Table 1. Dissociation constants of human and *L. casei* DHFR using the centrifugation versus the filtration methods

	Human DHFR (purified) K_D (nM)	*L. casei* DHFR K_D (nM)
pH 7.2		
Centrifugation method	3.8	2.1
Filtration method	0.058	0.57
pH 6.2		
Centrifugation method	0.5	1.04
Filtration method	0.094	0.54

All experiments were performed at 10^{-4} M NADPH. Dissociation constants were measured by Scatchard analysis. Conditions for complex formation and processing for both methods are detailed in the text.

actual amount of MTX bound to DHFR at a given ligand concentration. Exposure to charcoal for 1 min allowed complete adsorption of all unbound ligand while maintaining the maximum concentration of ternary complex, as the complex was relatively stable for 3 min following charcoal exposure (Fig. 2), We applied this new technique to a study of MTX binding constants (K_D) to DHFR purified from both a human and a bacterial source and compared these results to those obtained in parallel experiments utilizing centrifugation to separate bound from free drug. As Table 1 shows, the method used for measuring ternary complex formation produced constants of dissociation that differed by up to 70-fold, depending on the assay conditions and enzyme source. The lower affinity figure likely reflected the binding constant of MTX to DHFR at low concentrations of NADPH.

The filtration method not only improved the accuracy of determination of binding constants but also enhanced the sensitivity by at least 5-fold. A gain in sensitivity also occurred in the MTX concentration range of 10^{-7} to 10^{-9} M due to the greater steepness of the binding curve (Fig. 2). This range is important in that drug concentrations above 10^{-8} M are cytotoxic for bone marrow myeloid precursor and dividing cells of the gastrointestinal epithelium [7].

In summary, the use of rapid separation techniques that minimize the exposure of the DHFR–NADPH–MTX complex to activated charcoal allows a more accurate measurement of this complex. This technique facilitates the study of kinetic interactions of DHFR with MTX and the measurement of MTX concentrations.

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Genetic differences in inhibition of 2-aminofluorene *N*-acetyltransferase activity between C57BL/6J and A/J mice

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Competitive inhibition of one or more of the metabolic steps in the activation of chemical carcinogens is a potential chemopreventive mechanism. The first step in the metabolic activation of AF* to reactive electrophiles, and a potential point for control, is N-acetylation to AAF by liver NAT [1]. Differences in AF NAT activity are under genetic control in C57BL/6J and A/J mice [2], and differences in human arylamine N-acetylating capacity have been correlated with differences in susceptibility to urinary bladder cancer [3–5]. In the present study, we wanted to identify relatively non-toxic, preferably competitive inhibitors of liver AF NAT, and to determine if there was a differential susceptibility to the effect of these inhibitors between C57BL/6J and A/J mice. This paper presents evidence that the beta-carboline derivatives have no effect on NAT, and that PABA competitively inhibits, and folic acid and MTX noncompetitively inhibit NAT. In addition, there was a statistically significant difference in K_i values for folic acid and MTX between C57BL/6J and A/J NAT.

* Abbreviations: AF, 2-aminofluorene; AAF, 2-acetylaminofluorene; NAT, *N*-acetyltransferase; PABA, *p*-aminobenzoic acid; MTX, methotrexate; NF, 2-nitrofluorene; DTT, dithiothreitol; K_i, inhibition constant; TCA, trichloroacetic acid; TFA, trifluoracetic acid; DMSO, dimethyl sulfoxide; and HPLC, high performance liquid chromatography.

Materials and methods

Chemicals. AF was purchased from K & K Laboratories, Plainview, NY; AAF from the Aldrich Chemical Co., Milwaukee, WI; acetyl CoA from P-L Biochemicals, Inc., Milwaukee, WI; folic acid from the Nutritional Biochemical Corp., Cleveland, OH; and PABA, MTX, harmine, har-

maline, harmol, norharman and DTT from the Sigma Chemical Co., St. Louis, MO. NF was donated by Dr. Charles King of the Michigan Cancer Foundation, Detroit, MI. All solvents were HPLC grade.

Animals. Male C57BL/6J and A/J mice, ages 3–4 months, were obtained from Jackson Laboratories, Bar Harbor, ME. Mice were housed two to four to a cage, allowed mouse chow and water *ad lib.*, and maintained on a 12-hr light–dark schedule.

Enzyme preparation. Mice were killed by cervical dislocation, and livers were removed and homogenized by hand in glass homogenizers in 4 vol. of 50 mM potassium phosphate buffer, 1 mM DTT, pH 7.4. Homogenates were centrifuged at 10,000 *g* for 20 min; the supernatant fractions were centrifuged at 105,000 *g* for 1 hr. The cytosol was diluted 1:1 with 50 mM potassium phosphate buffer, 1 mM DTT, pH 7.4, and used for all assays. All preparations were carried out at 4°. Protein concentrations were determined by method of Lowry *et al.* [6].

Inhibition assay. PABA, folic acid, MTX, harmine, harmaline, harmol, and norharman were tested for their abilities to inhibit AF NAT activity. Initially, these inhibitors at a concentration of 5 mM were incubated with NAT and 0.2 mM AF for 0, 1, 2, and 3 min. The compounds with inhibitory capacity were studied further, and K_i values and types of inhibition were determined.

The reaction mixture (100 μl) consisted of 20 μl AF, 10 μl inhibitor, and 50 μl enzyme. Following a 2-min preincubation at 37°, 20 μl acetyl CoA (final concentration of 0.5 mM in reaction mixture) was added to begin the reaction. Acetyl CoA was omitted from controls. Tubes were incubated for 0, 1, 2, and 3 min, and the reaction was terminated by the addition of 10% cold TCA. Samples were spun for 1 min and frozen.

For K_i and type of inhibition determinations, six concentrations of AF, ranging from 0.01 to 2 mM, and four concentrations of inhibitors, 5, 0.5, 0.05, and 0.005 mM, were used. Neither AF nor the inhibitors were soluble at higher concentrations. All reaction tubes were incubated for 2 min and inhibitor was omitted from controls.

Sample preparation. Samples were thawed the following day and 1 N NaOH was added to increase the pH. NF (10^{-5} M) was added as an internal standard. Samples were extracted twice for 30 min each with ether (2 ml), evaporated under a gentle stream of N_2, and redissolved in 0.5 ml of acetonitrile/water (70:30, v/v).

HPLC analysis. AAF was determined by HPLC according to modifications of methods described by Stanley [7] and Raineri *et al.* [8]. A Varian instrument equipped with a variable wavelength detector set at 290 nm was used. Separations were performed on a Whatman C_{18} Partisil, ODS-3, 10 μm reversed-phase column. AAF was eluted at approximately 9.4 min and NF at approximately 12.5 min by a 10-min linear gradient of TFA (0.1%)/acetonitrile (60:40) to 100% acetonitrile at a flow rate of 1 ml/min. AAF was quantitated by reference to an AAF/NF vs AAF standard curve.

Treatment of data. Lineweaver–Burk plots were drawn for all inhibitors to determine the type of inhibition. Dixon plots were used to analyze and calculate K_i values. K_i differences were analyzed for significance by the chi-square test.

Results and discussion

N-Acetylation, being the first step in the metabolic activation of AF, is a potential point for control of its activation. Theoretically, if AF NAT could be inhibited, formation of reactive metabolites and resultant DNA damage would be prevented or delayed. Thus, we wanted to find non-toxic competitive inhibitors of AF NAT. In addition, since genetic differences in AF NAT activity exist between C57BL/6J and A/J mice, we wanted to determine whether the amount of inhibitor required to achieve inhibition differed between strains.

Inhibition of AF NAT activity by harmine, harmaline, harmol, and norharman. It has been demonstrated that nonharman, a beta-carboline derivative produced by the pyrolysis of tryptophan, activates S9-mediated mutagenicity of AAF in the Ames test [9]. Furthermore, Wright *et al.* [10] reported that the analogs, harmine and harmaline, inhibit NAT purified from rat and hamster liver. Thus, cytosolic preparations of NAT from C57BL/6J mice were incubated for 0, 1, 2, and 3 min with 0.2 mM AF in the presence of 5 mM harmine, harmaline, harmol, and norharman. However, none of these compounds inhibited AF NAT activity to any significant degree. In explanation of these discrepancies, it is possible that the mouse enzyme is different from the other two species and does not have the capability to bind these compounds. Furthermore, Wright *et al.* used purified enzyme, whereas we used a cytosolic preparation and the inhibitors may have undergone nonspecific binding to proteins other than NAT, thereby reducing the concentration available to inhibit NAT.

Inhibition of AF NAT activity by PABA, folic acid, and MTX. Mandelbaum-Shavit and Blondheim [11] showed that folic acid and MTX competitively inhibit PABA NAT activity in human blood. Furthermore, Andres *et al.* [12] reported that MTX is a noncompetitive inhibitor for both acetyl CoA and arylamines, and used MTX in an affinity column to isolate and purify NAT from pigeon liver. Thus, cytosolic preparations of NAT from C57BL/6J and A/J mice were incubated for 2 min with concentrations of AF ranging from 0.01 to 2 mM in the presence of 0, 5, 0.5, 0.05, and 0.005 mM PABA, folic acid, and MTX. Lineweaver–Burk plots show that PABA was a competitive inhibitor (Fig. 1), and folic acid and MTX were noncompetitive inhibitors of AF NAT (data not shown). Again, our results did not agree with those from previous investigations. However, a different species and tissue were used.

K_i values, calculated from Dixon plots, are shown in Table 1. The K_i for PABA was the same in both C57BL/6J and A/J mice, and was approximately 6–16 times lower than the K_i values for folic acid and MTX. The K_i values for folic acid and MTX were significantly lower (2.5 times) in C57BL/6J than A/J mice with the K_i values for MTX being slightly lower than those for folic acid.

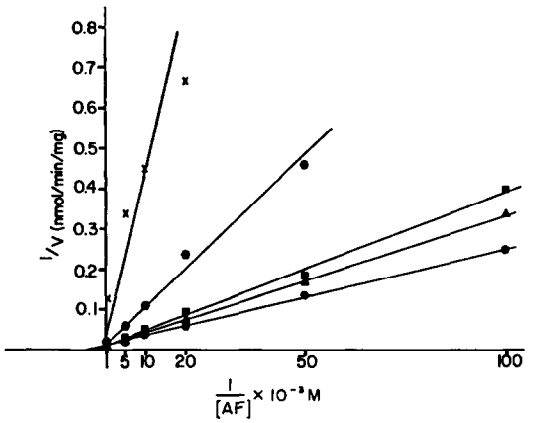

Fig. 1. Competitive inhibition of AF NAT activity by PABA. Cytosolic preparations of NAT were incubated for 2 min with concentrations of AF ranging from 0.01 to 2 mM in the absence of inhibitor (●) or in the presence of 0.005 mM PABA (▲), 0.05 mM PABA (■), 0.5 mM PABA (⬟), and 5 mM PABA (×). AAF was quantitated by HPLC.

Table 1. K_i values for inhibition of AF NAT activity

Strain	K_i (mM)		
	PABA	Folic acid	MTX
C57BL/6J (3)*	0.16 ± 0.01	1.06 ± 0.08	0.92 ± 0.16
A/J (3)	0.18 ± 0.01	2.80 ± 0.73†	2.36 ± 0.17†

Values are the mean ± S.E.M.
* Number of animals is given in parentheses.
† Significantly different from C57BL/6J mice, $P < 0.05$.

There are activity and structural differences in AF NAT between C57BL/6J and A/J mice [13] and, as a result, the A/J NAT required a higher concentration of inhibitor than the C57BL/6J NAT to achieve the same degree of inhibition. Recently, this same phenomenon has been observed by Szabo and Weber [14] who showed a differential response of C57BL/6J and A/J NAT to the inhibitor DMSO. However, PABA is a substrate with monomorphic NAT activity for mouse liver NAT and, therefore, it is not surprising that the K_i for PABA was the same in both strains. Nevertheless, NAT bound PABA with the greatest affinity and, thus, was the best inhibitor of AF NAT in both mouse strains.

In summary, the beta-carboline derivatives failed to inhibit AF NAT activity. PABA was a competitive inhibitor, and folic acid and MTX were noncompetitive inhibitors of AF NAT for the acceptor amine. NAT from C57BL/6J and A/J mice bound PABA with the same affinity and more tightly than the other inhibitors. There was a differential sensitivity to the effect of folic acid and MTX between C57BL/6J and A/J mice; the K_i value was significantly higher in A/J mice.

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