} and Asp^{122}. The active site Cys^{69} has been labelled with bromacetanilide (bright green). The His residue is behind the labelled Cys, and the Asp residue is in front. The pale pink, green and blue residues correspond to the three domains of the NAT structure.

NAT1 and human NAT2 (Goodfellow *et al.*, 2000). The importance of residues 125–127 were identified in a landmark study.

Comparison of the crystal structures of human NAT1 and the model of human NAT2 rationalized the substrate specificity profiles of the two enzymes. Interestingly, modifying only one residue in NAT1 (phenylalanine 125 to serine; F125S) alters the specificity such that it resembles human NAT2 in which residue 125 is serine. The same is true of the mouse equivalent enzyme to human NAT1, Nat2, its specificity is altered when residue 125 is mutated from phenylalanine to serine (F125S) (Laurieri *et al.*, 2010).

The NAT enzyme proteins, which are between 280 and 300 amino acids long (Sandy *et al.*, 2005a; Vagena *et al.*, 2008), consist of three almost equivalently sized domains, an α-domain; a β-domain and an α/β-C-terminal domain All active site Cys, His^{107} and Asp^{122} residues are within the first two domains and a third α-β lid folds over the active site. The length and role of the third C-terminal domain differs most among NATs. The C-terminus contributes to substrate recognition because in human NAT1, removal of the third domain results in a protein that hydrolyses acetyl CoA readily with no arylamine substrate present (Sinclair and Sim, 1997). The same is true of the enzyme from *S. typhimurium* (Mushtaq *et al.*, 2002) when either the entire third domain or even the C-terminal 11 amino acids are missing. Thus, the interaction with substrate and the interaction with cofactor can be separated. Folate, which mediates hydrolysis specifically in human NAT1 and mouse NAT2, may bind to effectively unpeel the third domain of NAT from the active site. More work is needed to confirm this interaction as there is no

crystal structure with folate bound. New modelling data support folate binding (Laurieri *et al*., 2014).

The role of the C-terminus also varies in relation to cofactor binding. The structure of human NAT 1 with CoA bound (Wu *et al*., 2007) and of an enzyme from *Mycobacterium marinum* with CoA bound (Fullam *et al*., 2008) and from *Bacillus anthracis* (Pluvinage *et al*., 2011) demonstrate that CoA can bind in different positions in relation to the C-terminus. Interestingly, the NAT enzyme from *B. anthracis* was shown as having CoA bound although the recombinant enzyme had been through several purification steps and had not been incubated with CoA (Pluvinage *et al*., 2011).

NATs have been crystallized from many organisms and in some cases with substrate bound including hydralazine (Abuhammad *et al*., 2011) or isoniazid (Sandy *et al*., 2005b). The ping-pong bi-bi mechanism implies that acetyl CoA binds first but there is clear evidence from crystallography and also from NMR (Delgoda *et al*., 2003; Kawamura *et al*., 2008) studies that substrate can be bound without prior binding of acetyl CoA.

NAT in bacteria

Finding NAT in bacteria prompted the question of whether there might be a NAT homologue in mycobacteria to metabolize the anti-tuberculosis agent isoniazid.

Isoniazid is a substrate of human NAT2 and isoniazid is a prodrug activated by katG in *M. tuberculosis*. Using gridded libraries from GlaxoSmithKline, we demonstrated that a *nat* gene was present and expressed in both *M. smegmatis* and *M. tuberculosis* (Payton *et al*., 1999b; 2001a,b). The genome of *M. tuberculosis* was published around the same time (Cole *et al*., 1998) and subsequent studies showed the gene in *M. bovis* BCG and *M. tuberculosis* were identical (see Sim *et al*., 2008b). The *nat* gene in mycobacteria is in a gene cluster, which is essential for cholesterol catabolism for intracellular fuel in these organisms (Van der Geize *et al*., 2007). The gene cluster, including the *nat* gene, is essential for intracellular survival of mycobacteria inside macrophage (Bhakta *et al*., 2004; Anderton *et al*., 2006; Yam *et al*., 2009).

A subgroup of *M. tuberculosis* strains, which had an accumulation of mutations, also had mutations in the *nat* gene, which contributed marginally to isoniaizid resistance in clinical isolates (Upton *et al*., 2001; Sholto-Douglas-Vernon *et al*., 2005). Overexpressing *nat* experimentally in mycobacteria, it was shown that NAT increases resistance to isoniazid (Payton *et al*., 1999a; Bhakta *et al*., 2004) but is not the sole factor. Making a *nat* gene deleted strain of *M. bovis* BCG, resulted in an approximately threefold increase in sensitivity to isoniazid. These gene deletion studies however showed a very exciting finding – that the *nat* deleted strain was defective in cell wall synthesis as well as being essential for intracellular survival of *M. bovis* BCG (Bhakta *et al*., 2004). These studies strongly suggested that the *nat* gene product from *M. tuberculosis* was a good drug target for anti-tuberculosis therapy.

We had extreme difficulty in generating large quantities of the NAT from *M. tuberculosis* (TBNAT) as a recombinant protein (Upton *et al*., 2001; Abuhammad *et al*., 2012) as did others (Sikora *et al*., 2008). While we were improving the yield of the TBNAT enzyme, we used NAT from *M. smegmatis* (Sandy *et al*., 2005b) and then *M. marinum* (Fullam *et al*., 2008), which is much closer in sequence to the NAT from *M. tuberculosis*. *M. marinum* NAT is very soluble while the *M. tuberculosis* NAT is not (Fullam *et al*., 2009; Abuhammad *et al*., 2013 in press) and they show very different physical properties (Lack *et al*., 2009), including melting at different temperatures. The melting property resides in the first two domains of the protein and may reflect the ability of TBNAT to remain active in inflammatory conditions associated with intracellular infection.

We have at last, after 15 years, obtained a crystal structure of the NAT from *M. tuberculosis* by seeding a preparation of *M. tuberculosis* NAT with a crystal of NAT from *M. marinum* (Abuhammad *et al*., 2013). The substrate specificities of the *M. marinum* and *M. tuberculosis* NAT enzymes are not identical and this illustrates the importance in drug discovery of using an appropriate model or at least understanding the differences when a surrogate has to be used. While *M. marinum* NAT has been useful as a model, it emphasizes the need to have the target protein available for developing specific inhibitors (Fullam *et al*., 2009).

NAT as drug target

Development of NAT ligands. We embarked on a search for specific NAT isoenzymes in 2003 to seek possible anti-tuberculosis agents and coupled that with a search for human NAT1 inhibitors. Up-regulation of human NAT1 in breast cancer made the search for NAT inhibitors an attractive prospect, which has recently been reinforced (Butcher and Minchin, 2012). We were also interested in IBD because the presence of NATs in the gut epithelium had been established by immunohistochemistry (Hickman *et al*., 1998) and NAT is involved in metabolic inactivation of the drug used to treat IBD, namely 5-AS. Therefore, there might be a benefit in IBD from co-administration of 5-AS and NAT inhibitors.

Screening for NAT inhibitors. An automatable assay was established (Brooke *et al*., 2003a) which could be adapted to detect inhibitors (Brooke *et al*., 2003b). A library of some 5000 compounds was available (Russell *et al*., 2009) and we assembled the other tools required – sufficient of all of the enzymes we wished to use. We produced hundreds of milligrams of each of human NAT1 (Wang *et al*., 2005), hamster NAT2 (Kawamura *et al*., 2004), *S. typhimurium* NAT (Sinclair *et al*., 1998), *M. smegmatis* NAT (Sandy *et al*., 2002) and *P. aeruginosa* NAT (Westwood *et al*., 2005). The mammalian enzymes were to allow any possible toxicity to be identified early but generated very interesting results.

We compared the substrate specificity profiles of each of these enzymes (Westwood *et al*., 2006) (Figure 6) and other NATs used in later stages of sophistication in the screen, which were available in lower quantities (Kawamura *et al*., 2004; 2008; Fullam *et al*., 2009). The screen (Westwood *et al*., 2011) identified compounds inhibiting either the eukaryotic enzymes or the prokaryotic enzymes specifically (Figure 7). All hits from the screen were resynthesized as in one case the isomer in the screen had been incorrectly annotated (Fullam *et al*., 2011).

Inhibitors and their development as potential antituberculars. Following duplicate screening, compounds inhibiting prokaryotic NAT enzymes specifically with an IC_{50} value of

less than 10 μM were tested for inhibition of growth of mycobacteria and of *Escherichia coli*. Of those that initially looked interesting (Fullam *et al.*, 2008; 2013; Westwood *et al.*, 2010; Abuhammad *et al.*, 2012), we have studied two of these classes in detail with structure activity analyses, identifying the mechanism of inhibition.

In the triazole class, which shows competitive inhibition with substrate, structure activity relationship (SAR) analysis of anti-mycobacterial activity and NAT inhibition are coincident and modelling of binding to the active site of TBNAT readily explains the SAR (Westwood *et al.*, 2010). The effects of the triazole class on *M. bovis* BCG phenotype was very similar to that of deleting the *nat* gene (Bhakta *et al.*, 2004), including inhibiting growth of *M. bovis* BCG inside macrophage without showing cytotoxicity for the macrophage. Based on these findings, a commercial company, now known as Summit plc, used a similar screening mechanism and generated compounds that were developed to the point of outlicencing as anti-tuberculosis leads.

The piperidinols have recently been shown to bind covalently, effectively acting in a prodrug-type manner (Figure 9). Covalent binding is a feature of excellent drugs for tuberculosis including the recently identified benzothiazines (Batt *et al.*, 2012) as well as isoniazid, the front line prodrug. The piperidinol class was our most effective hit in inhibiting growth of *M. tuberculosis* and was equally effective at blocking growth of *M. tuberculosis* in which the *nat* gene is deleted (Abuhammad *et al.*, 2012). This is now seen as a benefit as polypharmacy (as it is called) is a feature of many excellent and long-surviving drugs such as aspirin. It is good to see the demise of the dogma that a lead compound was only worth investigating if it could be demonstrated to be against only one identifiable target. A broader minded attitude is beginning to allow a flourishing pipeline for novel anti-tuberculosis agents (http://www.tballiance.org/). In view of the ever present possibility of the development of resistance, it is important that all promising targets are pursued as well as the return to the whole cell approach. Our own work has now begun to focus on another of the gene products of the '*nat*' gene cluster in *M. tuberculosis*, namely, HsaD (Lack *et al.*, 2009).

We have also looked at the effects of plant extracts and found a terpenoid from the traditional medicine, the pepper bark tree (*Warburgia salutaris*), which appears to act through inhibition of NAT from mycobacteria. However, the extract was not very potent (Madikane *et al.*, 2007).

Inhibitors and their development for use in cancer. Of the compounds identified as specific for eukaryotic NATs, we rescreened to identify those which inhibited human NAT1 and its homologues from other mammals. We pursued only two classes of compound that were effective at inhibiting human NAT1 in breast cancer cell extracts (Russell *et al.*, 2009). One of the compounds was found to be generally cytotoxic, a rhodanine (Russell *et al.*, 2009), but it has been exploited as a potential chemotherapeutic class (Tiang *et al.*, 2010; see Butcher and Minchin, 2012). The Australian group are pushing forward with this approach and have also identified NAT as a breast cancer target using siRNA (Tiang *et al.*, 2011). Using low MW inhibitors has proved difficult as a result of solubility issues.

In our own studies, a group of human NAT1-specific compounds, naphthoquinones, have proved very interesting. These compounds specifically change colour on binding to human NAT1 and to the mouse homologue mouse Nat2. The change of colour is due to a proton transfer between the protein and the compound and site-directed mutagenesis studies have allowed identification of the protein residues involved (Laurieri *et al.*, 2010; 2013). This compound shows the same colour change with mouse NAT2 but not with any of the other NAT enzymes. To use this for detecting human NAT1 as a biomarker sensitivity will need to be improved and we are currently making fluorescent versions of the naphthoquinone (Egleton, 2012).

Inhibitors of NAT and potential in IBD. 5-AS is used to treat IBD and it was reasoned that if the acetylation of 5-AS could be inhibited then this provides a method for improving the amount of p-AS, which has be generated in the treatment of IBD. 5-AS is metabolized by N-acetylation and while NATs in gut bacteria are very good at acetylating 5-AS (Delomenie *et al.*, 2001; Westwood *et al.*, 2006), the human enzyme human NAT1 as well as NAT2 also acetylate 5-AS.

As 5-AS is mostly given as a prodrug, balsalazide or sulphasalazine, with 5-AS released by azoreductase in the gut flora, it was necessary to understand whether potential NAT inhibitors would also inhibit the gut flora azoreductases. We commenced work on azoreductases during the sabbatical leave of the corresponding author in Dundee with Mike Coughtrie in 2005. Following bioinformatic analysis, we made recombinant azoreductases from *P. aeruginosa*, which are very soluble flavoproteins and crystallize readily (Wang *et al.*, 2007). Structures of azoreductase with balasalazide bound show clearly that the relative position of the substrate in relation to the isoalloxazine ring nitrogen atoms relies on a proton relay system within the substrate resulting in the reduction of the diazo bond in balsalazide (Ryan *et al.*, 2010a). We have also demonstrated that the antibiotic nitrofurazone is reduced by azoreducatse with the electron flow through the substrate from the flavin initially reducing the nitroso group (Ryan *et al.*, 2011; Figure 10).

It was proposed (Ryan *et al.*, 2010b) that the azoreductases would reduce quinones and we found in separate experiments that the naphthoquinone inhibitor of human NAT1 was indeed an inhibitor of azoreductase. To expand the diversity of NAT1 inhibitors, we have used the 3D shape of our best inhibitor, the naphthoquinone, to screen against shapes of all purchasable chemical space – some 35 million compounds. From a random selection of 23 compounds from the top 100 hits, almost half (10 compounds) had an IC_{50} of less than 10 μM (Ballester *et al.*, 2010). Although none was better than the probe compound, the diversity of chemical classes made this a positive approach to diversification in ligand identification.

Acknowledgements

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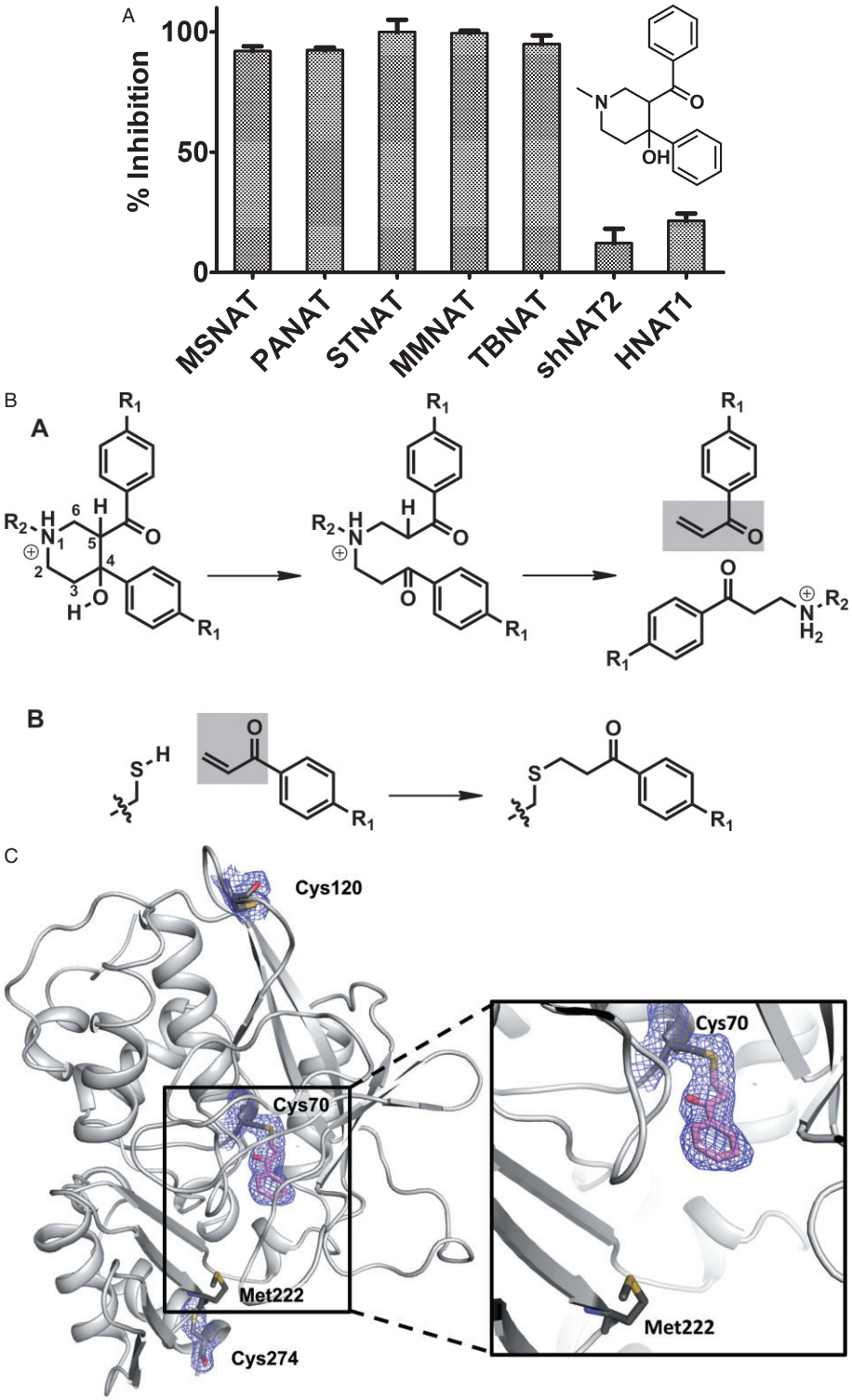

Figure 9

Inhibition of mycobacterial NAT by piperidinol. (A) The piperidinol inhibitor shows specificity for prokaryotic enzymes. (B) The mechanism of the reaction for the piperidinol inhibitor to NAT is through a chemical transformation to the corresponding phenyl vinyl ketone (PVK), which then binds to the active site Cys residue. C shows the inhibitor bound to the active site Cys residue. Electron density is shown around another Cys, which has no such modification.

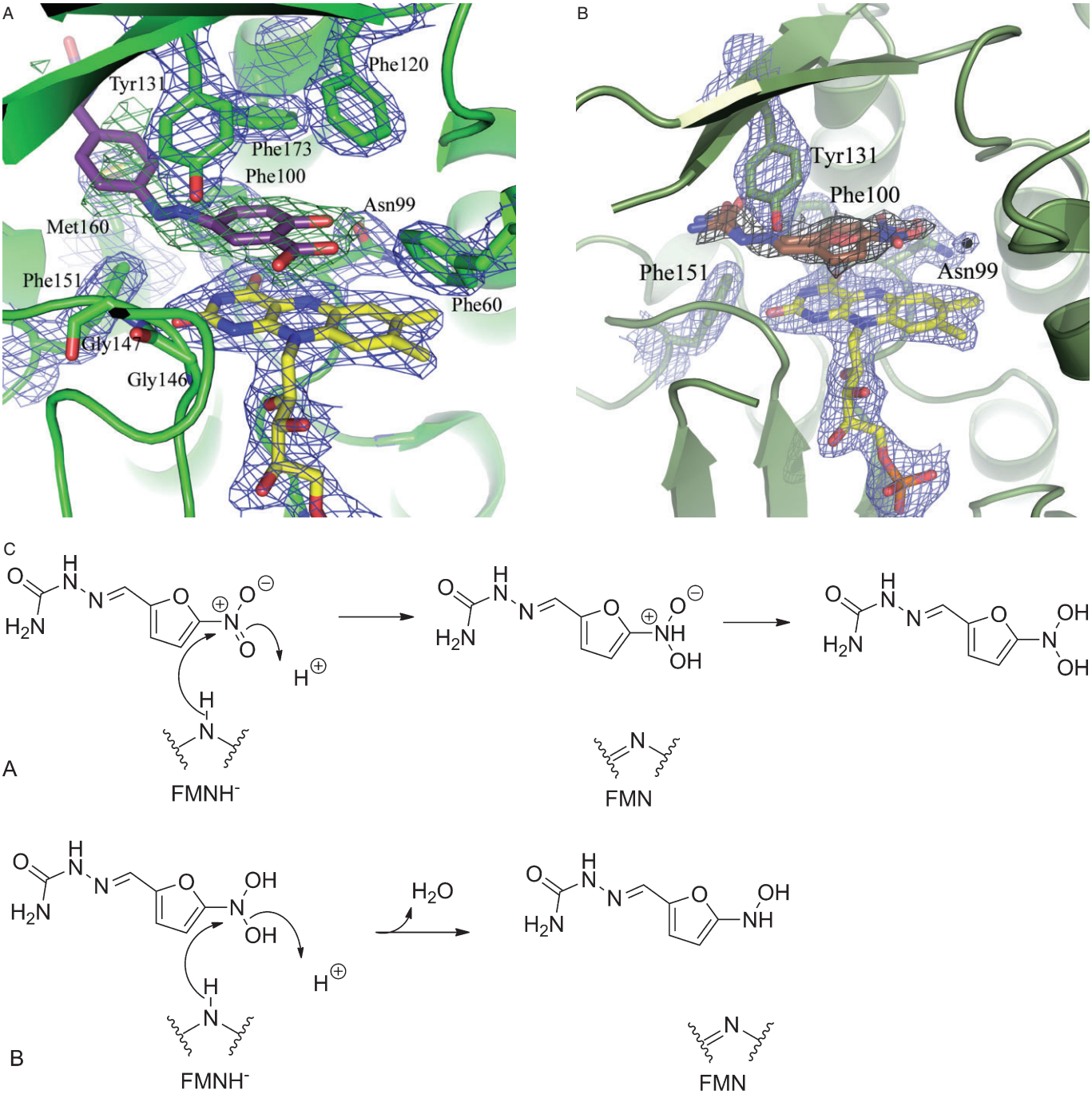

Figure 10

Binding of substrates to azoreductase paAzoR1 from *Pseudomonas aeruginosa*. (A) Balsalazide (from Ryan *et al.*, 2010a) structure was solved to 2.3 Å. Balsalazide is shown in purple and FMN cofactor is shown in yellow. (B) Nitrofurazone (Ryan *et al.*, 2011). Structure was solved at 2.08 Å. Nitrofurazone is in brown while stably bound water molecule involved in reduction is a black ball. C shows the tautomerization and charge relay leading to reduction of the nitro group.

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Conflict of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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Table S1 Collaborators and co-workers.