SPERMIDINE/SPERMINE N\textsuperscript{1}-ACETYLTRANSFERASE CATALYZES AMANTADINE ACETYLATION

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(Received November 11, 2000; accepted January 16, 2001)

This paper is available online at http://dmd.aspetjournals.org

ABSTRACT:

Amantadine acetylation was demonstrated to occur both in vivo and in vitro using transgenic male mice overexpressing spermidine/spermine \textsuperscript{N\textsubscript{1}}-acetyltransferase (SSAT). We previously reported that neither NAT1 nor NAT2 was responsible for catalyzing acetylation of the primary amine group of amantadine. We hypothesized that the inducible polyamine-catabolizing enzyme, SSAT, was an alternate pathway for acetylation amantadine. Transgenic mice injected s.c. with 3 mg/kg amantadine excreted 4.5 ± 1% (mean ± S.E.) of the administered dose as acetylamantadine in 24-h urine samples while, by contrast, nontransgenic control mice failed to excrete any detectable acetylamantadine in their urine. In vitro studies with the cytosolic liver fraction from transgenic mice as the source of SSAT demonstrated spermidine acetylation catalytic activity with an apparent $K_m = 267 \pm 46 \mu\text{M}$ and $V_{\text{max}} = 0.009 \pm 0.002 \text{nmol/min/mg of protein}$. Amantadine competitively inhibited spermidine acetylation with an apparent $K_i = 738 \pm 157 \mu\text{M}$. Incubation of amantadine, SSAT, and an acetyl CoA-regenerating system produced modest amounts of acetylamantadine. The NAT2 substrate, sulfamethazine, inhibited spermidine acetylation with a calculated $K_i = 3.5 \text{mM}$, suggesting that SSAT may be an alternate pathway for acetylation of NAT2 substrates. The NAT1 substrate, \textit{p}-aminobenzoic acid, had no inhibitory effect. These results provide evidence that amantadine can be acetylated by SSAT and may be a specific drug substrate for this enzyme. Further investigation of the role of SSAT as a potential drug-metabolizing pathway is warranted.

Many drugs are metabolized by acetylation in man, including procainamide, isoniazid, and sulfamethazine (SMZ)\textsuperscript{1} (Weber and Hein, 1985). The polymorphically expressed arylamine \textit{N}-acetyltransferases, NAT1 and NAT2, are the major contributors to this process (Vatsis et al., 1995). The interindividual variation observed in acetylation has been attributed to polymorphism. The ability to rapidly acetylate drugs is inherited as an autosomal dominant trait and is found in different frequencies in different ethnic groups (Kalow, 1982). However, in the presence of alcohol, both fast and slow acetylators increased the amount of acetylated sulfadimidine measured in blood and urine, which was reflected by a decreased serum half-life of sulfadimidine (Olsen and Mørland, 1978). This phenomenon could not be attributed solely to increases in acetic levels, and an alternate pathway not dependent on the NAT2 phenotype was suggested to explain the observed increase in acetylation in the presence of alcohol (Olsen and Mørland, 1982).

Amantadine hydrochloride is an achiral polycyclic aliphatic primary amine used in the prophylaxis and treatment of influenza A virus infection and to ameliorate Parkinson’s disease symptoms (Aoki and Sitar, 1988). Preliminary in vivo studies have indicated that 0.1 to 15% of an administered dose of amantadine is acetylated by some humans (Köppel and Tenczer, 1985; Bras et al., 1998). We demonstrated, in vitro, using various sources of enzymes, that amantadine acetylation is not catalyzed by NAT1 or NAT2 (Bras et al., 1998). We hypothesize that the \textit{N}-acetyltransferase associated with the conjugation of the naturally occurring polyamine, spermidine/spermine \textit{N\textsuperscript{1}}-acetyltransferase (SSAT), is participating in the acetylation of amantadine. Because substrates for NAT1 or NAT2 are selective and not specific, they may also be conjugated by SSAT and contribute to the overall occurrence of drug acetylation observed in vivo. The use of a transgenic mouse model overexpressing SSAT provided a convenient system to demonstrate not only in vivo but also in vitro activity. It was the purpose of the present study to demonstrate that transgenic mice that overexpress SSAT (Pietilä et al., 1997) could acetylate amantadine in vivo, to evaluate the ability of SSAT to catalyze the acetylation of amantadine in vitro, and to determine whether NAT1- or NAT2-selective substrates are able to inhibit spermidine acetylation in vitro.

Materials and Methods

Reagents. Sucrose, potassium chloride, dithiothreitol (DTT), magnesium chloride (MgCl\textsubscript{2}-6H\textsubscript{2}O), and hydroxylamine HCl were acquired from Fisher...

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weighing 25 to 38 g were anesthetized with pentobarbital sodium (200 mg/kg).

In Vivo Experiments. Experimental procedures involving the use of animals were approved by the University of Manitoba Protocol Management and Review Committee. Both transgenic and nontransgenic CD2F1 mice were injected s.c. with a dose of 3 mg/kg amantadine HCl (0.5 mg/ml) dissolved in normal saline. The mice were placed in separate metabolic cages. The total urine was harvested at 20°C until analyzed for acetylamantadine. Six hours after the last urine collection, the mice were injected s.c. with a dose of 80 mg/kg MGBG to induce SSAT (Pegg et al., 1985) and 18 h later injected with amantadine HCl (3 mg/kg). The total excreted urine was collected as described above.

Analytic Studies. Acetylamanalatine was quantified by gas-liquid chromatography as previously described (Bras et al., 1998) and modified to improve sensitivity. The modified procedures used solid phase extraction as follows: Supelclean ENVII-18 SPE tubes, 3 ml (Supelco, Bellafonte, PA.), were primed with 2 ml of methanol and 2 ml of DDW, followed by 2 ml of 0.2 M sodium phosphate buffer, pH 7.4. The mouse urine samples (1.0 ml), mixed with 2 ml of 1 M hydroxylamine HCl and placed on ice, were centrifuged at 30,000 g for 30 min. The supernatant was placed on a vacuum box (Whatman, Maidstone, UK). Negative pressure was applied and the discs were washed five times with DDW, followed by three washes with 1.0 ml of 95% v/v ethanol. The dried discs were placed into scintillation vials containing 4 ml of Ready Safe scintillation fluid and counted in a Beckman model LS6000TA scintillation counter (Beckman Instruments Inc., Fullerton, CA). To determine SSAT activity, the nonspecific radioactivity of the blanks was subtracted from the total radioactivity of the samples containing the added spermidine.

Inhibition Studies. Inhibition of spermidine acetylation by SSAT was determined by the addition of amantadine (1–100 μM) to fixed concentrations of spermidine (50 and 80 μM), the NAT1 selective substrate PABA (200–1000 μM), or the NAT2-selective substrate SMZ (200–700 μM) to tubes containing spermidine (200 μM) and the acetylation reagents as described above. Spermidine acetylation was quantified as described above. All assays were performed in triplicate.

Amarantadine Acylation in Vitro. Amantadine was incubated with transgenic mouse liver 100,000 g supernatant as our source of SSAT. An acetyl CoA-regenerating system was used as a source of acetate (Andre et al., 1985). The assay was performed in 1.5-ml microcentrifuge tubes. The assay buffer contained 225 mM Tris-HCl, 4.5 mM EDTA, and 4.5 mM DTT (pH 7.5 at 37°C). In brief, 100 μl of acetyl CoA (1 mM in DDW) and 100 μl of acetyl CoA-regenerating system (45 mM acetyl-CoA carnitine HCl) and 1 μM carnitine acetyltransferase dissolved in assay buffer) were added to the tubes. Then 200 μl of amantadine (50, 100, and 200 μM) dissolved in Tris-HCl buffer (pH 7.8) were added such that the final concentration of the buffer would be 100 mM in the final volume of 1000 μl. To start the reaction, 600 μl of supernatant were added and incubated for 10 min at 37°C. The reaction was stopped by the addition of 200 μl of ice-cold aqueous 1 M hydroxylamine HCl and placed on ice. The protein was removed as previously described for the spermidine acetylation procedures. The resultant incubation supernatant was frozen at −20°C until analyzed for acetylamantadine using gas-liquid chromatography.

Data Analysis. Data are expressed as mean ± S.E. of at least three experiments. Apparent K_m and V_max values were determined by nonlinear regression fit to the Michaelis-Menten equation with the computer program Fig P (version 6.0a, Biosoft, Ferguson, MO); IC_50 values for inhibition of spermidine acetylation were determined using regressive probit analysis (Cheng and Prusoff, 1973). The complementary graphical methods of Dixon (1953) and Cornish-Bowden (1974) were used to assess the type of inhibition caused by amantadine. Urinary acetylamantadine excretion between transgenic mice with or without induction by MGBG was evaluated by the two-tailed Student’s t test. Regression analysis was used to evaluate inhibitory activity of PABA.
and SMZ against spermidine acetylation. Differences between means with P values < 0.05 were considered significant.

Results

**In Vivo Studies of Amantadine Acetylation.** The chemical structure of amantadine is presented in Fig. 1 and illustrates its achiral amino group that is acetylated by transgenic mice overexpressing SSAT. Urine samples from amantadine-treated CD2F1 transgenic mice overexpressing SSAT consistently demonstrated metabolism of the parent compound to acetylamantadine in all timed collection periods (Fig. 2). The acetylamantadine excreted in the urine as a cumulative percent of administered dose at 24 h ranged between 2 to 6% with a mean of 4.5 ± 1.0%. Subsequently the CD2F1 transgenic mice were injected s.c. with a known inducer of SSAT, MGBG, and served as their own controls. At 24 h, the acetylamantadine excreted as a cumulative percentage of administered dose was 4.5 ± 1.0%, not different from the initial result without MGBG treatment. The urine samples from CD2F1 nontransgenic control mice and those treated with MGBG contained no acetylamantadine after a dose of amantadine.

**In Vitro Studies of Spermidine Acetylation.** A representative velocity versus substrate concentration plot for spermidine acetylation by SSAT is shown in Fig. 3. The kinetic parameters derived from these plots indicate an apparent K_m of 267 ± 46 μM and a V_max of 0.009 ± 0.002 nmol/min/mg of protein (n = 10). Using the nontransgenic mouse liver supernatant as a source of SSAT, we were not able to detect spermidine acetylation.

**Inhibition Studies.** We first evaluated the ability of amantadine to inhibit spermidine acetylation by including it with spermidine incubations. The addition of a therapeutic concentration of amantadine (2.5 μM) impeded the acetylation of spermidine, indicating it could serve as a substrate for the SSAT enzyme (Fig. 3). Subsequently, inhibition studies using fixed concentrations of spermidine (50 μM) and various concentrations of amantadine ranging from 1 μM to 10 mM were completed. Complete inhibition of the SSAT enzyme occurred by 10 mM amantadine. Amantadine inhibition profiles were used to determine IC_{50} values. Representative regressive probit plots of these data are shown in Fig. 4. From the IC_{50} values, the inhibitor dissociation constant (K_i) was calculated. Dixon and Cornish-Bowden analyses supported the interpretation that amantadine inhibition of spermidine acetylation was consistent with competitive inhibition (data not shown), and the use of IC_{50} values to calculate the K_i (Cheng and Prusoff, 1973). The IC_{50} and K_i values were 935 ± 191 and 738 ± 157 μM, respectively (n = 13).

Inhibition studies were undertaken to determine whether PABA and SMZ, selective substrates for NAT1 and NAT2, respectively, would inhibit SSAT acetylation of spermidine. SMZ demonstrated the ability to inhibit SSAT activity in a concentration-dependent manner (r^2 = 0.99, n = 4), which provides preliminary evidence that NAT2-selective substrates may also serve as substrate for SSAT (data not shown). However, the calculated K_i of 3.5 mM in this species suggests the effect will not be clinically relevant. In contrast, PABA, an NAT1-selective substrate, showed no SSAT inhibition (r^2 = 0.23, n = 5) (data not shown).

**Amantadine Acetylation.** Transgenic mouse liver supernatant, containing overexpressed SSAT and incubated with three concentrations of amantadine in the presence of an acetyl CoA-regenerating
system as the acetyl donor, produced modest amounts of acetylamantadine (Fig. 5) that did not increase with substrate concentration over the narrow range studied.

Discussion

This is the first demonstration of amantadine acetylation by SSAT, and indicates that SSAT may be a previously unrecognized drug-acetylating enzyme.

Our study demonstrated that acetylamantadine was excreted only in the urine of transgenic male mice overexpressing the SSAT enzyme. We have previously reported that male Sprague-Dawley rats also did not excrete acetylamantadine in their urine after the same therapeutic dose was administered (Goralski et al., 1999). These observations, together with the present finding that nontransgenic mice also fail to excrete acetylamantadine in their urine, strongly suggest that amantadine acetylation occurs only in the presence of increased levels of SSAT. The percentage of administered amantadine dose excreted as acetylamantadine by the transgenic mice in urine is in the range previously reported for humans (Köppel and Tenczer, 1985; Bras et al., 1998).

Mice that contain at least 20 copies of the SSAT transgene have basal activity in the liver that is 4-fold higher than nontransgenic mice (Pietilä et al., 1997), strongly implicating SSAT in the acetylation of amantadine, and possibly other primary amine-containing compounds. Under the experimental conditions described, treatment with MGBG did not show an increase in the percentage of acetylamantadine excreted in the urine of transgenic mice and did not result in detectable levels of acetylamantadine in the urine of the nontransgenic mice, although MGBG has been reported to be an inducer of SSAT in rat liver, increasing its activity by 7- to 700-fold (Karvonen and Pöösö, 1984; Persson and Pegg, 1984; Pegg et al., 1985). There are two possible explanations for our observation. First, mice may handle MGBG differently from rats. Second, by 24 h, up to three-quarters of MGBG is excreted by the mouse (Oliverio et al., 1963). The residual levels of MGBG may be reversed to a greater extent or more rapidly in the presence of spermidine than if there was depletion of spermidine (Porter et al., 1981). Previous attempts to induce SSAT in transgenic mice with MGBG have been unsuccessful (J. Jänne, unpublished observation).

Our in vitro data using transgenic mouse-derived liver supernatant support our in vivo observations and increase our belief that SSAT may be the enzyme that acetylates amantadine. This interpretation is further supported by our in vitro data, which demonstrated that neither NAT1 nor NAT2 could explain amantadine acetylation (Bras et al., 1998). Furthermore, amantadine is acetylated in modest amounts when it is incubated alone with transgenic mouse-derived liver supernatant, consistent with the observed modest amounts of acetylation occurring with amantadine when it is ingested by humans (Köppel and Tenczer, 1985; Bras et al., 1998). Our interpretation is further reinforced with respect to specificity, wherein the data indicate that amantadine may be a specific substrate for SSAT even though its capacity and affinity for the enzyme are rather limited.

In nontransgenic mouse-derived liver supernatant, no acetylation of spermidine was observed, probably due to the inherently low levels of SSAT. This observation is supported by previous observations where nontransgenic and noninduced rats did not produce detectable levels of acetylputrescine or acetylspermidine in liver supernatant (Seiler and al-Therib, 1974; Blankenship and Walle, 1977). Together, these data imply that in mammalian cells SSAT at higher than basal levels is required to demonstrate drug acetylation.

The small amount of metabolite that is excreted in urine after a dose of amantadine is reflected by the observed high $K_m$ and low $V_{max}$ values demonstrated in our in vitro studies. SSAT has been shown to acetylate other drugs that contain the substituted diaminopropane structure. Parry et al. (1995) showed that the antitumor and immunosuppressive agent 15-deoxyspergualin, a metabolite of amifostine, the radioprotective and chemoprotective agent S-2-(3-aminopropylamin-o)ethanethiol (WR-1065), and the spermine synthase inhibitor N-(n-
butyl-1,3-diaminopropane were acetylated in vitro by human SSAT expressed in Escherichia coli.

Interestingly, in our in vitro experiments, the prototypical NAT2 substrate SMZ, but not the NAT1 substrate PABA, was able to interact with SSAT in a concentration-dependent manner and inhibit spermidine acetylation. Although the concentrations used were several-fold higher than therapeutic doses, this observation suggests that substrates selective for NAT2 acetylation may interact with SSAT when it is induced by experimental or pathological processes.

SSAT appears to be ubiquitous in mammalian tissues, and it plays an important regulatory role in the cathodic pathway for maintaining spermidine and spermine homeostasis (Seiler, 1987). SSAT is normally present in very small amounts in the cell and is induced by a number of factors, including various toxic agents, hormones, drugs, growth factors, polyamines, and polyamine analogs (Casero and Pegg, 1993). When mammalian cells are induced with these factors, increased amounts of SSAT are present in the cell and may serve as a potential acetylator of primary amine-containing compounds and aryamine drugs.

Ethanol is known to increase basal levels of SSAT (Casero and Pegg, 1993; Perin and Sessa, 1993). Interestingly, Olsen and Mørland (1978) observed that slow and fast acetylator human volunteers exhibited enhanced acetylation of the NAT2 substrate sulfadimidine when they ingested alcohol concurrently with sulfadimidine. These authors also demonstrated the same phenomenon with procainamide when they ingested alcohol concurrently with sulfadimidine. These observations indicate a testable hypothesis that SSAT induction by ethanol explains increased acetylation of NAT2-selective substrates.

It is generally accepted that NATs are constitutive enzymes, and no induction is necessary to enhance the enzyme concentration before 100,000g tissue supernatant is used for in vitro experiments. SSAT, however, requires induction or overexpression from basal levels for sufficient concentrations to be present before the 100,000g supernatant is useful for in vitro experiments (Matsui and Pegg, 1980b; Seiler, 1987; Casero and Pegg, 1993; Pietilä et al., 1997). Cellular polyamine levels in several pathological states are increased along with increased levels of N′-acetylputrescine in the urine (Russell, 1971; Suh et al., 1997). Increased urine levels of N′-acetylserotonin suggest increased acetylation activity by SSAT enzyme. Interestingly, it has been demonstrated that NAT activity also increases in pathological states. These investigators showed that the growth of experimental transplantable tumors involved the intensification of acetylation of sulfadimidine; inhibition of the tumors caused a decreased rate of acetylation (Dilman et al., 1976). Furthermore, the level of N′-acetylation of sulfadimidine in cancer patients was relatively higher, both in rapid and slow acetylator phenotypes, than in the corresponding controls (Bulovskaya et al., 1978; Chekharina Ye et al., 1978). It has been assumed that the increased acetylation activity could be attributed to increased SSAT activity. Our data suggest that the increased acetylation seen in these pathological states may be a result of increased SSAT activity. Amantadine could be used to differentiate between acetylation by SSAT and NAT, since it is not subject to acetylation by NAT1 or NAT2 (Bras et al., 1998).

In conclusion, our results suggest that amantadine is a novel drug substrate that can be used to evaluate SSAT activity. Our results also suggest the potential for SSAT to contribute to acetylation of drugs that are substrates for NAT2. Further investigation of substrates that undergo acetylation remains to be completed to determine whether they either inhibit or undergo acetylation by SSAT. Since amantadine seems to be exclusively acetylated by SSAT, it may serve as a potential diagnostic tool for the presence of increased levels of this enzyme.

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