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SHORT COMMUNICATION

Acrolein, an *α,β*-unsaturated Aldehyde, Irreversibly Inhibits the Acetylation of Aromatic Amine Xenobiotics by Human NAT1

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Running Title: Inhibition of NAT1-dependent acetylation pathway by acrolein

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List of non standard abbreviations:

ACR: acrolein; NAT1: arylamine *N*-acetyltransferase 1; AA: aromatic amine; XME: xenobiotic-metabolizing enzyme

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ABSTRACT

Acrolein is an electrophilic α,β-unsaturated aldehyde of industrial, pharmaceutical and toxicological importance to which we are exposed in environmental, occupational and therapeutic situations. Acrolein is known to exert different biological effects through reactions with cellular macromolecules such as DNA, certain proteins or glutathione. In many situations (such as in tobacco smoke or other fumes), exposure to acrolein occurs concomitantly with other compounds such as aromatic amine chemicals. Interestingly, it has been shown that acrolein could impact the cellular metabolism of aromatic xenobiotics through an indirect mechanism based on the transcriptional induction of phase II xenobiotic-metabolizing enzymes. Here we report a novel mechanism by which acrolein acts on the metabolism of aromatic foreign chemicals. We provide molecular, kinetic and cellular evidence that acrolein can react directly and irreversibly with arylamine *N*-acetyltransferases, a major family of xenobiotic-metabolizing enzymes involved in the metabolization of aromatic amine chemicals. Formation of an acrolein adduct with a catalytic cysteine residue in the active site is responsible for the impairment of aromatic amine acetylation by the enzyme. This biochemical process may represent an additional mechanism by which acrolein impacts the metabolism and fate of aromatic amine drugs and pollutants.

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INTRODUCTION

Acrolein (ACR) is a three-carbon α,β-unsaturated aldehyde to which humans are widely exposed in environmental, occupational and therapeutic situations. ACR is highly abundant in tobacco smoke, cooking and automobile exhaust fumes (Kehrer and Biswal, 2000; Myers and Myers, 2009). In addition, ACR is used industrially as an herbicide and slimicide, as well as a starting material for acrylate polymers and acrylic acid. Although ACR is an ubiquitous pollutant and major component of cigarette smoke, it is also generated endogenously by cellular metabolism and lipid peroxidation (Kehrer and Biswal, 2000; Myers and Myers, 2009). Moreover, ACR is also a metabolite of the anti-cancer drug cyclophosphamide and is believed to be the main cause of its toxicity (Kehrer and Biswal, 2000). ACR is the most reactive electrophile among α,β-unsaturated aldehydes. Its cellular toxicity relies mainly on its ability to deplete glutathione and to form DNA and protein adducts (Cai et al., 2009). Formation of ACR-protein covalent adducts is principally due to Michael addition where the β-carbon of acrolein reacts with nucleophilic groups to form *1,4*-addition with the double bond (Cai et al., 2009; Martyniuk et al., 2011).

Exposure to ACR generally occurs mainly in the context of chemical mixtures (*e.g*. as tobacco smoke) containing other potentially toxic compounds such as aromatic amine (AAs) chemicals (Feng et al., 2006; Myers and Myers, 2009). AAs encompass several compounds with important pharmacological (such as sulfamide drugs) and industrial applications (such as in the manufacturing of drugs, pesticides, dyes, rubbers). AAs are also by-products of gasoline combustion and pyrolysis reactions. AAs represents one of the most important classes of occupational and/or environmental pollutants (Kim and Guengerich, 2005) and these aromatic molecules account for 12% of the chemicals which are either known or strongly suspected to be human carcinogens (NTP, 2011).

Human arylamine *N*-acetyltransferases (NAT1 and NAT2) are phase II xenobiotic-metabolizing enzymes (XME) that play a major role in the biotransformation of AA (Hein, 1988; Grant, 1993). The interrelationship between variable NAT activities and the biological effects of AA drugs and carcinogens has been known for years (Hein et al., 2000; Agundez, 2008). Indeed, NATs catalyze the acetyl-CoA-dependent *N*- and/or *O*-acetylation of AAs and their *N*-hydroxylated metabolites (Sim et al., 2008). NAT-dependent acetylation of AAs is a major biotransformation pathway which can have pharmacological and toxicological consequences (Hein, 1988; Butcher et al., 2008; Rodrigues-Lima et al., 2008).

Concomitant exposure to ACR and AAs occurs commonly (such as in cigarette smoke). It has been suggested that ACR could impact the cellular metabolism and hence the toxicological fate of chemicals such as AAs, in particular through the transcriptional regulation of phase II XME (Tirumalai et al., 2002). Herein, we provide molecular, kinetic and cellular evidence that ACR reacts irreversibly with NAT1 enzyme through covalent adducts at the active site. This modification leads to irreversible inhibition of NAT1 and subsequent alteration of AA acetylation. This biochemical process

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may represent an additional mechanism by which ACR modifies the metabolism of AA drugs and carcinogens.

MATERIALS AND METHODS

Materials. Acrolein (ACR), *2*-aminofluorene (2-AF), *p*-aminosalicylic acid (PAS), *p*-nitrophenylacetate (PNPA) acetyl-Coenzyme A (AcCoA), *1*,*4*-dithiothreitol (DTT), biotin hydrazide, fluorescein-iodoacetamide, reduced glutathione (GSH), *3*-(*4*,*5*-Dimethylthiazol-*2*-yl)*2*,*5*-diphenyltetrazolium bromide (MTT), protease inhibitor cocktail, and nickel agarose resin were obtained from Sigma-Aldrich. Bradford protein assay kit was supplied by Bio-Rad. All other reagents were purchased from Euromedex unless otherwise noted. Antibodies against ACR (rabbit polyclonal), biotin (rabbit polyclonal) and fluorescein (mouse monoclonal) were purchased from Lifespan Biosciences, Sigma-Aldrich and Roche, respectively. Rabbit polyclonal anti-human NAT1 antibody was raised in the laboratory against a synthetic peptide corresponding to the last 16 residues of human NAT1.

Production and purification of recombinant human NAT1. *Escherichia coli* BL21 (DE3) cells containing a pET28a-based plasmid were used to produce 6x-histidine-tagged human NAT1, as described previously (Dairou et al., 2004). Purified NAT1 was reduced by incubation with 10 mM DTT for 10 min at 4°C and dialyzed against Tris-HCl 25 mM, pH 7.5 (buffer A). Purity was assessed by SDS-PAGE, and protein concentrations were determined using the Bradford reagent following the manufacturer's instructions with bovine serum albumin as a standard.

Activity of recombinant NAT1. NAT1 activity was detected in a total volume of 100 µl. Samples containing recombinant enzyme were first incubated with PAS (500 µM final concentration) in PBS at 37°C for 5 min. PNPA (2 mM final concentration) was added to start the reaction, the samples were incubated for various periods (up to 30 min) at 37 °C and the absorbance was measured every 1 min at 450 nm. All assays were performed in triplicate under initial reaction rate. Enzyme activity is expressed as percentage of control. In all reaction mixtures, the final concentration of NAT1 was 30 nM.

Reaction of recombinant NAT1 with ACR. The effect of ACR on NAT1 activity was assessed by incubating the purified enzyme (3 µM final) with various concentrations of ACR in buffer A for 30 min at 37°C. Aliquots were then assayed for residual NAT1 activity.

Effects of reducing agents on ACR-dependent inhibition of recombinant NAT1. We tested whether reducing agents (reduced glutathione, GSH, and dithiotreitol, DTT) could restore the activity of the NAT1 inhibited by ACR. Recombinant NAT1 (3 µM) was first preincubated with ACR (final concentration 25 µM) for 10 min at 37°C. Mixtures were then incubated with different concentrations of DTT, GSH (up to 10 mM final concentration) for 10 min at 37°C. Residual enzyme

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activities were then assessed. Control assays were carried out in the conditions described above with GSH, DTT.

Effects of acetyl-CoA and CoA on ACR-dependent inhibition of recombinant NAT1.

To analyze the involvement of the catalytic cysteine residue of human NAT1 in the ACR-dependent inhibition of NAT1, recombinant enzyme (3 μM) was preincubated with different concentrations of AcCoA or CoA in 25 mM Tris-HCl, pH 7.5 for 5 min at 37°C (final concentration of AcCoA or CoA ranged from 0-3 mM). Mixtures were then incubated with ACR (20 μM) for 10 min at 37°C and then assayed for NAT1 activity after dilution (100 fold) with Tris-HCl buffer.

Kinetic analysis of ACR-dependent NAT1 inhibition. NAT1 (3 μM) was incubated with ACR (final concentration ranging from 5 to 20 μM) at 37°C. At various time intervals, aliquots were removed and assayed for residual activity. Semilog plot of time courses for the inhibition of NAT1 by various concentrations of ACR and Kitz-Wilson replot were carried out for the kinetic analysis of the data (Cornish-Bowden, 2001).

SDS-PAGE and Western Blotting. ACR-treated or non treated samples were mixed with SDS sample buffer, boiled 5 min at 95°C, and separated by SDS-PAGE. For Western blotting, proteins were electrotransferred onto nitrocellulose membrane. The membrane was blocked by incubation with Tris-buffered saline/Tween 20 (TBS) supplemented with 5 % non fat milk powder for 1h in TBS. Antibodies were added and the membranes were incubated for overnight in TBS at 4°C. Amersham ECL was used for detection.

Dot-blotting. Recombinant NAT1 (3 μM) was incubated with ACR (25 μM) for 30 min at 37 °C. Treated and non treated samples were mixed with TBS buffer (up to 200 μl) and dotted on nitrocellulose sheets using a Bio-Rad dot-blot apparatus. The membrane was blocked by incubation with Tris-buffered saline/Tween 20 (TBS) supplemented with 5 % non fat milk powder for 1h in TBS. Antibodies against ACR-adducts were added (1:1000) and the membranes were incubated for overnight in TBS at 4°C. Amersham ECL was used for detection. Ponceau stains were carried out to ensure that equal amounts of NAT1 were dotted on the membrane.

To detect the free carbonyl group of ACR-adducts, treated and non treated NAT1 aliquots were further incubated with 2 mM biotin-hydrazide (a reagent that covalently labels carbonyl groups) for 2 h at room temperature prior to dot-blotting and detection with a anti-biotin antibody.

To detect ACR-dependent modification of cysteines residues, treated and non treated NAT1 aliquots were further incubated with 20 μM fluorescein-conjugated iodoacetamide (a reagent that covalently labels free reduced cysteine residues) for 30 minutes at room temperature prior to dot-blotting and detection with an anti-fluorescein antibody.

Cell culture. NCI-H292 human lung epithelial cells were obtained from ATCC (USA). Cells were grown at 37 °C as monolayers in 6 well plates or in 100 mm Petri dish in DMEM supplemented with 10% (v/v) fetal calf serum. No significant toxicity (>85% cell survival) was found after exposure (30 min) to ACR (up to 100 μM).

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Detection of ACR-adducted NAT1 in cells. Cells in 10 cm Petri dishes were exposed to 25 or 100 µM ACR for 30 min at 37 °C. After washing with PBS (phosphate buffered-saline) buffer, cells were scraped in 500 µl of PBS containing 0.1% Triton X-100 and protease inhibitors prior to sonication and centrifugation at 17,000 x g for 15 minutes at 4°C. The supernatant was saved and used for immunoprecipitation of endogenous NAT1. To this end, equal amounts of cell extracts (1.3 mg protein) were incubated with anti-NAT1 antibodies bound to protein A agarose beads overnight at 4°C. After several washes with PBS containing 0.1% Triton X-100, immunoprecipitated NAT1 was eluted with SDS sample buffer (non reducing). Eluted proteins were analysed by Western-blot with specific antibodies against ACR-adducts.

Cellular NAT activity assay. NAT activity was measured in cell extracts using reverse-phase HPLC as described previously (Grant et al., 1991). Samples (50 µl) were first incubated with AA substrate (1 mM final) in assay buffer (25 mM Tris-HCl, pH 7.5) at 37 °C for 5 min. AcCoA (1 mM final) was added and the samples (100 µl final volume) were incubated at 37 °C for various periods of time (up to 30 min). The reaction was quenched by adding 100 µl of ice-cold acetic acid (15% w/v), and proteins were recovered by centrifugation for 5 min at 12,000 g. 20 µl of the supernatant were injected into the C18 reverse-phase HPLC column. All assays were performed in triplicate under initial reaction rate conditions.

Acetylation of 2-AF by intact lung epithelial cells in culture. Acetylation of *2*-AF by endogenous NAT in intact lung epithelial cells was measured by reverse-phase HPLC as described previously (Wu et al., 2000). Briefly, after exposure to ACR (up to 100 µM), the monolayers (6 well-plates, 10 cm^2 per well, ~ 100 µg total protein) were washed with PBS and grown in presence of 500 µM *2*-AF in DMEM culture medium in a 37 °C incubator. At different time points (3, 4, 5 and 6 h), aliquots (100 µl) of culture medium were collected and added to 100 µl of ice-cold aqueous acetic acid (15% w/v). Samples were then centrifuged and the amount of acetylated-*2*-AF quantitated by HPLC analysis. Controls were done in the same conditions with cell monolayer not exposed to ACR. Appearance of *N*-acetylated AA in the culture medium was found to be linear with time.

RESULTS AND DISCUSSION

Exposures to ACR occur mainly through inhalation of smokes and fumes where ACR concentrations can reach high levels. For instance, it has been shown that 1 cigarette bubbled through 10 ml of an aqueous buffer can generate ACR concentrations up to 500 µM (Lambert et al., 2005). In the respiratory tract fluid of smokers ACR was estimated to reach concentrations close to 100 µM (Jia et al., 2007). Most of the adverse effects of ACR on humans are likely due to direct interaction with the lung epithelial cells leading to the alterations of key cellular functions (Myers and Myers, 2009; Spiess et al., 2011). These cells are known to express xenobiotic-metabolizing enzymes (such as NAT1) which are involved in the biotransformation of inhaled chemicals (Zhang et al., 2006; Dairou et al., 2009). We first tested the effect of ACR exposure on the cellular acetylation of AA chemicals.

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To this end, we exposed human lung epithelial cell (NCI-H292) monolayers to different concentrations of ACR and measured the amount of acetylated *2*-AF (a well known AA that is specifically acetylated by NAT enzymes) in culture medium by HPLC. Brief exposure (30 min) of epithelial cells to ACR (up to 100 µM) leads to a dose-dependent decrease in the amount of acetylated *2*-AF in the culture medium (Figure 1A). Accordingly, enzyme assays with the extracts of the cells exposed or not to ACR, showed that cellular NAT1 is inhibited by ACR (with 80% inhibition of the enzyme activity at 100 µM ACR (Figure 1B). ACR has a strong electrophilic character and forms covalent adducts with biological molecules such as proteins, DNA or GSH (Cai et al., 2009). Immunoprecipitation and Western-blotting experiments using specific antibodies against human NAT1 enzyme and ACR-protein adducts (Luo et al., 2007) indicated that exposure of epithelial cells to ACR leads to the formation of ACR adducts on cellular NAT1 enzyme (Figure 1C). Taken together these data show that in lung epithelial cells, ACR reacts with NAT1 through covalent protein adducts and impairs the NAT-dependent biotransformation of AA chemicals.

Further mechanistic and kinetics studies were carried out to better understand the molecular basis for the effects of ACR on the enzymatic acetylation of AA chemicals. To this end, recombinant NAT1 enzyme (0.5 µM final concentration) was incubated with various concentrations of ACR (0-100 µM final concentration) and residual NAT1 activity was measured by following the AcCoA-dependent acetylation of PAS. Figure 2A shows that NAT1 activity was significantly inhibited by ACR in a dose-dependent manner (Figure 2A).

As observed for cellular NAT1, the recombinant enzyme was inhibited by biologically-achievable concentrations of ACR (<100 µM) with an IC_{50} value of 8 µM (Fig. 2A). The lack of reactivation of the enzyme by dilution with assay buffer or by dialysis (data not shown) indicated that the ACR-dependent inhibition of NAT1 activity was irreversible. In addition, we found that NAT1 activity could not be recovered by high concentrations of reducing agents such as DTT or GSH (data not shown). This data suggested that ACR-dependent inhibition of recombinant NAT1 could be due to the formation of covalent adducts as observed when lung epithelial cells were exposed to ACR (Figure 1). Dot-blot experiments using recombinant NAT1 inhibited by ACR (25 µM final concentration) confirmed the formation of covalent ACR-enzyme adducts which were readily detected by a specific antibody against ACR-adducts (Figure 2B). To confirm the presence of these adducts on NAT1, we used biotin-hydrazide, a reagent that reacts specifically with the free aldehyde carbonyl group of covalently bound ACR (Spiess et al., 2011). As shown in Figure 2B, biotin-hydrazide was found to react with NAT1 enzyme exposed to ACR thus confirming the presence of ACR-adducts on NAT1. ACR, through its α,β-unsaturated moiety, is known to react covalently with cysteine residues to form covalent adducts (Seiner et al., 2007). We used fluorescein-conjugated iodoacetamide (which reacts only with reduced cysteines) to detect the covalent modification of NAT1 cysteines by ACR. As shown in Figure 2B, exposure to ACR decreased the labeling of NAT1 by fluorescein-iodoacetamide thus demonstrating that ACR reacts with NAT1 cysteine residues. Interestingly, the acetyltransferase

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activity of NAT1 relies on a cysteine residue present in the active site of the enzyme. This catalytic cysteine can be specifically acetylated by AcCoA (the physiological acetyl donor) to form a covalent acetyl-enzyme intermediate. AcCoA-protection approaches takes advantage of this property and have been used to identify whether the inhibition of NAT enzymes by chemicals involves reactions with the active site cysteine (Liu et al., 2008; Ragunathan et al., 2008). As shown in Figure 2C, AcCoA provided significant protection (≈80% ± 5 with 500 µM of AcCoA). CoA, a product resulting from the hydrolysis of AcCoA, which does not form an acetyl enzyme intermediate provided no protection. These data support that inhibition of NAT1-dependent acetylation is due to irreversible adduction at the active site cysteine of the enzyme. Similar results have been recently reported for other families of enzymes such as human protein tyrosine phosphatase 1B, protein disulfide isomerase and glyceraldehyde *3*-phosphate dehydrogenase which are irreversibly inhibited by ACR through covalent Michael adducts with their catalytic cysteine residue (Carbone et al., 2005; Seiner et al., 2007; Martyniuk et al., 2011; Nakamura et al., 2012). In aqueous solution, thiols react with ACR through conjugate addition to the double bond of the α,β-unsaturated aldehyde (thus forming a Michael adduct) rather than by addition to the aldehyde moiety (Seiner et al., 2007). Consistent with this, analysis of a series of structurally related aldehydes indicate that the double bond found in ACR is critical for its properties as a NAT inhibitor (Figure 3). In contrast, aliphatic aldehydes such as glyoxal, acetaldehyde and propanal, which lack the unsaturation found in ACR, are poor inhibitors of NAT1 (Figure 3).

Kinetics analysis further showed that ACR is a potent time-dependent irreversible inhibitor of NAT1 (Figure 4A). A Kitz-Wilson plot of the inhibition data indicated that k_{inact}, the rate of inhibition at saturating concentrations of ACR, was 0.01±0.0008 s^{-1} (Figure 4B). K_i, the concentration of ACR required to achieve half-maximal rate of inhibition was 182±24 µM. The second-order rate constant for the inhibition of NAT1 by ACR (k_{inact}/K_i) was 57 $M^{-1}. s^{-1}$. This value is similar to the k_{inact}/K_i obtained for the inhibition of human protein tyrosine phosphatase 1B by ACR (87 $M^{-1}. s^{-1}$) which has been suggested to be a physiological inhibitor of this enzyme (Seiner et al., 2007). In addition to our cellular studies, the rate of inhibition of NAT1 by ACR (57 $M^{-1}.s^{-1}$) is compatible with *in vivo* effects of ACR on NAT1 enzyme and hence on acetylation of aromatic amine chemicals. For biologically-achievable concentrations of ACR ranging from 10 to 100 µM (Lambert et al., 2005; Jia et al., 2007), $t_{1/2}$ of inhibition (time taken to inhibit half of NAT activity) would range from 13 to 1 minute, respectively.

Deciphering the biochemical mechanisms that are involved in the interaction of chemicals is of pharmacological and toxicological interest. ACR is an aldehyde compound of great industrial and toxicological importance. Exposure to ACR and other chemicals occurs frequently concomitantly and it has been suggested that ACR impacts the metabolism and biological fate of certain aromatic chemicals (Tirumalai et al., 2002; Feng et al., 2006). We have provided molecular, cellular and kinetic evidence that ACR irreversibly impairs the acetylation of aromatic chemical by inhibiting covalently a key family of XME. It is well known that alteration of AA metabolism and in particular of the NAT-

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dependent acetylation can impact the pharmacological and/or toxicological fate of these aromatic chemicals (Hein et al., 2000; Agundez, 2008; Butcher et al., 2008). The toxicity of AAs depends mainly on the activity of different XME such as certain cytochromes P450, NAT, sulfotransferases or glutathione *S*-transferases (Kim and Guengerich, 2005). Interestingly, ACR has been shown to modify the metabolic pathways governed by certain phase II XME through their Nrf2-dependent transcriptional induction. Therefore our data show a novel mechanism by which ACR directly impacts the metabolism of AA chemicals, an important group of drugs and carcinogens.

AUTHORSHIP CONTRIBUTIONS

Participated in research design: Bui, Dupret, Rodrigues-Lima, Dairou

Conducted experiments: Bui, Manaa, Xu, Duval, Rodrigues-Lima, Dairou

Contributed new reagents or analytic tools: Xu, Duval

Performed data analysis: Bui, Manaa, Busi, Dupret, Rodrigues-Lima, Dairou

Wrote or contributed to the writing of the manuscript: Busi, Dupret, Rodrigues-Lima, Dairou

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FOOTNOTES

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LEGENDS TO FIGURES

Figure 1: Impairment of *2*-AF acetylation by NCIH-292 cells upon exposure to ACR and inhibition of endogenous NAT activity NCIH-292 cells. **A** Cells in 6-well plates (90% confluence) were exposed to different concentrations of ACR for 30 min at 37 °C. Following ACR treatment, cells were washed and grown in fresh culture medium in presence of 500 µM *2*-AF. At given time points (2, 4 and 6 h), the amount of acetylated-*2*-AF was quantitated in culture medium by HPLC. Error bars indicate the S.D. values (*, $p<0.05$). **B.** NCIH-292 cells in 6-well plates were exposed to different concentrations of ACR for 30 min. After washing, cell extracts were made (15 mg/mL protein concentration) and residual NAT activity was assessed by HPLC using *2*-AF as a substrate. Error bars indicate the S.D. values (*, $p<0.05$). **C.** NCIH-292 in Petri dishes were exposed or not to 25 or 100 µM ACR for 30 min at 37 °C. Endogenous NAT1 enzyme was immunoprecipitated from the cell extracts using anti-NAT1 antibodies. Bound enzyme was eluted and analysed by Western-blot using anti-NAT1 and anti-ACR-adducts (lower panel). Equivalent amounts of NAT1 enzyme were present in the three different cell extracts used for immunoprecipitation as shown by Western-blot using anti-NAT1 (upper panel).

Figure 2: Effect of ACR on purified recombinant human NAT1. A. NAT1 (3 µM) was incubated with different concentrations of ACR (1 to 100 µM) at 37 °C for 30 minutes and residual activity measured. NAT1 exposed only to buffer was used as control. Error bars indicate the S.D. values. **B.** For Dot blot experiments, purified recombinant NAT1 (3 µM final) was exposed or not to ACR (final concentration of 25 µM) for 30 minutes at 37 °C and spotted on nitrocellulose sheets using a dot-blot Bio-Rad apparatus. Detection of ACR-protein adducts was carried out using specific anti-ACR adducts antibodies. To further detect ACR adducts on NAT1 enzyme, we carried out labelling experiments with biotin-hydrazide a compound that reacts with the carbonyl group of covalently bound ACR. To this end, NAT1 samples were incubated with biotin-hydrazide (2 mM) for 2 h at room temperature, prior to dot-blotting and immunodetection using anti-biotin antibodies. Fluorescein-iodoacetamide, a compound that reacts reduced cysteines was used to detect ACR-dependent modification of NAT1 cysteine residues. To this end, NAT1 samples were incubated with fluorescein-iodoacetamide (20 µM) for 30 minutes at room temperature, prior to dot-blotting and immunodetection with anti-fluorescein antibodies. All membranes were stained with Ponceau S to ensure that equal amounts of NAT1 were dotted. **C.** Effects of AcCoA and CoA on the ACR-dependent inhibition of NAT1 was carried out by incubating NAT1 (3 µM) with ACR (20 µM) in presence of different concentrations of AcCoA or CoA for 30 minutes at 37 °C prior to NAT1 activity determination. The data are means of experiments done in triplicate where NAT1 activity was determined in triplicate. Errors bars indicate S.D. values. * $p<0.05$ versus ACR-inhibited NAT1

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Figure 3: Effect of various aldehydes on human recombinant NAT1. NAT1 (3 μM) was incubated with different aldehydes (100 μM) for 10 minutes at 37 °C prior to residual activity determination. Error bars indicate the S.D. values.

Figure 4: Kinetic analysis of ACR-dependent inhibition of NAT1.

NAT1 (3 μM final) was treated with ACR at different concentrations 37 °C and the residual activity assayed in aliquots taken at various times. **A.** Plots of the natural logarithm of percent residual activity *versus* time for each ACR concentration. The apparent first-order inactivation constants (k_{obs}) were calculated from linear regressions. **B.** Kitz-Wilson replot of the inactivation data.

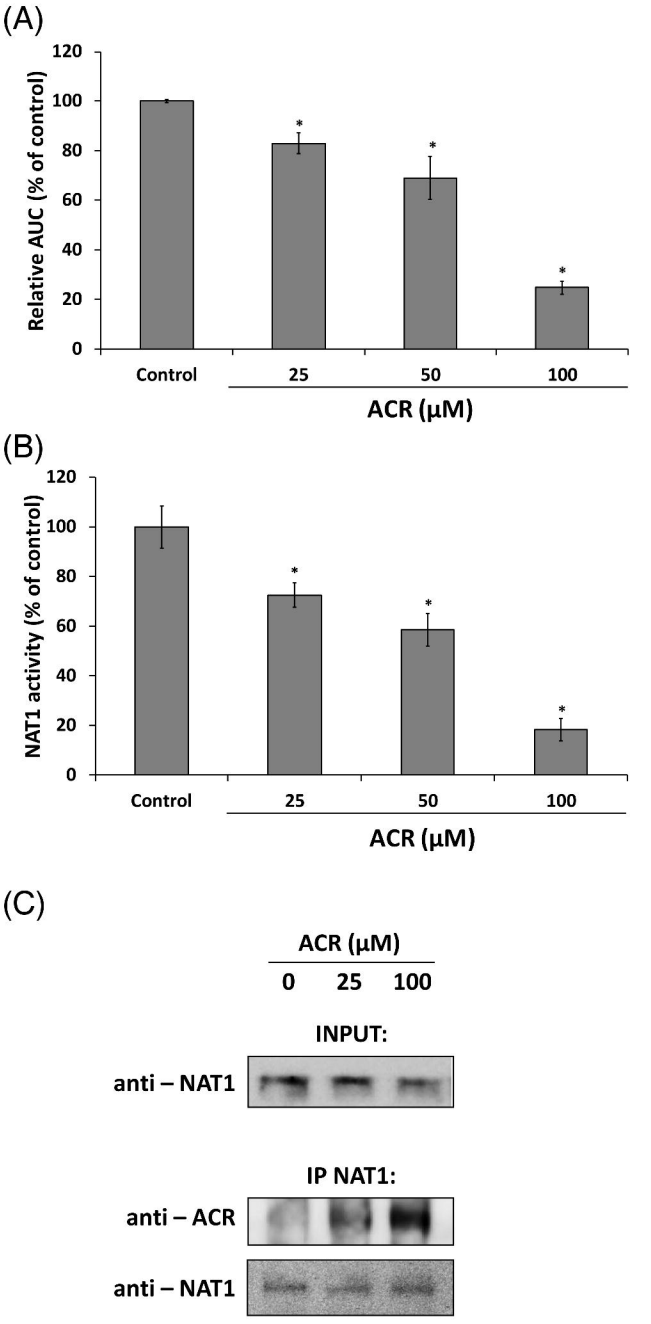
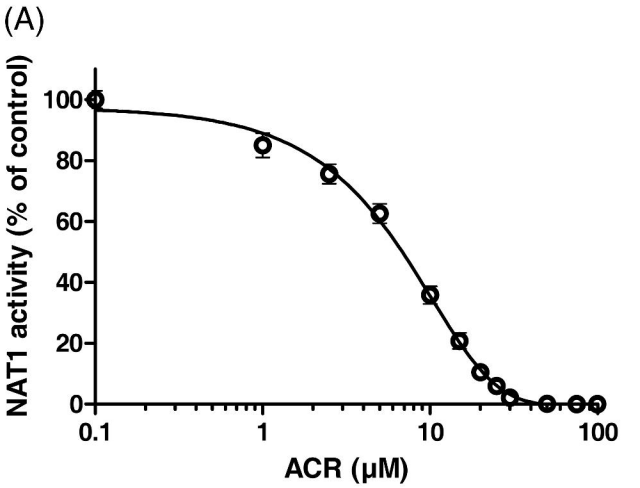
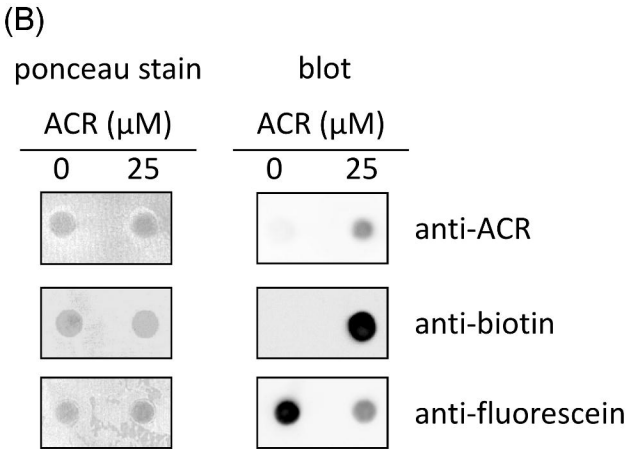

(A)
Relative AUC (% of control)
120
100
80
60
40
20
0
*
*
*
Control
25
50
100
ACR (μM)
(B)
NAT1 activity (% of control)
120
100
80
60
40
20
0
*
*
*
Control
25
50
100
ACR (μM)
(C)
ACR (μM)
0
25
100
INPUT:
anti – NAT1
IP NAT1:
anti – ACR
anti – NAT1

Figure 1


(A)
NAT1 activity (% of control)
100
80
60
40
20
0
0.1
1
10
100
ACR (μM)


(B)
ponceau stain
blot
ACR (μM)
0
25
ACR (μM)
0
25
anti-ACR
anti-biotin
anti-fluorescein

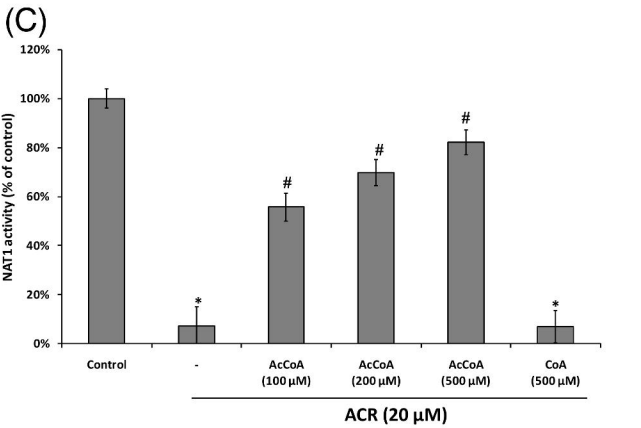

(C)
NAT1 activity (% of control)
120%
100%
80%
60%
40%
20%
0%
Control
-
AcCoA (100 μM)
AcCoA (200 μM)
AcCoA (500 μM)
CoA (500 μM)
ACR (20 μM)

Figure 2

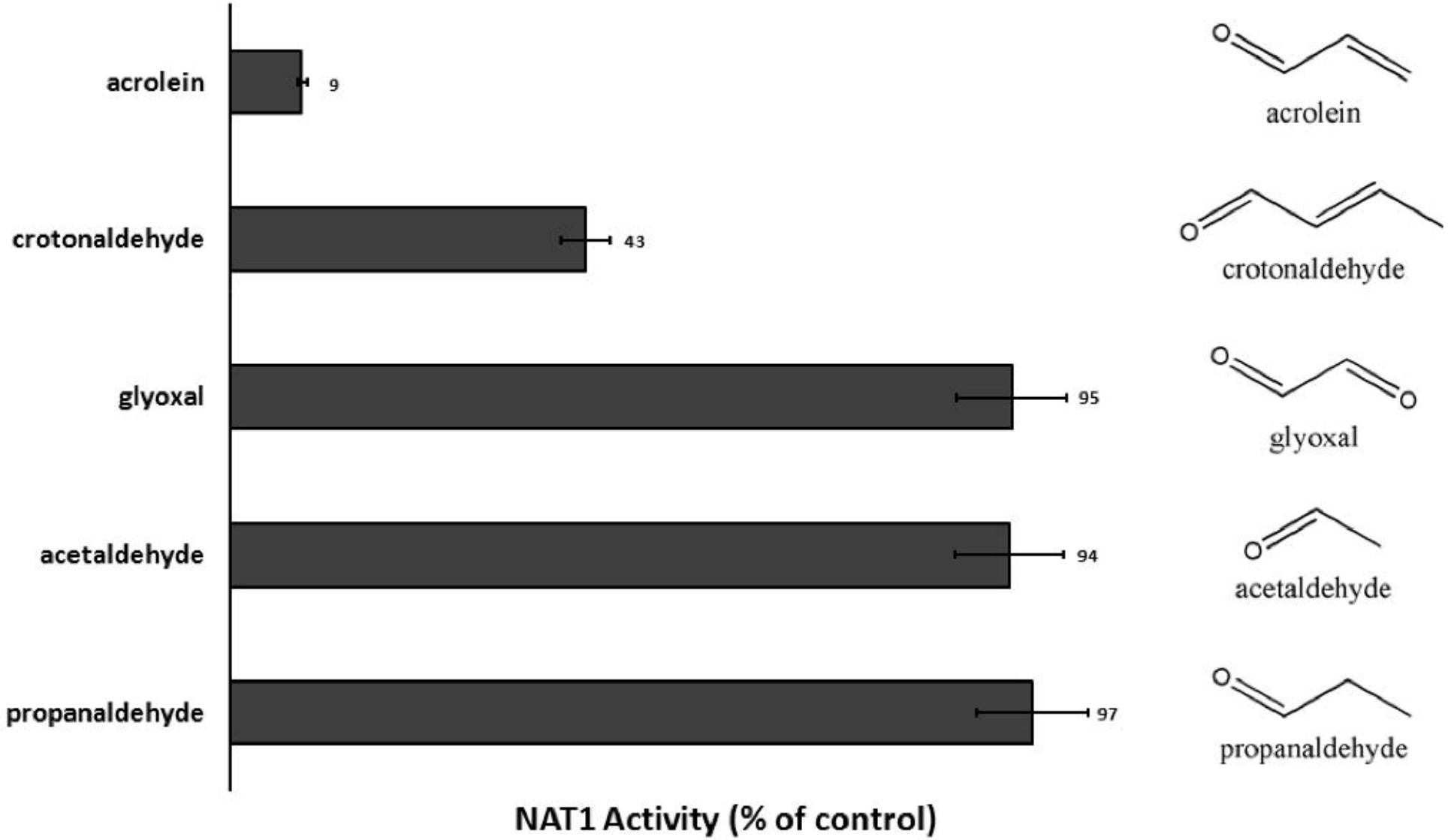
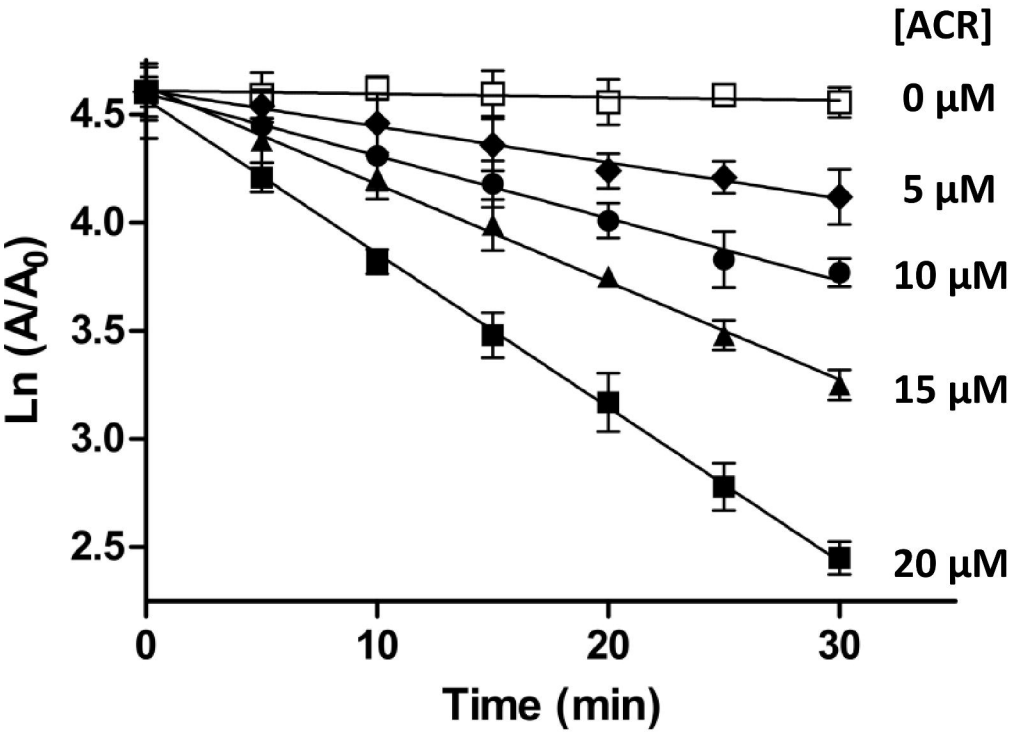

acrolein
9
crotonaldehyde
43
glyoxal
95
acetaldehyde
94
propanaldehyde
97
NAT1 Activity (% of control)
acrolein
crotonaldehyde
glyoxal
acetaldehyde
propanaldehyde

Figure 3

(A)


[ACR]
0 µM
5 µM
10 µM
15 µM
20 µM
Ln (A/A0)
4.5
4.0
3.5
3.0
2.5
0
10
20
30
Time (min)

(B)

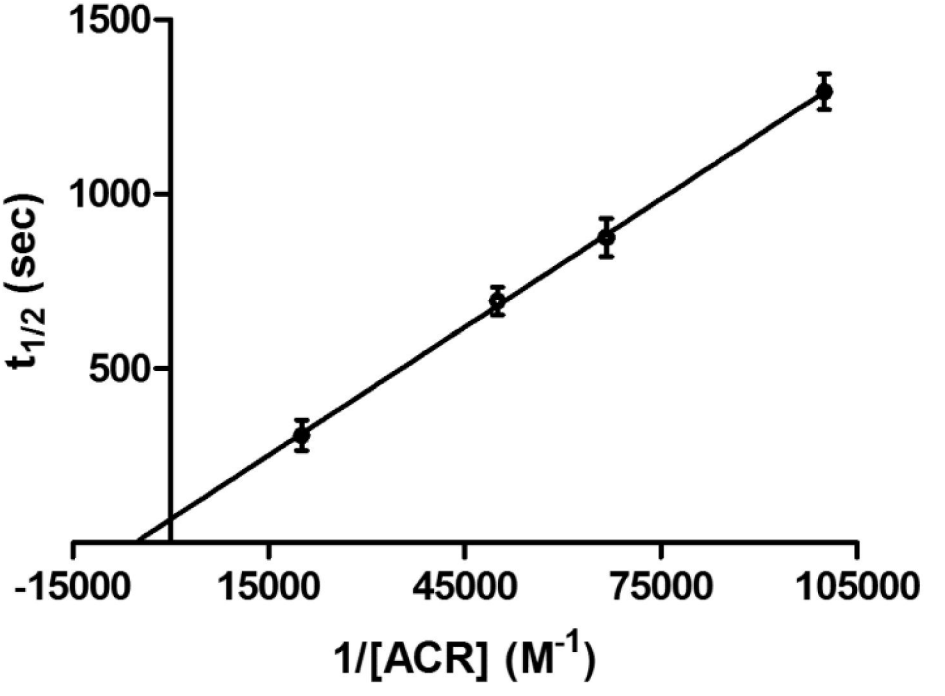

1500
1000
500
t1/2 (sec)
-15000
15000
45000
75000
105000
1/[ACR] (M-1)

Figure 4