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Ellagica acid inhibits arylamine N-acetyltransferase activity and DNA adduct formation in human bladder tumor cell lines (T24 and TSGH 8301)

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Abstract The fact that vitamin C (ascorbic acid) exhibits a protective effect in certain types of cancer is well documented. Our previous studies demonstrated that human bladder tumor cell line (T24) has N-acetyltransferase (NAT) activity in cytosols and intact cells. The present studies examined the inhibition of arylamine NAT activity and carcinogen (2-aminofluorene)-DNA adduct formation by ellagic acid (EA) in human bladder tumor cell lines (T24 and TSGH 8301). Two assay systems were performed, one with cellular cytosols (9,000 g supernatant), the other with intact bladder tumor cell suspensions. NAT activity and 2-aminofluorene-DNA adduct formation in T24 and TSGH 8301 cells was inhibited by EA in a dose-dependent manner in both systems, i.e., the greater the concentration of EA in the reaction the greater the inhibition of NAT activity (dose- and time-course dependent effects). The data also indicated that EA decreased the apparent Km and Vmax of NAT enzymes from T24 and TSGH 8301 cells in cytosols. NAT activity and 2-aminofluorene-DNA adducts in T24 is higher than in TSGH 8301. This report is the first to demonstrate that EA affects human bladder tumor cell NAT activity.

Keywords N-acetyltransferase · 2-aminofluorene · N-acetyl-2-aminofluorene · *P*-aminobenzoic acid · N-acetyl-*P*-aminobenzoic acid · DNA adduct · Bladder cancer

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Introduction

Arylamine N-acetyltransferases (NATs) catalyze the metabolism of xenobiotics and carcinogens by transferring an acetyl group from the acetyl coenzyme A to the amine nitrogen atom of these agents. NAT localizes in the cytosol fraction of the liver and other organs or tissues of mammals [27]. Two distinct NAT genes (*NAT1* and *NAT2*) in humans have been identified and sequenced that encode for two different arylamine NATs – NAT1 and NAT2; [4, 23]. The acetylation process can be either rapid or slow based on NAT activity. Statistical and epidemiological studies in humans show that rapid acetylation has been linked to increased risk of colorectal cancer [20, 17] and slow acetylation to increased susceptibility of occupational bladder cancer [5]. NAT activity has been reported in human colon [18] and bladder [19] tissues. Thus, the genetically mediated variation in NAT activity in target organs or tissues may lead to different risks for arylamine-induced neoplasm in humans.

Ellagic acid (EA) is a naturally occurring plant polyphenol. Dietary treatment with EA has been shown to inhibit the incidence of hepatocellular neoplasms [25], and decrease the number of esophageal preneoplastic and neoplastic lesions induced by N-nitrosomethylbenzylamine in rats [21, 8] . EA has also demonstrated anticarcinogenic activity [24], and orally administered EA during or after carcinogen treatment significantly reduces the incidence and number of small intestinal tumors [2]. Studies have also demonstrated that dietary EA affects rat hepatic and esophageal mucosal cytochromes P450 and phase II enzymes [1]. However, there

is no available information to address EA's effect on NAT activity on human bladder tumor cells. Therefore, we investigated the chemopreventive mechanisms of EA using human bladder tumor cells as the test system.

Materials and methods

Chemicals and reagents

EA, ethylenediaminetetraacetic acid (EDTA), 2-aminofluorene (AF), N-acetyl-2-aminofluorene (AAF), P-aminobenzoic acid (PABA), N-acetyl-P-aminobenzoic acid (N-Ac-PABA), acetylcarnitine, leupeptin, bovine serum albumin (BSA), phenylmethylsulfonylfluoride (PMSF), Tris, dimethyl sulfoxide (DMSO), dithiothreitol (DTT), and carnitine acetyltransferase were obtained from Sigma Chemical Co. (St. Louis, MO). Acetyl-CoA was obtained from P-L Biochemicals Inc. (Milwaukee, WI). All of the chemicals used were reagent grade.

Human bladder tumor cell lines T24 and TSGH 8301

Human bladder tumor (carcinoma) cell lines (T24 – female Caucasian, 81 years old, and TSGH 8301 – male Chinese, 56 years old) were obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The cells were placed separately into 75-cm^2 tissue culture flasks and grown at 37°C under a humidified 5% CO_2 atmosphere in RPMI 1640 medium with glutamine (Sigma Chemical Company, St. Louis. MO) and supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY) and 2% penicillin-streptomycin (10,000 U/ml penicillin and 10 mg/ml streptomycin).

Preparations of cell cytosols

About 6×10^8 cells were placed in 2 ml of the lysis buffer (20 mM Tris/HCl, pH 7.5 at 4°C, 1 mM DTT, 1 mM EDTA, 50 μM PMSF, and 10 μM leupeptin), as previously described [28]. The suspensions were centrifuged at 9,000 *g* for 1 min in a model 3200 Eppendorf/Brinkman centrifuge, and the supernatant fraction was subsequently centrifuged at 10,000 *g* for 60 min. The supernatant was kept on ice for NAT activity and protein determinations.

NAT activity determinations

The determination of acetyl-CoA-dependent N-acetylation of PABA and AF were performed, as previously described [28].

Protein determination

Protein concentrations in cell cytosols were determined by the Bradford method (1976) with bovine serum albumin as the standard. All of the samples were assayed in triplicate.

Intact cell NAT activity determination

Cells (in 1 ml RPMI 1640 media with glutamine and 10% fetal calf serum) were incubated with arylamine substrate at 1×10^6 cells/ml in individual wells of 24-well cell culture plates with or without EA cotreatment for the time indicated at 37°C in 95% air and 5% CO_2. At the conclusion of 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 acetyl-AF, as described above. For experiments with PABA, aliquots of the supernatant were assayed directly for N-Ac-PABA [6, 28].

Effects of various concentrations of EA on NAT activity in intact cells

Cells (in 1 ml RPMI 1640 media with glutamine and 10% fetal calf serum) were incubated with various concentrations of arylamine substrate (AF or PABA) at 1×10^6 cells/ml in individual wells of 24-well cell culture plates with or without EA (0.4, 4, 40, and 400 μM) cotreatment for the time indicated at 37°C in 95% air and 5% CO_2. At the end of 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 acetyl-AF, as described above. For experiments with PABA, aliquots of the supernatant were assayed directly for N-Ac-PABA [28].

Time-course effects of EA on NAT activity in intact cells

Cells were incubated with 60 μM AF at 5×10^5 cells/ml in individual wells of 24-well cell culture plates with or without EA cotreatment for the 6-, 12-, 18-, and 24-h incubation time intervals. At the end of incubation the acetylated AF or PABA and unacetylated AF or PABA were determined by using HPLC, as described above [28].

Effects of EA on kinetic constants of NAT

Cell cystols cotreated with or without 40 μM EA and various concentrations of AF or PABA were determined for NAT activity, as described above [28]. All reactions were run in triplicate.

Detection and measurement of DNA adducts in cells

Cells were incubated with 30 and 60 μM AF at 5×10^6 cells/ml in individual wells of 6-well cell culture plates with or without 40 μM EA cotreatment for the 24-h incubation period. At the end of incubation the cells were collected and detection and measurement of DNA adducts were performed, as described previously [28].

Statistical treatment of data

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] that performs linear regression using a least-squares method.

Results

The possible effects of EA on NAT activity in human bladder tumor cell lines T24 and TSGH 8301 were examined by high-performance liquid chromatography (HPLC), assessing the levels of acetylation and non-acetylation of AF and PABA. The means ± SD (standard deviation) of NAT activity cotreated with or without various concentrations of EA, with various concentrations of both substrates in intact cells, are given in Fig. 1A, B, and Fig. 2A, B. The data indicated that there was decreased NAT activity associated with increased EA in examined cells.

To determine the time-course effects of 40 μM EA on NAT activity in intact cells, cells with or without 40 μM EA were incubated at 37°C and assayed at different time intervals (6, 12, 18, 24, and 48 h). The results are shown in Fig. 3A ,B. In the presence of 40 μM EA the amounts of acetylated produced decreased by 28–44% (T24) and

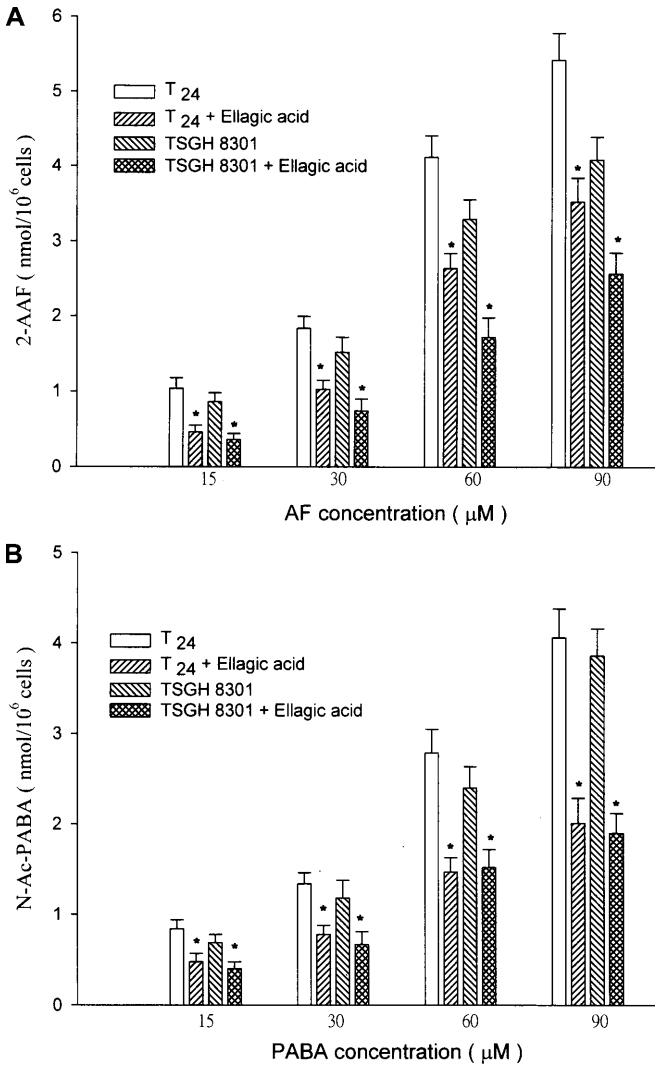

Fig. 1A,B Effects of EA on production of 2-AAF and N-Ac-PABA by human bladder tumor cells (T24 and TSGH 8301). T24 and TSGH 8301 cells were incubated separately, as described, for 18h at the various concentrations of AF (**A**) and PABA (**B**) cotreatment with 40 μM EA. AAF and N-Ac-PABA were measured by HPLC assay. Each *point* represents the mean of triplicate assays of three incubations of cells. Mean differences between EA and control – $P < 0.05$

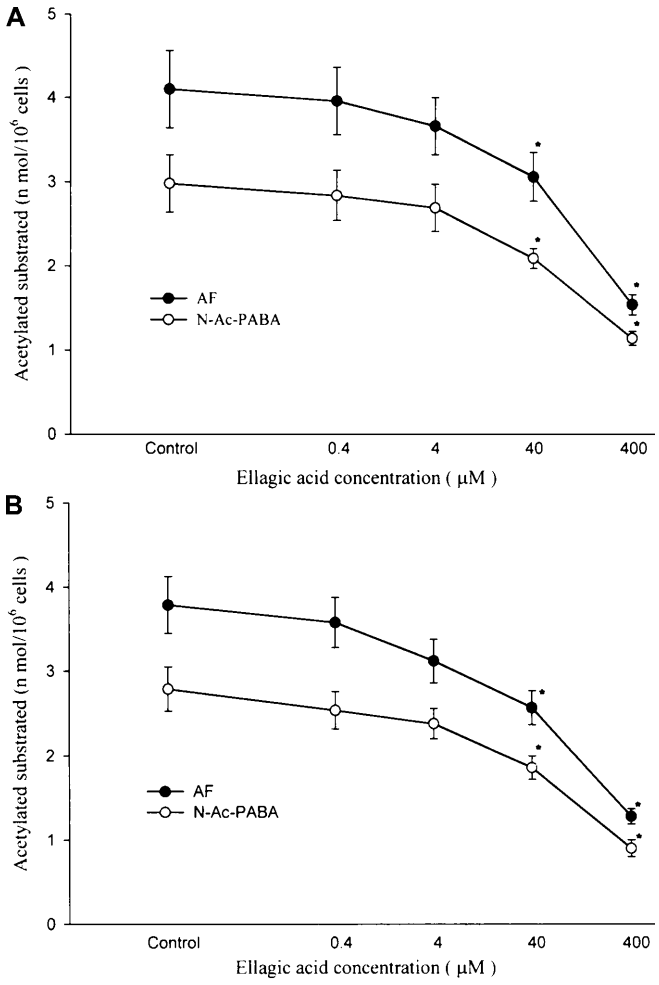

Fig. 2A,B Effects of various concentrations of EA on production of 2-AAF and N-Ac-PABA by human bladder tumor cells (T24 and TSGH 8301). T24 (**A**) and TSGH 8301 (**B**) cells were incubated separately, as described, for 18 h at 60 μM of AF cotreatment with 40 μM EA. AAF and N-Ac-PABA were measured by HPLC assay. Each *point* represents the mean of triplicate assays of three incubations of cells. Mean differences between EA and control –$P < 0.05$

38–50% (TSGH 8301) for AF, and 19–38% (T24) and 42–56% (TSGH 8301) for PABA after 6-, 12-, 18-, 24-, and 48-h incubation.

In the presence or absence of EA specific concentrations of AF and PABA (0.373, 0.435, 0.543, 0.745, 1.102, and 2.205 mM) were added to the recycling mixtures for determining cell NAT kinetic constants. For the cytosol examinations, when 40 μM EA were added to the reaction mixtures, the apparent K_m and V_{max} values were decreased by 52.2% and 51.9% (T24) and 50.0% and 56% (TSGH8301) for acetylation of AF, and by 44.2% and 60.0% (T24) and 57% and 61% (TSGH 8301) for acetylation of PABA (Fig. 4A–D, and Tables 1 and 2). Clearly, both K_m and V_{max} values on cell NAT were decreased in the presence of EA.

After 24-h incubation of T24 and TSGH 8301 cells separately with AF (30 and 60 μM) in the presence or absence of EA, cells were harvested, DNA was isolated and hydrolyzed to nucleotides, and adducted nucleotides were extracted into butanol and analyzed by HPLC. The data indicated that EA affected DNA-AF adduct formation in all examined AF doses (Fig. 5).

Discussion

Although an EA effect on NAT activity in human colon tumor and normal mononuclear leukocytes has been reported [15, 16], an EA effect on NAT in human bladder tumor cells has not. Therefore, the purpose of this study was to determine whether EA could affect NAT activity in human bladder tumor cells. The results indicate that EA did affect the T24 and TSGH 8301

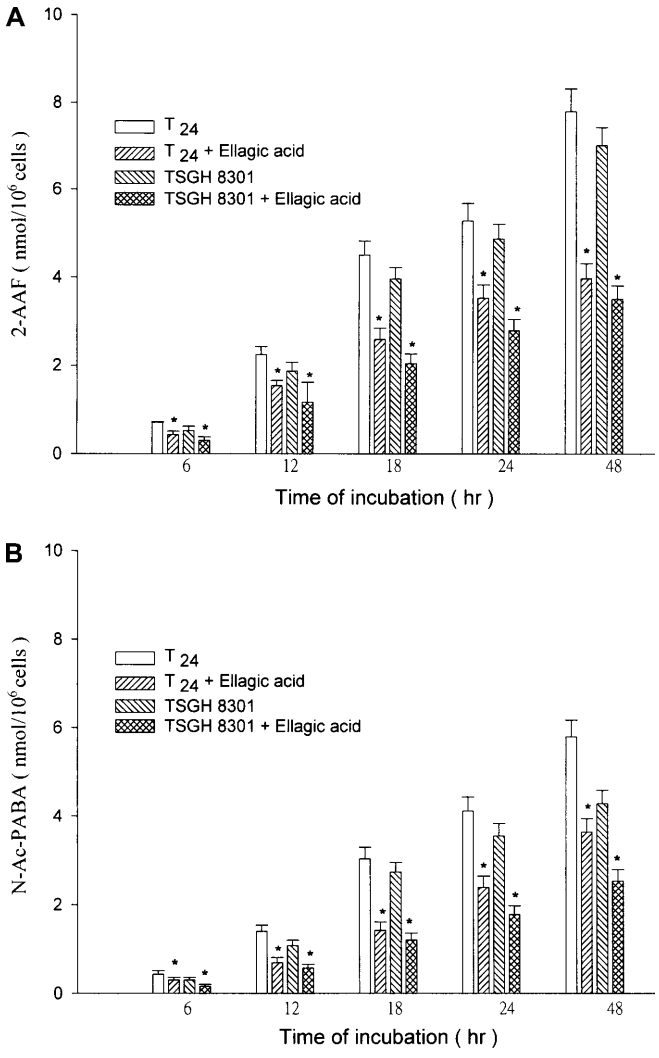

Fig. 3A,B Time-course effects of EA on production of 2-AAF and N-Ac-PABA by human bladder tumor cells (T24 and TSGH 8301). T24 cells were incubated, as described, for 6, 12, 18, 24, and 48 h at 60 μM of AF (**A**) and PABA (**B**) cotreatment with 40 μM EA. AAF and N-Ac-PABA were measured by HPLC assay. Each *point* represents the mean of triplicate assays of three incubations of cells. Mean differences between EA and control – $P < 0.05$

cells, based on the following observations: (1) NAT activity was inhibited, (2) K_m and V_{max} of NAT was decreased, and (3) AF-DNA adduct was decreased after cells were cotreated with EA.

Many other observations may be prerequisites for the observed effects of EA on NAT activity in T24 and TSGH 8301 cells. First, NAT enzyme and activity is exhibited in tissues or organs of laboratory animals (including frogs), and also in humans; and NAT has been demonstrated to play a role in chemical carcinogenesis [11, 22, 14]. Second, slow acetylation seems to predispose to bladder carcinogenesis[5]. Third, an inherited phenotype in the rapid and slow acetylators is classified by drug metabolism [20] or genotype [13]. Fourth, arylamine NAT activity toward AF and PABA was detected in T24 cells [28] but there is no information on TSGH 8301 cells. Fifth, AF-DNA adduct has been

Fig. 4A–D Lineweaver-Burk double reciprocal plot of NAT in human bladder tumor cells (T24 and TSGH 8301) cotreatement with or without EA. About 5×10^5 cells/ml containing 0.044, 0.088, 0.163, 0.303, and 0.709 mM AF for 18 h of incubation. The amounts of AF and AAF, PABA, and N-Ac-PABA were determined, as described. Lineweaver-Burk double reciprocal plots were measured by using linear regression analysis of reciprocal substrate concentrations plotted against reciprocal initial velocities: T24 for AF (**A**), TSGH 8301 for AF (**B**), T24 for PABA (**C**), and TSGH 8301 for PABA (**D**)

found in T24 intact cells, but there is no information on TSGH 8301 cells. Sixth, it was reported that EA has anticarcinogenic activity [24]. Therefore, it was of interest to study whether EA would affect NAT activity in human bladder tumor cells.

The data from the cytosol and intact cells of T24 and TSGH 8301 studies indicated that there were significant differences in NAT activity and AF-DNA adducts between the control and EA treatment groups based on the *P* values of Student's *t*-test. EA can markedly inhibit NAT activity and AF-DNA adducts in T24 and TSGH 8301 cells in both systems examined, and the inhibition showed a dose-dependent effect. The results also demonstrated that, with an increase in the concentration of AF and PABA, the production of AAF and N-Ac-PABA was increased in the intact cells. More interesting was that increasing the incubation time of intact cells with substrates led to increased acetylation of substrates in both cell lines examined. EA also decreased NAT kinetic constants (Km and Vmax) in cell cytosols.

There are several reasons for choosing 40 μM EA for the reported examinations. First, NAT activity in T24 and TSGH 8301 cells was inhibited by more than 50% after cotreated with EA. Second, 400 μM EA already inhibited cell NAT activity by more than 80%. Third, the amount of EA (40 μM) was close to the amounts of dietary EA (0.4 g/kg) in the studies in rats [3].

The association of acetylation phenotypes with bladder cancer is still controversial. At first investigations demonstrated that slow acetylators are at a higher risk for bladder cancers, based on epidemiological statistics [5]. However, subsequent investigations demonstrated that NAT2-related slow acetylators might not be at increased risk of bladder cancer related to benzidine, and may even benefit from a protective effect [12].

Frazier et al.[10] pointed out the age-associated risk of colorectal cancer among individuals with *NAT2* mutations and mutations in DNA mismatch repair genes. It is not known whether decreasing NAT activity and AF-DNA adducts will lead to decreased tumor production or whether EA would prevent the development of bladder cancer. Our results demonstrated that EA can decrease DNA-AF adduct formation in T24 and TSGH 8301 cells. DNA-carcinogen adduct is an important step for carcinogen-induced carcinogenesis. Therefore, whether or not DNA-AF adduct decreased by EA will lead to decreased bladder cancers needs further investigation. Our studies did not determine the exact mechanism by which NAT activity decrease could lead to

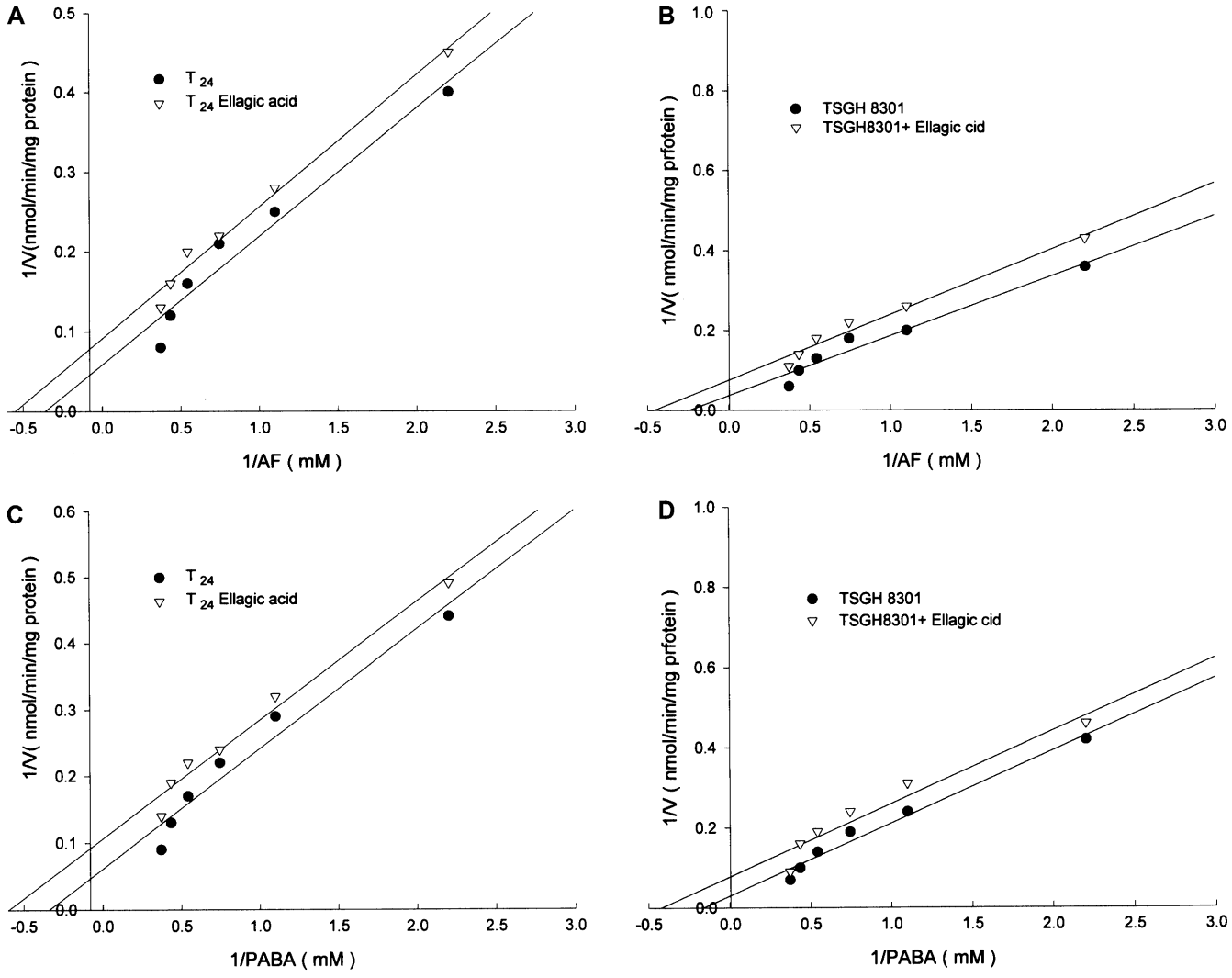

Table 1 Kinetic data for acetylation of AF and PABA in T24 cells. Values are mean ± SD $n=3$. The Acetyl CoA and ellagic acid concentrations were 0.1 mM and 40 μM, and the kinetic constants were calculated from the modified HYPER Program of Cleland (1967)

	AF		PABA	
	K_m (mM)	V_{max} (nmol/mg protein)	K_m (mM)	V_{max} (nmol/min/mg protein)
Control	4.02 ± 0.42	20.12 ± 3.68	5.56 ± 0.59	25.08 ± 4.98
Ellagic acid	2.22 ± 0.24 [a]	11.06 ± 2.43 [b]	2.33 ± 0.28 [c]	11.11 ± 2.56 [d]

[a] Differs between 40 μM ellagic acid and control. $P < 0.05$
[b] Differs between 40 μM ellagic acid and control. $P < 0.01$
[c] Differs between 40 μM ellagic acid and control. $P < 0.01$
[d] Differs between 40 μM ellagic acid and control. $P < 0.01$

Table 2 Kinetic data for acetylation of AF and PABA in TSGH 8301 cells. Values are mean ± SD $n=3$. The Acetyl CoA and ellagic acid concentrations were 0.1 mM and 40 μM, and the kinetic constants were calculated from the modified HYPER Program of Cleland (1967)

	AF		PABA	
	K_m (mM)	V_{max} (nmol/mg protein)	K_m (mM)	V_{max} (nmol/min/mg protein)
Control	4.35 ± 0.86	25.16 ± 5.94	6.67 ± 0.78	31.68 ± 7.28
Ellagic acid	2.24 ± 0.43 [a]	12.56 ± 3.84 [b]	2.63 ± 0.48 [c]	14.29 ± 4.84 [d]

[a] Differs between 40 μM ellagic acid and control. $P < 0.05$
[b] Differs between 40 μM ellagic acid and control. $P < 0.01$
[c] Differs between 40 μM ellagic acid and control. $P < 0.01$
[d] Differs between 40 μM ellagic acid and control. $P < 0.01$

DNA-AF adduct formation in T24 and TSGH 8301 cells. However, it has also been demonstrated that decreasing NAT activity in liver is associated with bladder and breast cancer processes [26, 27]. More important is that increased levels of NAT activity are related to increased sensitivity to the mutagenic effects of

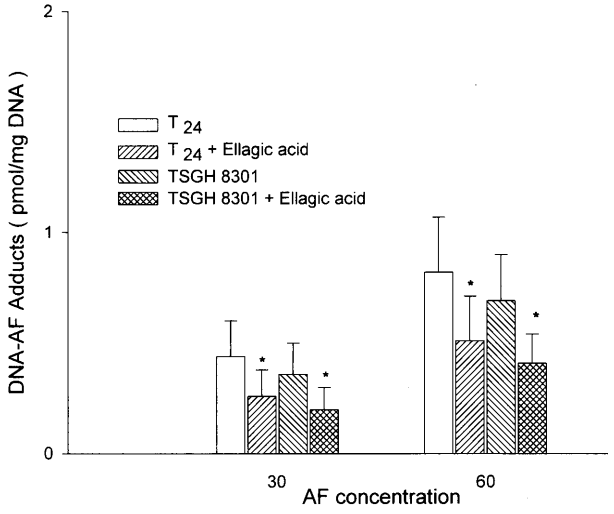

Fig. 5 Effects of EA on DNA-AF adduct formation by human bladder tumor cells (T24 and TSGH 8301). Following 24-h incubation of T24 and TSGH 8301 cells with AF (30 and 90 μM) in the presence of EA (50 μM), cells were isolated and DNA was prepared, hydrolyzed to nucleotides, and adducted nucleotides were extracted into butanol and analyzed by HPLC. Values are means ± SD of six separate preparations. Values are mean ± SD ($n = 3$). Difference between 40 μM EA and control – $P < 0.05$

many arylamines [9]. While further investigation is needed the present results offer some information that EA decreased NAT activity in vitro and in intact human bladder tumor cells (T24 and TSGH 8301).

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