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Inhibition by Vitamin C of DNA adduct formation and arylamine *N*-acetyltransferase activity in human bladder tumor cells

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Abstract Previous studies have already demonstrated the protective role of vitamin C (ascorbic acid) in certain types of cancer. This study reports on the effects of vitamin C on arylamine *N*-acetyltransferase (NAT) activity and DNA adduct formation in a human bladder tumor cell (T24) line. The activity of NAT was measured using high-performance liquid chromatography (HPLC), by assaying for the amounts of acetylated 2-aminofluorene (AF) and *p*-aminobenzoic acid (PABA) and the remaining amounts of AF and PABA. T24 cells were used for examining NAT activity and carcinogen–DNA adduct formation. The results demonstrated that NAT activity and 2-aminofluorene–DNA adduct formation in T24 cells were inhibited and decreased by vitamin C in a dose-dependent manner. The apparent kinetic parameters (apparent values of K_m and V_{max}) from T24 cells were also determined with and without vitamin C cotreatment. The data also indicated that vitamin C decreased the apparent values of K_m and V_{max} from T24 cells.

Key words Vitamin C · *N*-acetyltransferase · 2-aminofluorene · DNA adduct · Bladder cancer

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Introduction

The formation of DNA adducts is an initial stage event for chemical carcinogens in chemical carcinogenesis, which can be used for measuring the target dose as a biomarker for genotoxic risk. However, most chemical carcinogens need to be metabolized in order to form reactive metabolites, which are capable of binding to target tissue macromolecules, such as DNA, which then form DNA adducts [25]. *N*-Acetylation is a major metabolic pathway for arylamine carcinogens, which is catalyzed by host cytosolic arylamine *N*-acetyltransferase (NAT) using acetyl coenzyme A (Co-A) as an acetyl group donor [32]. DNA–carcinogen adduct formation is an important step in the initiation of the arylamine-induced carcinogenesis in target tissues [27]. Arylamine carcinogens, such as 2-aminofluorene (AF), are *N*-acetylated to become 2-acetylaminofluorene (AAF), and can undergo further activation or detoxification reactions to finally form DNA adducts. NAT activity in human and several laboratory animal species is genetically determined and the rapid acetylator phenotype has been shown to predispose humans to colorectal and breast cancer, whereas the slow acetylator phenotype is related to arylamine-induced bladder cancer [3, 16, 20, 32]. NAT activity has been reported in the colon [9, 18] and bladder [19] in humans. Thus, the genetic variations in NAT activities may indicate different risks for arylamine-induced tumor in human populations.

Vitamin C is increasingly being recognized as an agent with broad biologic functions and importance. Vitamin C has been reported to either delay or prevent the development of gastric [26, 28] and liver cancers [17]. However, other investigations did show that vitamin C could promote tumor formation in mammals [29, 30], or enhance chemical mutagenicity in *Salmonella* [33]. Vitamin C may reduce the cytotoxic effects of *N*-hydroxyacetylaminofluorene and reduce the covalent binding of AAF to cellular protein [15]. Vitamin C also expresses

antimutagenic effects in *Salmonella typhiurium*, against mutagens such as AF, by inactivating the mutagen outside the cells [12]. Recently, we have reported that human bladder tumor cells (T24) contain NAT activity and are also inhibited by berberine [6]. T24 cells exposed to vitamin C exhibited membranous damage [10]. However, there is no available information to address vitamin C effects on NAT activity and AF–DNA adduct formation of the T24 cells. Thus, our initial studies were focused on the effects of vitamin C on the NAT activity and DNA adduct formation of a human T24 cell line. The results of the present study demonstrated that, with the use of AF and PABA as substrates for NAT activity determinations, vitamin C decreased NAT activity in T24 cells.

Materials and methods

Chemicals and reagents

Vitamin C, ethylenediaminetetraacetic acid (EDTA), PABA, *N*-Ac-PABA, acetylcarnitine, Tris, leupeptin, bovine serum albumin (BSA), phenylmethylsulfonylfluoride (PMSF), dimethylsulfoxide (DMSO), dithiothreitol (DTT), acetyl-CoA, and carnitine acetyltransferase were obtained from Sigma Chemical (St. Louis, Mo., USA). AF and AAF were obtained from K and K Laboratories (Plainview, N.Y., USA). All chemicals used were of reagent grade.

Human bladder tumor cell line

The human bladder tumor (carcinoma) cell line (T24: human, female, Caucasian, 81 years old) was obtained from the National Taiwan University Hospital (Taipei, Taiwan). It has been cultured for several generations and throughout the years we have checked the cells for viability. The cells were placed into 75-cm^2 tissue culture flasks and grown at 37 °C under a humidified 5% CO_2 atmosphere in RPMI 1640 medium (Sigma Chemical, St. Louis, Mo., USA) supplemented with 10% fetal bovine serum (FBS; Gibco BRL, Grand Island, N.Y., USA), 2% penicillin–streptomycin (101,000 U/ml penicillin and 10 mg/ml streptomycin).

NAT activity determinations

The determination of acetyl-CoA-dependent *N*-acetylation of PABA and AF was performed as described by Chung et al. [4, 5]. Protein concentrations in the T24 cells were determined by the method of Bradford [2], with BSA as the standard. All of the samples were assayed in triplicate.

Effect of various concentrations of vitamin C on NAT activity in T24 cells

T24 cells (in 1 ml of RPMI 1640 media with glutamine and 10% FBS) were incubated with 60 μM AF or PABA at 1×10^6 cells/ml in individual wells of a 24-well cell culture plate, with or without vitamin C co-treatment (final concentrations were 50, 100, 200, and 400 μM) for an incubation time of 18 h at 37 °C in 95% air, 5% CO_2. At the conclusion of the incubation, the cells and media were removed and centrifuged. For experiments with AF, the supernatant was immediately extracted with ethyl acetate: methanol (95:5), the solvent evaporated, and the residue redissolved in methanol and assayed for AAF as described above. For experiments with PABA, aliquots of the supernatant were assayed directly for *N*-Ac-PABA.

Effects of incubation time of vitamin C on NAT activity in T24 cells

T24 cells (in 1 ml of RPMI 1640 media with glutamine and 10% FBS) were incubated with selected 60 μM AF or PABA at 1×10^6 cells/ml in individual wells of a 24-well cell culture plate, with or without vitamin C co-treatments (final concentration was 200 μM) for various incubation times (6, 12, 18, and 24 h) at 37 °C in 95% air, 5% CO_2. At the conclusion of the incubation, the cells and media were removed and centrifuged. The amounts of acetylated substrates were determined as described above.

Effects of vitamin C on the kinetic constants of NAT from T24 cells

For the intact cell studies, 1×10^6 T24 cells were incubated with selected concentrations (0.373, 0.435, 0.543, 0.745, 1.102, and 2.205 mM) of AF or PABA, with or without 200 μM vitamin C for 18 h in a 37 °C incubator. Following incubation, the cells and media suspensions were removed and centrifuged. In the experiment with AF, the supernatant was immediately extracted with ethyl acetate:methanol (95:5), the solvent evaporated, and the residue redissolved in methanol and assayed by high-performance liquid chromatography (HPLC). For the experiment with PABA, aliquots of the supernatant were assayed directly. All samples were run in triplicate.

Detection and measurement of DNA adducts in T24 cells

Detection and measurement of DNA adducts were performed as follows. T24 cells were incubated with AF (30 and 60 μM), with or without vitamin C (200 μM), and recovered by centrifugation. The DNA was prepared using a G NOME DNA isolation kit protocol (BIO 101, La Jolla, Calif., USA) as described in previous studies [21, 22]. The isolated genomic DNA was dissolved in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). DNA (5 mg each) in 5 ml of TE buffer was mixed with 5 ml of 10 × T4 polymerase buffer (0.33 M Tris acetate, 0.66 M potassium acetate, 0.10 M magnesium acetate, 5.0 mM DTT, pH 7.5) and 40 ml H_2O. Seventeen picomoles of γ-[^{32}P]-dATP (3000 Ci/mmol) along with 22 units of T4 DNA polymerase were added to the above reaction buffer and incubated at 30 °C for 60 min. The reaction was stopped by the addition of 10 ml of 0.5 M EDTA. The non-incorporated and the incorporated isotopes were separated by sephadex G-50 chromatography. Post-labeled adducted nucleotides were separated using a Beckman HPLC (pump 168 and detector 126) and an Ultrasphere C18 reversed phase ion-pairing column (4.6 cm × 25 cm). They were eluted at a flow rate of 1.5 ml/min, with 30 mM KPO_4, pH 6.0, containing 10% CH_3CN for 10 min followed by a linear gradient of 90% 30 mM KPO_4, pH 6.0, 5 mM tetrabutylammonium phosphate, and 50% CH_3CN at 65 min. UV absorbance at 254 nm followed. Samples (1 min = 1.5 ml) were collected and quantitated by scintillation spectrometry [21]. The calculation of adduct formation was performed by dividing the radioactivity in the adduct peak (after correction for recovery and efficiency of counting) by the specific activity of the ATP used in the labeling. Adduct levels are reported as pmol adduct/mg DNA analyzed [21, 22].

Statistical analysis

Statistical analysis of the data was performed with an unpaired Student's *t*-test. The kinetic constants were calculated with the Cleland HYPER Program [7], which performs linear regressions using a least-squares method.

Results

The possible effects of vitamin C on NAT activity in T24 cells were examined by HPLC, assessing the percentage acetylation of AF and PABA. The means ± SD of NAT activity co-treated with or without vitamin C with both substrates are given in Figs. 1 and 2. The data indicate that NAT activity decreased with increased concentrations of vitamin C in intact cells.

To determine the time course effect of 200 μM vitamin C on the acetylation of AF and PABA in T24 cells, the cells were incubated at 37 °C with or without vitamin C and harvested at 6, 12, 18, and 24 h. An increased time of incubation led to an increased AAF and *N*-Ac-PABA production, up to 24 h (Fig. 3a, b). These figures also demonstrate that vitamin C decreased the production of AAF and *N*-Ac-PABA during the time periods examined.

In the presence or absence of vitamin C (200 μM), specific concentrations of AF and PABA (0.373, 0.435, 0.543, 0.745, 1.102, and 2.205 mM) were added to the culture media for determining T24 cell NAT kinetic constants. The effects of vitamin C on the K_m and V_{max} values in examined cells are presented in Table 1. Clearly, K_m and V_{max} values for the T24 cells NAT were decreased in the presence of vitamin C.

After an 18-h incubation of T24 cells with AF, cells were recovered and DNA was prepared, hydroylzed to nucleotides, and adducted nucleotides were extracted with butanol and analyzed using HPLC. The results indicate that bladder tumor cells activate AF to a metabolite that is capable of binding covalently with DNA; AF also induced dose-dependent AF–DNA adduct formation (Table 2). In the presence of 200 μM vitamin C, the AF–DNA adduct formation decreased in both AF concentrations ($P < 0.05$).

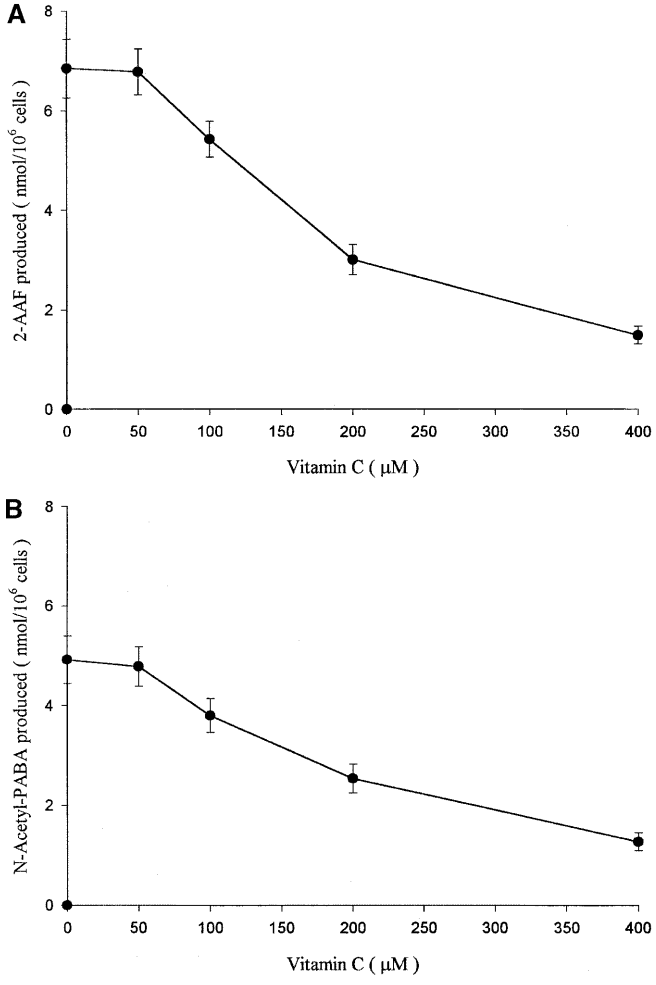

Fig. 1A, B Effects of various concentrations of vitamin C on production of AAF and *N*-Ac-PABA by T24 cells. T24 cells were incubated as described for 18 h with 60 μM AF (**A**) and PABA (**B**) co-treatment with 200 μM vitamin C. AAF and *N*-Ac-PABA were measured by HPLC assay. *Each point* represents the mean of triplicate assays of three incubations of cells

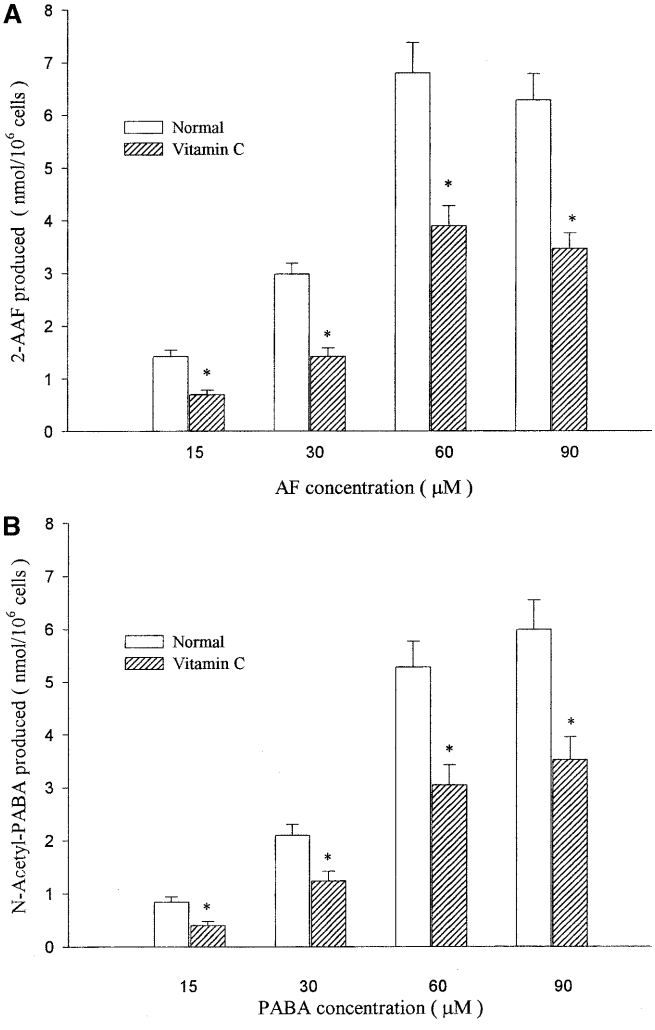

Fig. 2A, B Effects of vitamin C on acetylation of various concentrations of AF and PABA by T24 cells. T24 cells were incubated as described for 18 h at various concentrations (15, 30, 60, and 90 μM) of AF (**A**) and PABA (**B**) co-treatment with 200 μM vitamin C. AAF and *N*-Ac-PABA were measured using a HPLC assay. Each point represents the mean of triplicate assays of three incubations of cells. * Mean differences between vitamin C and control, $P < 0.05$

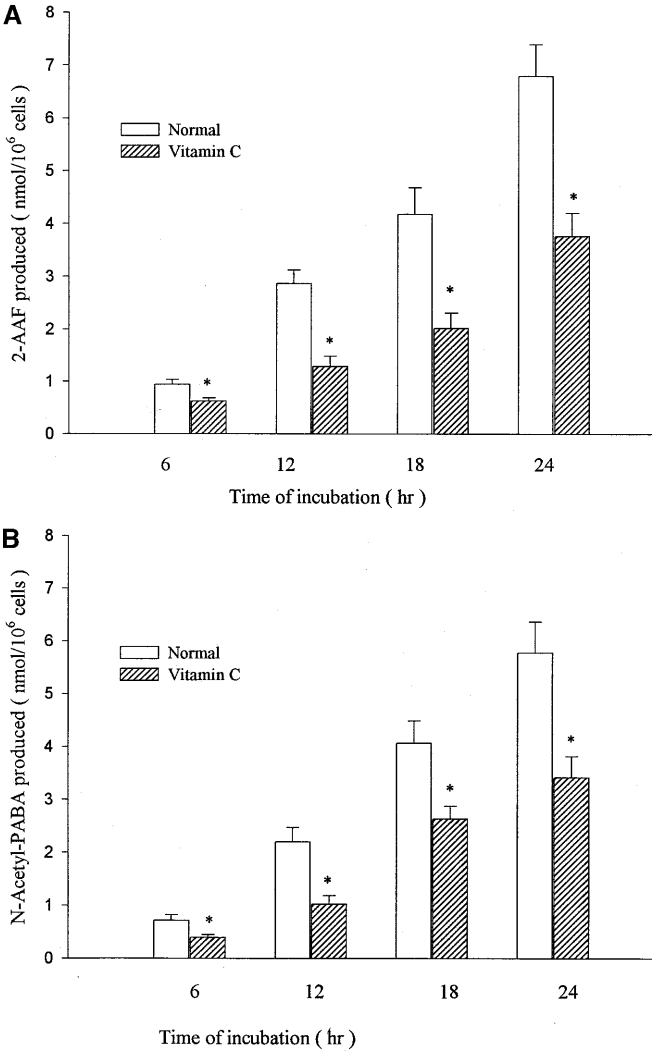

Fig. 3A, B Effects of incubation time on AAF and *N*-Ac-PABA production by T24 cells. T24 cells were incubated with AF (**A**) and PABA (**B**) at 60 μM with 200 μM vitamin C co-treatment for the time shown. AAF and *N*-Ac-PABA were measured using a HPLC assay. *Each point* represents the mean of triplicate assays of three incubations of cells. * Mean differences between vitamin C and control, $P < 0.05$

Discussion

The NAT activity in human T24 cells is presented. However, it is not known whether this NAT is NAT1 or NAT2. This requires further investigation, such as using the molecular method for amino acid sequences and DNA sequences. Laboratory animals, microorganisms, fish, common fruits, vegetables, and humans have been shown to contain the NAT enzyme. NAT has been shown to associate with some chemical carcinogens. The substrate specificity for NAT1 is different from that for NAT2 in humans. AF was chosen in the present study because it is the common substrate for both NAT1 and NAT2, while PABA is a substrate for NAT1 only [32]. Based on the NAT activity in T24 cells it is impossible to conclude whether this NAT belongs to rapid or slow acetylation; therefore, it also needs further investigation. Although, rapid and slow acetylations have been shown to have a predisposing factor for the sensitivity of individuals to the toxicity during exposure to many arylamine drugs and carcinogens [32]. This *N*-acetylation is a primary determinant in the elimination of several therapeutic compounds and arylamines.

NAT activity has been reported in the bladder tissue [19]. Our earlier studies had demonstrated that human T24-cells also contained NAT activity and can acetylated arylamine substrates [4]. Vitamin C had been shown to induce membranous damage in T24 cells [10]. Therefore, it is of interest to this study whether or not vitamin C would affect the NAT activity of T24 cells. The reason for selecting AF and PABA for determining T24-cell NAT activity is to compare the metabolism of carcinogen (AF) and non-carcinogen (PABA).

The present study demonstrated that vitamin C can markedly inhibit the NAT activity in T24 cells. The inhibition has a dose-dependent effect. As vitamin C inhibited NAT activity, the values of the apparent K_m and V_{max} from NAT acetylation with or without vitamin C co-treatment were also determined. Both kinetic constants were decreased; thus, vitamin C acts as a non-competitive inhibitor in this reaction. The results also show that vitamin C decreased AF–DNA adduct formation in T24 cells. Carcinogen–DNA adduct formation is the important step in chemical carcinogenesis. The levels of AF–DNA adduct formation is reported to be related to the initiation of carcinogenesis [4, 31]. The bacterial and animal studies both indicated that formation of *N*-(dGus-8-yl)-AF adducts in DNA is essential to the induction of mutations and in the initiation of hepatocarcinogenesis [24]. It has been reported that *N*-hydroxyarylamines are ultimate bladder carcinogens, and that C8-deoxyguanosine substitution may represent an initiating lesion in bladder tumor formation [1].

Table 1 Kinetic data for acetylation of AF and PABA in T24 cells. Values are means ± SD; $n = 3$. The acetyl CoA and vitamin C concentrations were 0.5 mM and 200 μM, respectively, and the kinetic constants were calculated from the modified HYPER Program of Cleland [25]

	AF		PABA	
	K_m (mM)	V_{max} (nmol/10^6 cells)	K_m (mM)	V_{max} (nmol/10^6 cells)
Control	3.90 ± 0.56	27.16 ± 5.38	5.12 ± 0.68	36.68 ± 6.82
Vitamin C	2.20 ± 0.34*	12.08 ± 2.36**	2.56 ± 0.48***	22.18 ± 5.48$^{\#}$

Differences between 200 μM vitamin C and control: *$P < 0.005$; **$P < 0.001$; ***$P < 0.001$; $^{\#}P < 0.001$

Table 2 AF–DNA adduct formation (pmol adduct/mg DNA) following 18 h incubation of T24 cells with or without 200 μM vitamin C. Values are means ± SD of six separate preparations (T24 cells, incubation with AF, DNA preparation, postlabeling, and HPLC)

Cancer cell (mM)	Adducts (pmol/mg DNA)	
	30	60
T24 cells	0.52 ± 0.14	0.88 ± 0.18
T24 cells + vitamin C	0.24 ± 0.06*	0.40 ± 0.10*

*Differs from T24 cells, $P < 0.05$, by two-tailed Student's *t*-test

It is difficult at this point to extrapolate the quantity of vitamin C that would be needed to be consumed by humans to potentially reduce the NAT activity and AF–DNA adduct formation in target cells in vivo. However, for adult men and women, the recommended dietary allowances for vitamin C was set at 60 mg/day based on a consideration of (i) the observed turnover rate and depletion rate of an initial body vitamin C pool of 1500 mg; (ii) an assumed absorption of vitamin C of about 85% at usual intakes; and (iii) the variable loss of vitamin C during food preparation [28]. The concentration (200 μM) of vitamin C, selected for all of the studies, was based upon: (i) it was close to normal intakes per day; and (ii) it induced a 50% decrease in NAT activity of examined T24 cells. It is still controversial in relation to acetylator and bladder cancer. On the one hand, some reports have raised the possibility that slow acetylators raise the risk for bladder cancer [1, 25]. On the other hand, Hayes et al. [13] demonstrated that the NAT2 related slow acetylator might not be associated with an increased risk of bladder cancer in workers exposed to benzidine and may have a protective effect [13]. Thus, in current studies, whether or not T24 cells NAT belongs to the slow acetylator phenotype needs further investigation.

A metabolic characteristic of potential relevance to bladder carcinogenesis involved polymorphic NAT2, an inherited phenotype in the rapid and slow acetylators classified by drug metabolism [25] or genotype [14]. Humans show considerable genetic variability in their ability to *N*-acetylate arylamine [11], and this variation has been implicated in their etiology of arylamine-induced bladder tumor [23] and colon cancer [15, 20]. Currently, our data (Table 2) also demonstrated that vitamin C decreased AF–DNA adduct formation in T24 cells. Although currently it is not known whether or not this decrease of NAT activity and AF–DNA adduct formation would result in the decrease of tumor production, or whether vitamin C could prevent the development of bladder cancer, other investigators have already reported that promoted NAT activity is associated with the increased sensitivity to the mutagenic effects of many arylamines [8]. The attenuation of liver NAT activity is shown to be associated with several disease processes, such as breast and bladder cancer [32]. We also added sulfamethazine to the T24 cell culture but we did not detect acetylate sulfamethazine. Therefore, NAT activity from T24 cells was NAT1 and was affected by vitamin C (data not shown). Apparently, the changes of NAT activity are related to several diseases. Further investigation is needed to examine whether or not the decrease of NAT activity led to the decrease of the target organ malignancy after being exposed to arylamine carcinogens. We also added sulfamethazine to the T24 cell culture, but we did not detect acetylate sulfamethazine. On the other hand, we could find the acetylated PABA and AF. PABA is a NAT1 substrate, sulfamethazine is a NAT2 substrate, AF is both a NAT1 and a NAT2 substrate. Therefore, NAT activity from T24 cells was NAT1, which is affected by vitamin C (data not shown). The exact mechanism still needs further investigation.

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