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The role of the human acetylation polymorphism in the metabolic activation of the food carcinogen 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ)

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The metabolic activation of the heterocyclic food carcinogen 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) by two human cytochrome P450 monooxygenases (P4501A1 and P4501A2) and two human *N*-acetyltransferases (NAT1 and NAT2) was investigated. Various combinations of these enzymes were functionally expressed in COS-1 cells. DNA adducts resulting from the activation of IQ were assayed quantitatively by the ^{32}P-postlabeling procedure. The highest adduct frequency was observed in cells expressing both CYP1A2 and NAT2. CYP1A2 in combination with NAT1 was 3–6 times less active. When expressed alone these enzymes gave rise to low adduct frequencies. Experiments with *N*-acetyl-IQ as substrate suggest that NAT1 and NAT2 in addition to their known role in *N*-acetylation display arylhydroxamic acid *N,O*-acetyltransferase (AHAT) activity. Quantitative differences in adduct formation between IQ and *N*-acetyl-IQ indicated that metabolic activation of these arylamines preferentially occurs by P4501A2-catalyzed *N*-hydroxylation followed by *O*-acetylation mediated through NAT1 and/or NAT2. These data, in combination with the known genetic polymorphism of NAT2, may explain the clinical observation that the acetylation polymorphism constitutes a risk factor in the carcinogenic activation of environmental mutagens.

Introduction

Frying of meat and fish results in the generation of heterocyclic arylamines (1), which present a major source of dietary carcinogens. Among a number of food-derived heterocyclic amines, 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ*) was found to be highly mutagenic in *Salmonella typhimurium*, and its carcinogenic potential was demonstrated in various rodent species and a primate (2,3).

The effects of heterocyclic arylamines, like those of other arylamines, require metabolic activation by drug-metabolizing enzymes. It is generally accepted that the ultimate mutagens/carcinogens are electrophilic arylnitrenium ions, which can react with DNA and form adducts (4–6). Reactions involved in the metabolic activation include *N*-hydroxylation and *N*- and *O*-acetylation (Figure 1), catalyzed by microsomal monooxygenase enzymes of the cytochrome P4501A family (for review see 7,8), and acetyl coenzyme A-dependent arylamine *N*-acetyltransferases (NATs) in liver cytosol (9). In the case of IQ and related heterocyclic arylamines, *N*-acetylation appears to be a minor metabolic pathway (10,11). Considerable interindividual variations in the activities of these enzymes has been observed caused either by environmental conditions or genetic polymorphisms. P4501A1 is induced by cigarette smoking, and individuals with genetically controlled high inducibility are at higher risk of developing cigarette-induced bronchogenic carcinoma (12). A restriction fragment length polymorphism (RFLP) has been reported in the human CYP1A1 gene downstream of the coding region (13–15). The association of this RFLP with an increased incidence of bronchogenic carcinoma has been observed in some but not all populations tested (12). Point mutations in the NAT2 gene, resulting in decreased enzyme protein levels (16), are responsible for the acetylation polymorphism, which divides the population into so-called 'rapid' and 'slow' acetylators. Between 40 and 70% of individuals in Caucasian populations are slow acetylators (17). Slow acetylators display impaired metabolism of a variety of arylamine and hydrazine drugs and chemicals. These include many therapeutically useful drugs (for review see 17,18), as well as potential arylamine carcinogens present in dyes, antioxidants, pesticides and explosives (19).

There is circumstantial evidence for a link between rapid acetylator status and certain diseases, e.g. colorectal cancer (20–22). A second human NAT gene, NAT1, is not affected by the acetylation polymorphism, and does not contribute to the

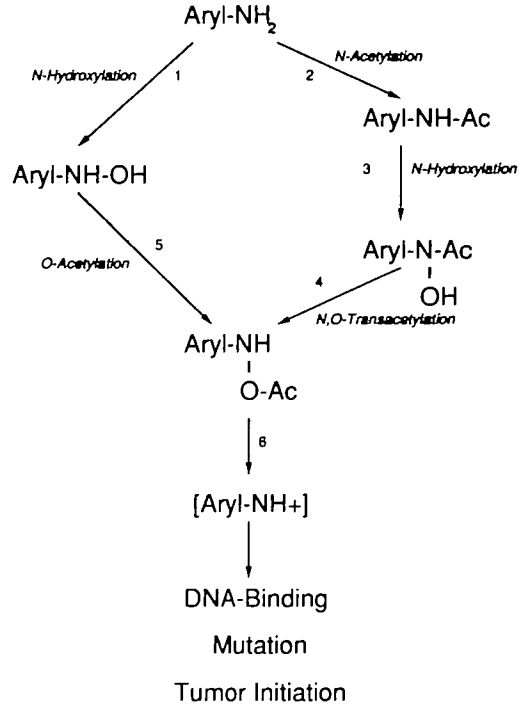

Fig. 1. Metabolic activation of arylamine carcinogens.

*Abbreviations: IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoline; NAT, *N*-acetyltransferase; RFLP, restriction fragment length polymorphism; AHAT, arylhydroxamic acid *N,O*-acetyltransferase; OAT, *O*-acetyltransferase.

acetylator status (23,24). The availability of the cloned NAT1/NAT2 genes allows us to test in a cell culture situation, which one of these genes is involved, if at all, in the metabolic activation of IQ. Upon transfection of different combinations of NAT and CYP1A genes, P4501A2 and NAT2 resulted in the highest IQ-DNA adduct frequencies. This suggests that the human acetylation polymorphism may affect the carcinogenic action of the food mutagen IQ.

Materials and methods

Chemicals

IQ was purchased from WAKO Pure Chem. Industries, Ltd (Neuss, Germany). *N*-Acetyl-IQ was prepared from IQ and acetic anhydride, and was further purified by chromatography on silica gel. Phenoxazone ethers and resorufin were purchased from Pierce Chemicals. All other chemicals were of HPLC or analytical grade quality and obtained from local suppliers.

Functional expression of NAT1, NAT2 CYP1A1 and CYP1A2 in monkey kidney COS-1 cells

COS-1 cells were cultured and transfected by the DEAE–dextran/chloroquine method as described (25). The human NAT1 and NAT2 expression constructs in the vector p91023(B) were as described (23). cDNA clones encoding human CYP1A1 (26) and CYP1A2 (27) were kindly provided by D.W.Nebert and F.J.Gonzales (NCI, NIH Bethesda, MD), and were subcloned into p91023(B). Cultures were treated with IQ or *N*-acetyl-IQ (dissolved in ethanol) 50 h after transfection, and cells were harvested 9–12 h later.

Enzyme assays and detection of expressed proteins on Western blots

Enzyme activity of expressed NAT proteins was determined using sulfamethazine as a substrate and quantitated using a HPLC assay as described (28).

P4501A1 and P4501A2 proteins in transfected COS-1 cultures and in microsomes prepared from kidney donor liver (KDL35) were identified by immunoreaction on Western blots with a rabbit antiserum raised against the corresponding rat P4501A1 (P450c) enzyme. This antiserum recognizes both human P4501A1 and P4501A2 proteins. Their enzymatic activities were determined by dealkylation of phenoxazone ethers 7-methoxy- and 7-ethoxy-resorufin as described by Burke *et al.* (29). Deacetylation of *N*-acetyl-IQ was measured by reversed-phase HPLC (MN 5-C18 Macherey-Nagel, Germany) by isocratic elution using 50 mM sodium acetate/methanol (40:60) pH 6.3 as mobile phase and UV detection at λ = 254 nm.

DNA isolation and ^{32}P-postlabeling

Cell pellets were homogenized by means of an Ultra-Turrax, and treated with RNase A, RNase T_1 and proteinase K. DNA was extracted with phenol/chloroform and precipitated with ethanol. After digestion of the DNA to 3′-nucleotides with micrococcus endonuclease and spleen exonuclease (30), an aliquot of the digest equivalent to 4 μg was labelled with 100–120 μCi [γ-^{32}P]ATP (sp. act. 3000 Ci/mmol; Amersham, Braunschweig, Germany) and by use of T4 polynucleotide kinase (Pharmacia). The intensification procedure was applied according to Randerath *et al.* (31). Four-directional TLC separation of the adducts was performed on PEI–cellulose sheets (Macherey-Nagel) with the following solvents: D1, 1 M sodium phosphate, pH 6.8; D3, 3.8 M lithium formate, 6.8 M urea, pH 3.4; D4, 0.6 M sodium phosphate, 0.38 M Tris–HCl, 6.5 M urea, pH 8.2; D5, 1.7 M sodium phosphate, pH 6.0. After detection of adducts by autoradiography, spots were excised and quantitated by Cerenkov counting; intensification factors required for the quantitative analysis were determined in each experiment by use of a highly IQ-modified DNA (32).

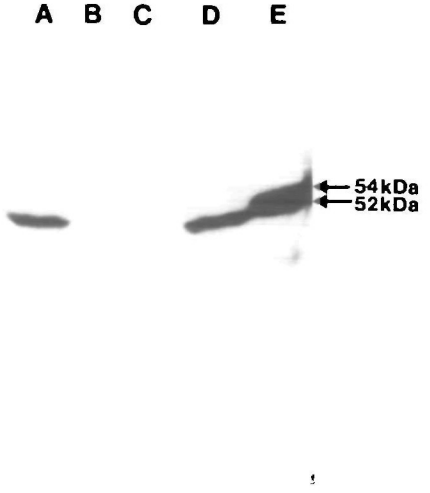

Fig. 2. Immunoreaction on Western blots of CYP1A1 and CYP1A2 in human liver microsomes and upon expression of the respective cDNAS in COS-1 cells. Homogenates of COS-1 cells transfected with cDNAS encoding CYP1A1 in sense (lane A) and antisense orientation (lane B), and CYP1A2 in antisense (lane C) and sense (lane D) orientation, as well as human liver microsomes (lane E) were subjected to SDS–PAGE (150 μg of COS-1 homogenates and 50 μg microsomal protein), transferred to nitrocellulose and immunoreacted with a polyclonal rabbit antiserum raised against purified rat P4501A1.

Results

Metabolic activation of IQ

Figure 1 shows the proposed enzyme reactions involved in the metabolic activation of arylamines such as IQ. The first step of metabolism of arylamines can either be an *N*-hydroxylation (reaction 1 in Figure 1) or an *N*-acetylation (reaction 2 in Figure 1). Reaction 1 may be catalyzed by either P4501A1 or P4501A2 (7,8), and it has been shown that liver cytosolic NAT accounts for the *N*-acetylation reaction (24). The *N*-arylacetamide resulting from reaction 2 serves as substrate for P4501A1 or P4501A2, and *N*-hydroxylation yields the respective aryl-hydroxamic acid (reaction 3, Figure 1). The two pathways converge through reactions 4 (*N,O*-transacetylation) and 5 (*O*-

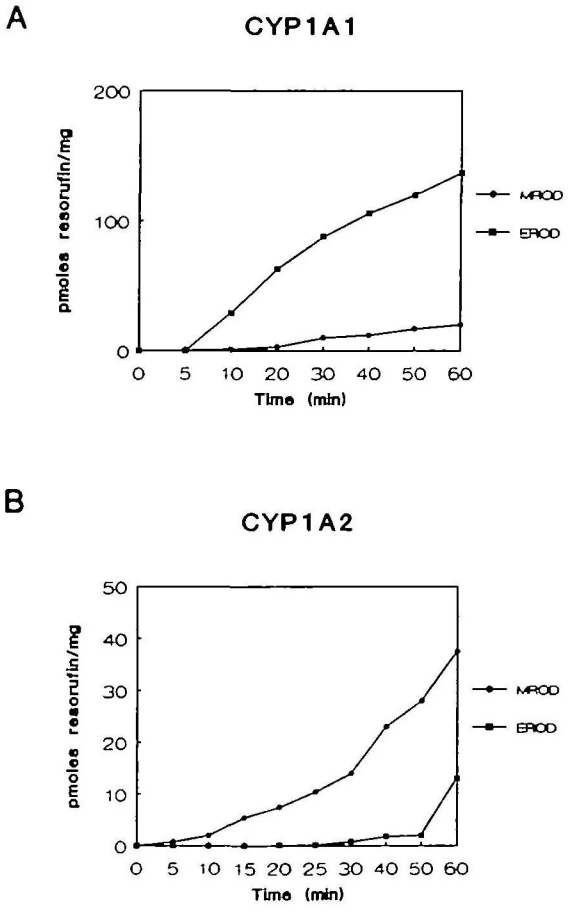

Fig. 3. Expressed cytochrome P4501A1 and cytochrome P4501A2 are functionally active in COS-1 cells. Homogenates of COS-1 cells transfected with CYP1A1 (A) and CYP1A2 (B) were incubated with methoxyresorufin (●) and ethoxyresorufin (■), and formation of the dealkylated products was determined at the time points indicated. Note: P4501A1 preferentially metabolized ethoxyresorufin, whereas P4501A2 displayed higher activity with methoxyresorufin.

acetylation) to yield the product, *N*-acetoxy-*N*-arylamine. Both of these reactions have been attributed to the activity of cytosolic NAT in hamster and rabbit (33,34), whereas no such correlation could as yet be established for the human NAT enzymes. Spontaneous degradation of *N*-acetoxy-*N*-arylamines (reaction 6 in Figure 1) leads, presumably via unstable arylnitrenium ions (4–6), to the formation of DNA adducts.

COS-1 cell cultures provide an in vitro system to analyze IQ-adduct formation

COS-1 cells were used to analyze the involvement of the proposed enzymes in the above-mentioned reactions in an *in vitro* system, both individually and in combination. We have previously demonstrated that NAT1 and NAT2 can be functionally expressed in COS-1 cells, and that COS-1 cells have no detectable endogenous *N*-acetylation activity (25). In this study, we have additionally analyzed the expression of CYP1A1 and CYP1A2 cDNAs in COS-1 cells. Immunoreaction on a Western blot of proteins expressed from the cDNAs using a polyclonal rabbit antiserum raised against the rat P4501A1 is shown in Figure 2. Protein bands of the expected mol. wts of 52 and 54 kDa in cultures transfected with CYP1A1 (lane A, Figure 2) and CYP1A2 (lane D, Figure 2) respectively were detected. These were indistinguishable in electrophoretic mobility from microsomal proteins isolated from human liver (lane E, Figure 2), suggesting that in this particular liver both P4501A proteins were present. No immunoreactive proteins were detected in cultures transfected with the constructs in antisense orientation (lanes B and C, Figure 2). Cell homogenates of cultures transfected with CYP1A1 or CYP1A2 both displayed enzyme activity with the substrates 7-methoxy- and 7-ethoxyresorufin (Figure 3). Homogenates of untransfected cells and cells transfected with cDNAs in antisense orientation did not display any detectable activities with either substrate (not shown).

Table I. Formation of IQ–nucleotide adducts in COS-1 cells transfected with cytochrome P450 monooxygenase and *N*-acetyltransferase genes

Gene combinations	Adducts/10^8 nucleotides (±SD)		
	IQ 100 μM	IQ 10 μM	*N*-Acetyl-IQ 10 μM
CYP1A1	7.3 ± 0.2	ND	ND
CYP1A2	13.6 ± 2.3	0	0
NAT1	1.7 ± 0.4	ND	0
NAT2	1.6 ± 0.2	ND	0
CYP1A2 + NAT1	81.6 ± 5.9	9.3 ± 0.7	1.0 ± 0.2
CYP1A2 + NAT2	250.3 ± 28.8	57.2 ± 5.8	14.4 ± 1.3

COS-1 cells were transfected with CYP1A1, CYP1A2, NAT1 and NAT2, either alone or in the combination indicated. Cultures were incubated in the presence of 10 or 100 μM IQ or *N*-acetyl-IQ for 12 h, followed by isolation of the DNA and analysis of IQ-adducts.
Note: the combination of CYP1A2 and NAT2 resulted in the highest adduct frequencies in all three experiments. Adduct formation with the acetylated IQ substrate still required the NAT enzyme(s), indicating their inherent *N,O*-transacetylase activities.
ND, not done; 0, no adducts detectable.

The highest DNA adduct frequency occurs in COS-1 cells transfected with CYP1A2 and NAT2

COS-1 cells were transfected with NAT and CYP1A genes individually and in the combinations shown in Table I. Transfected cultures were treated with IQ (at a concentration of 100 μM) and the resulting adduct frequencies determined (Table I, left-hand column). Transfections with NAT1 or NAT2 alone resulted in low levels of IQ–DNA adducts (1–2 adducts/10^8 nucleotides; see Table I). Slightly more DNA adducts were found after transfection of COS-1 cells with either CYP1A1 or CYP1A2 (7–14 adducts/10^8 nucleotides; Table I). From these experiments we conclude that COS-1 cells have virtually no endogenous enzyme activity for the acetylation and hydroxylation of arylamines. Because expressed P4501A2 was twice as active as P4501A1, combinations of CYP1A2 and NAT genes were used in all further experiments. As shown in Table I, these combinations resulted in adduct frequencies 5–20 times higher than those obtained with single genes, and NAT2 was three times more effective than NAT1.

A lower IQ concentration (10 μM) was applied in a second series of transfection experiments (Table I). Qualitatively, the same results were obtained, namely highest adduct frequencies following transfection of CYP1A2 in combination with NAT2. The chromatographic patterns of IQ–nucleotide adducts obtained after activation by P4501A2 and NAT1 and by P4501A2 and NAT2 were qualitatively identical (Figure 4a and b); the difference was merely quantitative. The same pattern had been found previously in liver DNA of rats that had received an oral dose of IQ (Figure 4d), and with that obtained from the IQ-related

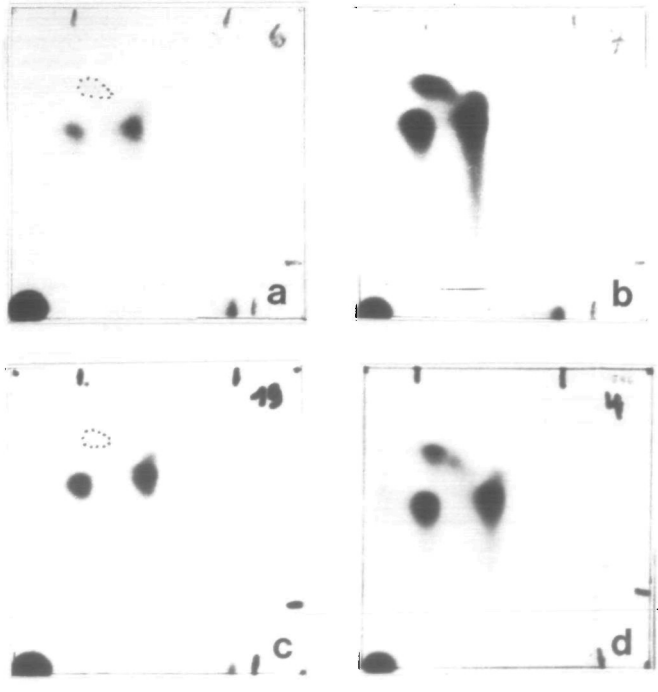

Fig. 4. Identical patterns of IQ and *N*-acetyl-IQ adducts in COS-1 cells expressing CYP1A2 + NAT1 and CYP1A2 + NAT2. Autoradiographs of IQ–DNA adducts separated by TLC. (a) COS-1 cells transfected with CYP1A2 + NAT1 (substrate: IQ, 10 μM); (b) COS-1 cells transfected with CYP1A2 + NAT2 (IQ, 10 μM); (c) COS-1 cells transfected with CYP1A2 + NAT2 (substrate: *N*-acetyl-IQ, 10 μM); (d) IQ adducts formed in the liver of a F344 rat 24 h after a 30 mg/kg oral dose of IQ; adduct frequency 123/10^8 nucleotides. The origin of the chromatograms is in the lower left-hand corner.

Table II. COS-1 cells have no endogenous *N*-acetyl-IQ deacetylase activity

N-Acetyl-IQ concentration (μM)	COS-1 cell homogenate	Human liver microsomes
100	0.002[a]	0.017[a]
200	0.002	0.085
400	0.019	0.232

Untransfected COS-1 cells were homogenized and assayed for endogenous deacetylase enzyme activity using chemically synthesized *N*-acetyl-IQ as substrate. Microsomes isolated from human liver were similarly analyzed.
[a]Numbers represent nmol IQ/mg protein/min.

compounds nitro-IQ in *Salmonella typhimurium* and azido-IQ in calf thymus DNA (32,35). The quantitatively most prominent, slow migrating adduct has been identified as *N*-(deoxyguanosin-8-yl)-IQ (36).

NAT1 and NAT2 both display N,O-transacetylase (AHAT) enzyme activity

Kinetic characterization of purified NAT enzyme activities from rabbit and mouse liver have suggested that the same enzyme has NAT, arylhydroxamic acid *N,O*-acetyltransferase (AHAT) and *O*-acetyltransferase (OAT) activities (33,34).

We used the COS-1 expression system to address the question of whether human NATs have AHAT activity. The adduct formation by *N*-acetyl-IQ was studied, which, provided that this substrate is not deacetylated by COS-1 cells, can be converted to an arylnitrenium ion only in the presence of AHAT activity (Figure 1). Therefore the *N*-acetyl-IQ deacetylase activity of COS-1 cells was tested and compared with that of human liver microsomes. As is evident from Table II, COS-1 cells display only minute endogenous deacetylase activities at all substrate concentrations tested, whereas significant activities were found in human liver microsomes.

In another series of transfection experiments *N*-acetyl-IQ was added to cultures and the resulting adducts were determined (Table I). Adducts were detected with combinations of CYP1A2 and either NAT1 or NAT2. NAT2 was 14 times as effective as NAT1, and overall frequencies were considerably lower (1/4–1/9) than those observed with IQ at the same concentration (10 μM). The chromatographic pattern of the *N*-acetyl-IQ–DNA adducts (Figure 4c) was the same as that of the IQ–DNA adducts. Thus, the same arylnitrenium ion was formed from IQ and *N*-acetyl-IQ and it can be concluded that both NAT1 and NAT2 display AHAT enzyme activity. The lower adduct frequency observed with *N*-acetyl-IQ compared to that observed with IQ is probably due to an efficient direct activation of IQ (via *N*-hydroxylation and *O*-acetylation) and a less efficient activation of *N*-acetyl-IQ (via *N*-hydroxylation and *N,O*-transacetylation).

Discussion

In the present report we used transient expression in COS-1 cells of each of four drug-metabolizing enzymes to study their role in the activation of the food mutagen and carcinogen IQ. Our experiments suggest that (i) human P4501A2 is more efficient than P4501A1 for *N*-hydroxylation of this heterocyclic amine; (ii) *N*-hydroxy-IQ is only weakly reactive towards DNA; (iii) the most efficient metabolic activation and DNA adduct formation is achieved with P4501A2 in combination with NAT2. Finally, we propose that NAT1 and NAT2 in addition to their known role in *N*-acetylation display *N,O*-transacetylase activity.

Our first finding is in keeping with published results obtained in rat and rabbit. When purified rat (37) and rabbit (38) P4501A1 and P4501A2 were used in the *Salmonella* mutagenicity assay with IQ, the rat P4501A2 enzyme was found to be 12 times, and the corresponding rabbit enzyme 8 times more efficient than P4501A1. Similarly, when mouse P4501A1 and P4501A2 enzymes were expressed in Hep G2 human hepatoma cells and used in the *Salmonella* assay with IQ, P4501A2 was nine times as active as P4501A1 (39). Thus, the available evidence points to P4501A2 as the predominant physiological activator of IQ. *N*-Hydroxylation of IQ, obtained by expression of CYP1A2 alone (Table I), results in only minor formation of IQ-nitrenium ions and DNA binding: an acetylation step is required as well. This is in agreement with mutagenicity data obtained in *Salmonella* strains with low, normal and high acetyltransferase activity, where the mutagenic activity of IQ (in the presence of rat liver S9) strongly depends on the acetylator status of the strain used (40).

The data presented indicate that the human acetylation polymorphism has to be considered an important factor in the metabolic activation of arylamine carcinogens. This becomes evident because (i) the polymorphic NAT2 in our analysis was about five times as effective as NAT1 in activating IQ into the arylnitrenium ion; and (ii) NAT2 possesses ten times as much *N,O*-transacetylase (AHAT) activity as NAT1, thus broadening the spectrum of reactions affected by the polymorphism.

In addition we suggest that NAT1 and NAT2 can catalyze the hydroxylamine *O*-acetylation. This was concluded from the very low rate of IQ *N*-acetylation by rodent NATs (10,41), and from the relatively high adduct frequencies found in our IQ experiments, which can only be explained by the presence of OAT activity (see Figure 1).

In conclusion, this study provides a mechanistic basis for the clinical observation that the acetylation polymorphism constitutes a risk factor in the metabolic activation of environmental and dietary mutagens/carcinogens.

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