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Title page

Metabolism of Triethylenetetramine (TETA) and 1,12-diamino-3,6,9-triazadodecane (SpmTrien) by the Spermidine/spermine-N^1-acetyltransferase and Thialysine Acetyltransferase

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Running tile page

Running title: Metabolism of TETA and SpmTrien by SSAT1 and SSAT2

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Abbreviations

APAO, acetylpolyamine oxidase; DENSpm, N^1,N^{11}-diethylnorspermine; FBS, fetal bovine serum; MAT, N^1-AcTETA; Put, putrescine; Spd, spermidine; Spm, spermine; SpmTrien, 1,12-diamino-3,6,9-triazadodecane; SSAT1, spermidine/spermine-N^1-acetyltransferase; SSAT2, thialysine acetyltransferase; TETA, triethylenetetramine (Trientine, Syprine®)

ABSTRACT:

Triethylenetetramine (TETA), a drug for Wilson's disease, is a copper chelator and a charge-deficient analog of polyamine spermidine. We recently showed that TETA is metabolized *in vitro* by polyamine catabolic enzyme spermidine/spermine-N^1-acetyltransferase (SSAT1) and by thialysine acetyltransferase (SSAT2) to its monoacetylated derivative (MAT). The acetylation of TETA is increased in SSAT1-overexpressing mice as compared to wild-type mice. However, SSAT1-deficient mice metabolize TETA at the same rate as the wild-type mice, indicating the existence of another N-acetylase responsible for its metabolism in mice. Here, we show that siRNA-mediated knockdown of SSAT2 in HEPG2 cells and in primary hepatocytes from the SSAT1-deficient or wild-type mice reduced the metabolism of TETA to MAT. By contrast, 1,12-diamino-3,6,9-triazadodecane (SpmTrien), a charge-deficient spermine analog, was an extremely poor substrate of human recombinant SSAT2, and was metabolized by SSAT1 in HEPG2 cells and in wild-type primary hepatocytes. Thus, despite the similar structures of TETA and SpmTrien, SSAT2 is the main acetylator of TETA, whereas SpmTrien is primarily acetylated by SSAT1.

INTRODUCTION

Triethylenetetramine (TETA, Trientine, Supplemental Fig. 1) is an alternative drug for Wilson's disease if the patient becomes intolerant to the primary medication with penicillamine (Walshe, 1969). TETA is a very efficient copper chelator, and very well tolerated. However, it is rapidly metabolized to its mono- (MAT) and diacetylated (DAT) derivatives (Lu et al., 2007). Recently, TETA has also shown potency in the treatment of other diseases such as diabetes-associated end-organ damage (Cooper, 2011), cancer (Yin et al., 2003) and Alzheimer's disease (Cooper, 2011).

TETA resembles the biogenic polyamine spermidine (Spd) in structure (Supplemental Fig. 1), but possesses only about + 2 net charge at the physiological pH as compared with + 3 that of Spd (Bencini et al., 1999; Frassineti et al., 2003). The polyamines, Spd and spermine (Spm) are present at high concentrations in almost all cell types and are considered as mediators of important cellular functions such as proliferation, differentiation and tissue integrity (Pegg, 2009). We recently found that spermidine/spermine N^1-acetyltransferase (SSAT1), the key enzyme of polyamine catabolism, acetylates TETA *in vitro* and *in vivo* (Weisell et al., 2010; Cerrada-Gimenez et al., 2011). However, SSAT1-deficient (SSAT1-KO) mice metabolized TETA at the same rate as the wild-type mice did, indicating the presence of other enzyme(s) mainly responsible for its metabolism. Based on a databank/literature search, we found a structurally similar enzyme, thialysine acetyltransferase (SSAT2), which is encoded by a different gene than SSAT1 and metabolizes thialysine instead of polyamines (Coleman et al., 2004). Further assays with human recombinant enzyme showed that SSAT2 acetylated TETA at the same velocity as thialysine *in vitro* (Cerrada-Gimenez et al., 2011).

We report here that SSAT2 is the primary enzyme metabolizing TETA in mouse primary hepatocytes and in human HEPG2 cells, whereas the charge-deficient Spm analog, 1,12-diamino-3,6,9-triazadodecane (SpmTrien, Supplemental Fig. 1) (Khomutov et al., 2007) is metabolized by SSAT1.

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MATERIALS AND METHODS

Hepatocyte isolation and culture

Hepatocytes were isolated from SSAT1-KO (Niiranen et al., 2006) and wild-type mice by collagenase digestion (detailed protocol in Supplemental data). The cells were transfected with control or SSAT2 siRNA (100 nM; Sigma-Aldrich) using siPORT NeoFX (Ambion).

Lentiviral-mediated RNA interference in HEPG2 cells

The cells were cultured in DMEM containing 15 % fetal bovine serum (FBS) and 50 μg/ml gentamycin at + 37 °C 5 % CO_2. The cells were transduced with control, SSAT1 or SSAT2 shRNA lentiviral particles ($0.25x10^6$ IFU/6-well plate, Santa Cruz Biotechnology) and stable clones selected using puromycin hydrochloride (5 μg/ml, Sigma-Aldrich) for one week. The cells were plated onto 6-well plates and incubated overnight before treatments.

Recombinant protein assays

SpmTrien (100-5000 μM) was incubated with 10-100 ng of human recombinant SSAT1 or SSAT2 for 10 min at + 37 °C and analyzed as described previously (Weisell et al., 2010; Cerrada-Gimenez et al., 2011). The end products were also analyzed by HPLC (Hyvönen et al., 1992) (Detailed protocol in Supplemental data).

Analytical methods

Polyamines and SSAT1 activity were measured from cell lysates according to published methods (Libby, 1978; Hyvönen et al., 1992) (Detailed protocols in Supplemental data). The amount of DNA was measured using the PicoGreen reagent (Invitrogen). RNA was extracted using TRI

Reagent (Sigma Aldrich), Dnase-treated (DNA-free, Ambion) and reverse transcribed (High Capacity cDNA synthesis kit, Applied Biosystems). SSAT1, SSAT2 and 18S rRNA expression levels were measured from cDNA using DynaZyme PCR kit (Finnzymes). For statistical analysis, one-way ANOVA with Tuckey's post-hoc test was used with the aid of GraphPad Prism 5.03 (GraphPad Software Inc.).

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RESULTS AND DISCUSSION

Metabolism of SpmTrien by human recombinant SSAT2

Previously determined kinetic values for TETA with human recombinant SSAT2 are K_m of 2.5 ± 0.3 mM and V_{max} of 3.96 ± 0.15 µmol /min/mg, and with human SSAT1 K_m of 83 ± 7 µM and a V_{max} of 0.90 ± 0.02 µmol /min/mg (Cerrada-Gimenez et al., 2011). For SpmTrien, the kinetic values with mouse SSAT1 are K_m of 106 ± 6 µM and V_{max} of 1.17 ± 0.02 µmol /min/mg (Weisell et al., 2010). Here we tested SpmTrien as a substrate for human recombinant SSAT2 by using P81 disk method (Libby, 1978). It was a very poor substrate, as the incorporation of C^{14}-label to N-AcSpmTrien was <0.4 pmol/10 min/ng (vs. TETA to MAT 15 pmol/10 min/ng) when using 5 mM substrate concentration and 100 ng of the enzyme. As analyzed by the HPLC method (Hyvönen et al., 1992), the incubation of SpmTrien with SSAT1 yielded 12.8 nmol of N^1-AcSpmTrien and 4.2 nmol of N^{12}-AcSpmTrien when using 100 ng of enzyme with 1 mM SpmTrien for 50 min. Under the same conditions SSAT2 produced both N^1- and N^{12}-AcSpmTrien in trace quantities, yielding 0.65 nmol of N^1-AcSpmTrien and 1.1 nmol of N^{12}-AcSpmTrien. Thus, SSAT1 readily metabolized SpmTrien, preferring acetylation of the N^1-end (aminoethyl) of the molecule.

Metabolism of TETA and SpmTrien in mouse primary hepatocytes

We next investigated the metabolism of TETA and SpmTrien in primary hepatocytes isolated from SSAT1-KO (devoid of SSAT1 activity) or wild-type mice. The cells were treated with control or SSAT2 siRNA for 3d, yielding knockdown efficiency of ~50% in both genotypes (Fig 1A). As indicated in Fig. 1C, SSAT2 siRNA-treated cells showed reduced (~50%) metabolism of TETA (1 mM) to MAT as compared to control siRNA-treated cells, and the reduction correlated

with the knockdown efficiency, suggesting that SSAT2 is the main enzyme metabolizing TETA. By contrast, no metabolism of SpmTrien (100 μM) was detectable in SSAT1-KO cells. In wild-type cells, SSAT2 knockdown did not reduce the metabolism of SpmTrien (100 μM) to N^1-AcSpmTrien (Fig. 1D). In fact it led to the induction of SSAT1 activity (Fig. 1B) and increased metabolism of SpmTrien to N^1-AcSpmTrien (Fig. 1C).

Metabolism of TETA and SpmTrien in HEPG2 cells

The metabolism of TETA and SpmTrien was then investigated in human HEPG2 cells with functional SSAT1. Interestingly, HEPG2 cells showed apparently very low acetylpolyamine oxidase (APAO) activity, since large amounts of N^1-AcSpd and especially N^1-AcSpm, which is rarely detectable in cell samples, were found at the basal state (Supplemental Table 1). TETA was metabolized to MAT, and SpmTrien to N^1-AcSpmTrien, TETA and MAT, but at slower rate than in mouse primary hepatocytes. The metabolizing rates for TETA were 10 pmol MAT/h per μg DNA in HEPG2 cells vs. 40 pmol MAT/h per μg DNA in primary hepatocytes, and for SpmTrien 0.8 pmol N^1-AcSpmTrien/h per μg DNA in HEPG2 cells vs. 3.4 pmol N^1-AcSpmTrien /h per μg DNA in primary hepatocytes. When SSAT1 activity was induced by 24-h pretreatment with 50 μM N^1,N^{11}-diethylnorspermine (DENSpm; a N^1N^{11}-diethylated Spm analog which induces SSAT1 activity at all levels of gene expression (Pegg, 2008)) before the addition of TETA or SpmTrien, the amounts of intracellular MAT and N^1-AcSpmTrien, respectively, were increased (Fig 2), indicating that SSAT1 could metabolize both TETA and SpmTrien. In addition, SpmTrien's other metabolite, N^{12}-AcSpmTrien, became detectable in DENSpm-pretreated cells (data not shown).

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Next, the expression of SSAT1 or SSAT2 was knocked down in HEPG2 cells using stable lentiviral shRNA, which reduced SSAT1 and SSAT2 mRNA ~90% and ~35%, respectively, as compared to control siRNA (Fig. 3A). Like in primary hepatocytes, SSAT2 knockdown induced SSAT1 mRNA (Fig. 3A) and SSAT1 activity (Fig. 3B), but SSAT1 knockdown did not significantly affect SSAT2 mRNA level. SSAT1 knockdown led to a dramatic reduction of N^1-AcSpd and N^1-AcSpm pools, while SSAT2 knockdown did not affect acetylpolyamine levels (Fig. 3C). As indicated in Fig 3D, the metabolism of TETA to MAT was reduced with SSAT2 but not with SSAT1 shRNA. As expected, the metabolism of SpmTrien to N^1-AcSpmTrien was reduced with SSAT1 but not with SSAT2 shRNA (Fig. 3D).

Taken together, these data demonstrate that SSAT2 is the primary metabolizing enzyme for TETA, while SSAT1 is responsible for the metabolism of SpmTrien. However, SSAT1 can also contribute to the metabolism of TETA when it is induced at relatively high level, which occurs under various conditions such as inflammation and oxidative stress (Pegg, 2008). The cell culture data are in agreement with our previous findings where SSAT1-deficient mice metabolized TETA at the same rate as wild-type mice did, but SSAT1 overexpressing mice displayed increased acetylation (Cerrada-Gimenez et al., 2011). The higher cellular accumulation of SpmTrien and its metabolism to TETA may offer means to improve achieving the effective therapeutic level *in vivo* by using SpmTrien as a bioactive precursor of TETA. The suggested metabolic pathways of SpmTrien and TETA based on our current knowledge are summarized in (Supplemental Figure 2).

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Authorship contribution

Participated in research design: Hyvönen and Keinänen.

Conducted experiments: Hyvönen

Contributed to new reagents or analytic tool: Alhonen, Khomutov, Weisell, and Vepsäläinen.

Performed data analysis: Hyvönen and Keinänen.

Wrote or contributed to the writing of manuscript: Hyvönen, Alhonen, Khomutov, Weisell, Vepsäläinen and Keinänen.

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Footnotes

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[‡]The authors declare no competing financial interest

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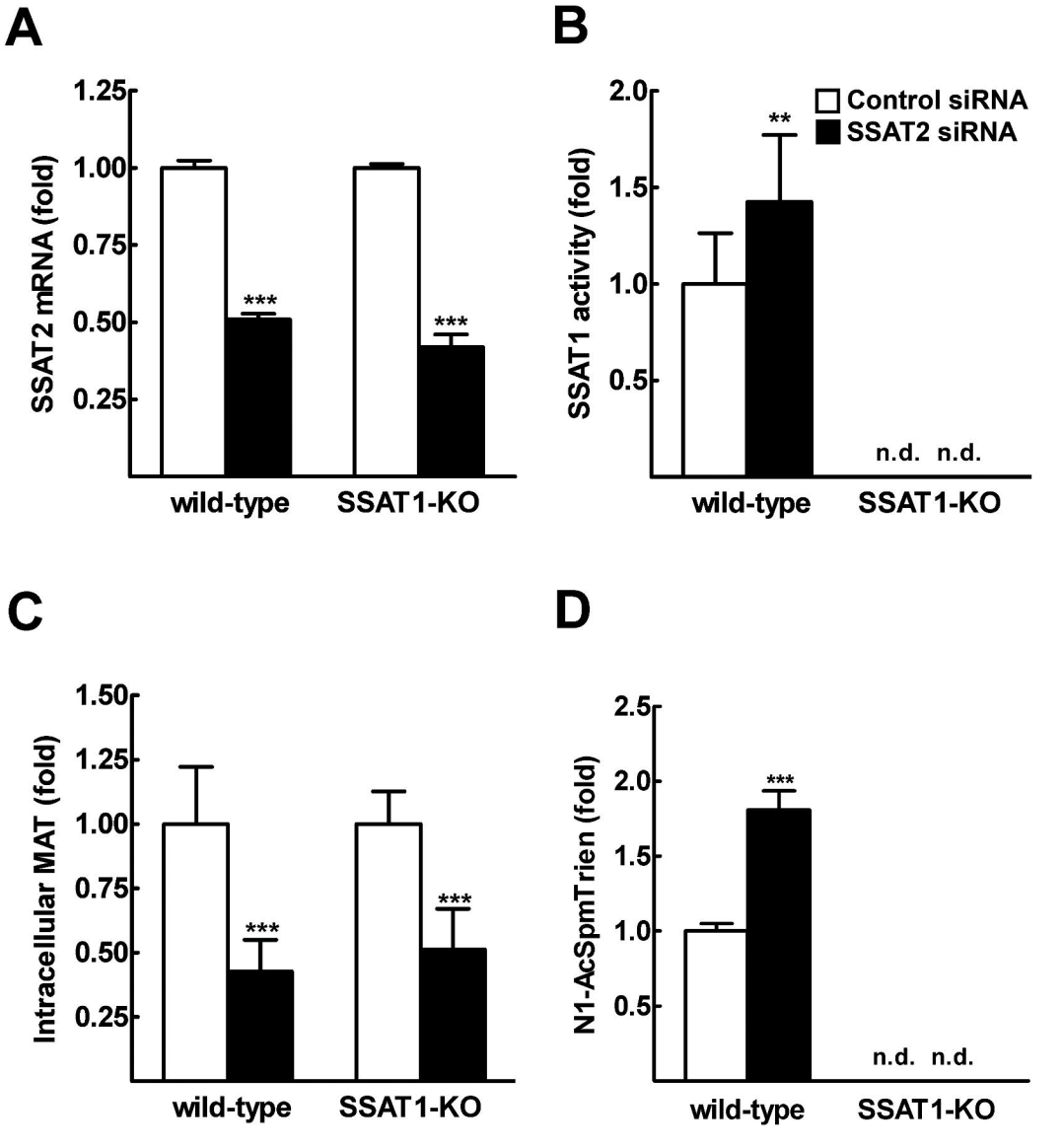
Figure legends

FIG. 1. SSAT2 knockdown in primary hepatocytes from wild-type and SSAT1-KO mice A) SSAT2 mRNA level. B) SSAT1 activity. Amounts of intracellular C) MAT and D) N^1-AcSpmTrien after 4 h incubation with 1 mM TETA or 100 μM SpmTrien, respectively. SSAT2 mRNA was knocked down by 3-d siRNA treatment. Data are means ± SD, n=3.

FIG. 2. Effect of SSAT1 induction on the metabolism of A) TETA and B) SpmTrien in HEPG2 cells. The cells were pretreated with 50 μM DENSpm or medium only for 24 h. Then, the plates were washed and cells incubated with 1 mM TETA or 100 μM SpmTrien for 24 h, and samples analyzed for metabolites. Data are means ± SD, n=3.

FIG.3. SSAT1 or SSAT2 knockdown in HEPG2 cells. A) SSAT1 and SSAT2 mRNA levels. B) SSAT1 activity. C) Amounts of intracellular N^1-AcSpd and N^1-AcSpm. D) Amounts of MAT and N^1-AcSpmTrien after 24 h of incubation with 1 mM TETA or 100 μM SpmTrien, respectively. SSAT1 and SSAT2 mRNA were knocked down with stable lentiviral shRNA. Data are means ± SD, n=3.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wild-type
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B
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Control siRNA
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n.d. n.d.
wild-type
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C
Intracellular MAT (fold)
1.50
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1.00
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***
wild-type
SSAT1-KO
D
N1-AcSpmTrien (fold)
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1.0
0.5
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n.d. n.d.
wild-type
SSAT1-KO

Figure 1

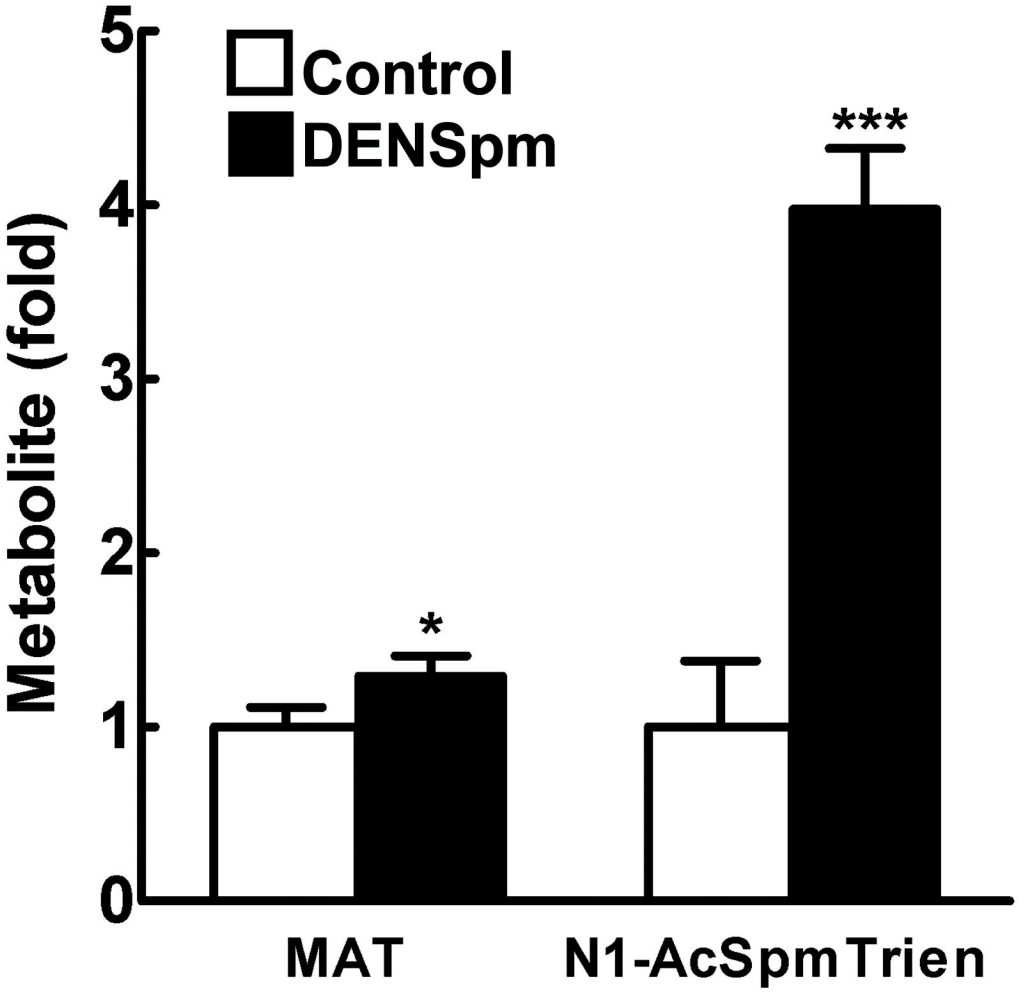
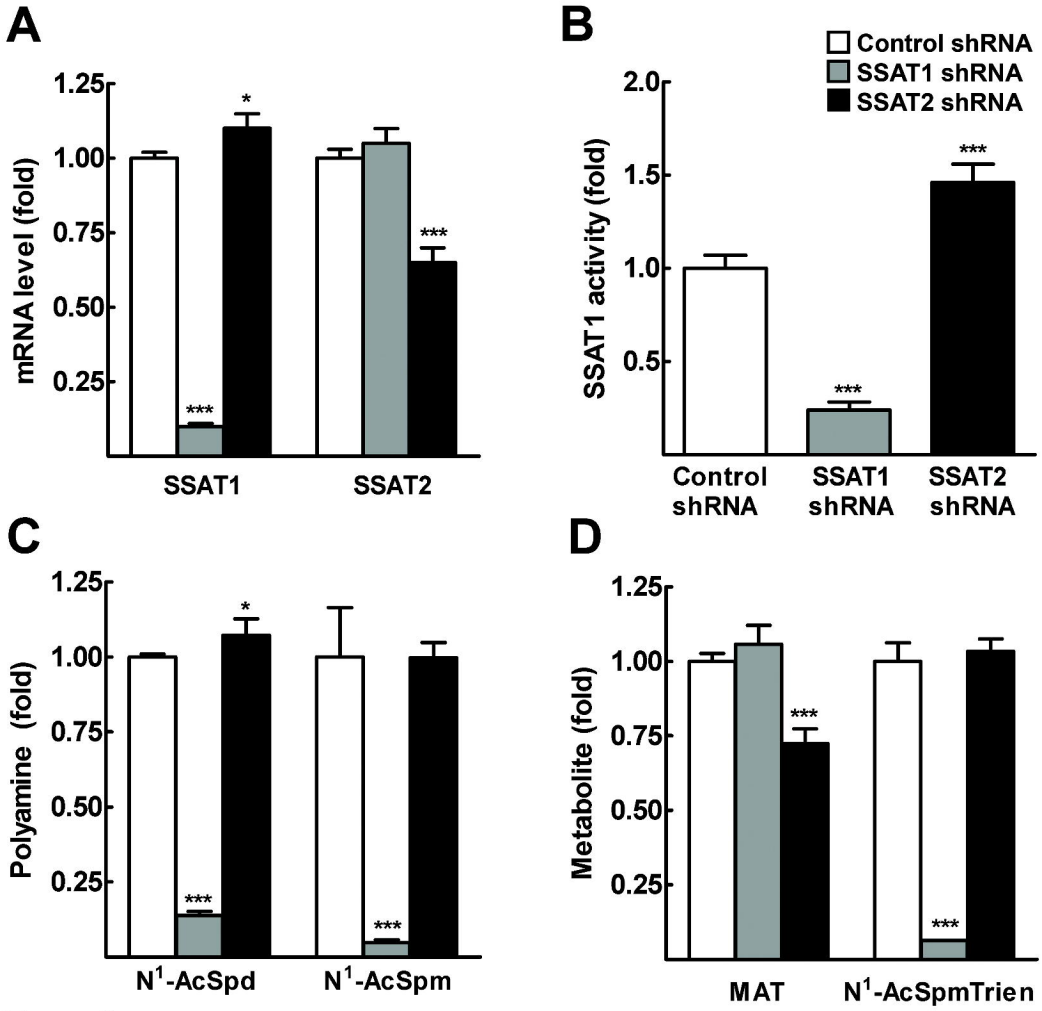


Figure 2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Polyamine (fold)
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N1-AcSpm
D
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0.75
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MAT
N1-AcSpmTrien

Figure 3

Metabolism of Triethylenetetramine (TETA) and 1,12-diamino-3,6,9-triazadodecane (SpmTrien) by the Spermidine/spermine-N^1-acetyltransferase and Thialysine Acetyltransferase

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Drug Metabolism and Disposition; Supplemental Data

MATERIALS AND METHODS

Hepatocyte isolation and culture

SSAT1-KO (Niiranen et al., 2006) and wid-type mice were euthanized, and circulation perfused with warm Krebs-Ringer buffer (4.76 g/l HEPES pH 7.4, 7.14 g/l NaCl, 0.42 g/l KCl, 2.1 g/l Na_2HPO_4 and 0.995 g/l D-glucose). The livers were perfused through portal vein with warm collagenase solution (100 U/ml collagenase type I (Sigma-Aldrich), 5 mM $CaCl_2$ and 0.8 mM $MgCl_2$ in Krebs-Ringer buffer), cut into small pieces, incubated for 5 min at + 37 °C 10 % CO_2 in collagenase solution and carefully suspended in 50 ml of washing medium (10 % fetal bovine serum (FBS) in Dulbecco's Modified Eagles medium (DMEM)). Suspensions were filtered through 100 μm filter and tubes centrifuged at 200 g for 10 min. The cell pellets were washed, resuspended in enriched medium (William's E containing 1 μM dexamethasone, 10 % FBS and 10 μg/ml bovine insulin) and plated onto 6-well plates coated with collagen I from rat tail (BD). Control siRNA or SSAT2 siRNA (100 nM; both from Sigma-Aldrich) were mixed with siPORT NeoFX transfection reagent (Ambion) according to manufacturer's instructions and pipetted to cell suspension. The plates were incubated overnight at + 37 °C 10 % CO_2, the medium was changed, and the plates were further incubated for 2 d. Then, the cells were treated with TETA (1 mM) or SpmTrien (100 μM) for 4 h whereafter the samples were collected for analyses.

Recombinant protein assays

Human recombinant SSAT1 and SSAT2 were a kind gift from Dr. Myung Hee Park, Oral and Pharyngeal Cancer Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, USA. SpmTrien (100-5000 μM) was incubated with 10-100 ng SSAT1 or SSAT2 for 10 min at + 37 °C and analyzed as described previously (Weisell et al., 2010; Cerrada-Gimenez et al., 2011). To determine the acetylated products, SpmTrien (1 mM) was incubated with 50-100 ng SSAT1 or SSAT2 for 10-50 min at + 37 °C in a buffer containing 100 mM Tris-HCl pH 7.8, 1 mM acetyl coenzyme A and 1 mM DTT in a total volume of 90 μl. Reaction was terminated by the addition of 10 μl 50 % sulphosalicylic acid containing 100 μM diaminoheptane, and the mixtures were analyzed by HPLC.

Analytical methods

Cell pellets (~$0.5x10^6$ cells) were resuspended in 300 μl of lysis buffer (25 mM Tris-HCl pH 7.4, 0.1 mM EDTA, 1 mM DTT, 0.1% Triton X-100 and 1 x Complete EDTA-free protease inhibitor cocktail (Roche Diagnostics)), and 90 μl of the lysate was taken for polyamine measurement. The remaining lysate was incubated for 15 min on ice, centrifuged for 15 min at 13000 rpm at +4°C, and the supernatant was used for SSAT activity measurement (Libby, 1978). Polyamines were measured with HPLC according to the published method (Hyvönen et al., 1992). The HPLC retention times (min) for the compounds were: putrescine 7.8, N^1-AcSpd 16.2, diaminoheptane 18.9, spermidine 20.4, MAT 20.6, N^1-AcSpm 21.7, TETA 22.1, N^{12}-AcSpmTrien 22.9, N^1-AcSpmTrien 23.3, spermine 23.7 and SpmTrien 24.4. To validate the observed peaks, selected set of HPLC samples were also spiked with the synthesized reference compound, because sample matrix changed the retention time of some analytes.

Acid-precipitated pellets were dissolved to 0.1 M NaOH, and the amount of DNA was measured using the PicoGreen reagent (Invitrogen) according to the manufacturer's instructions using dilutions of calf thymus DNA (Sigma-Aldrich) as the standards. RNA was extracted using TRI Reagent (Sigma Aldrich), Dnase-treated (DNA free, Ambion) and reverse transcribed (High Capacity cDNA synthesis kit, Applied Biosystems) according to the manufacturers' instructions. SSAT1, SSAT2 and 18S rRNA (control) expression levels were measured from cDNA using

TaqMan PCR system (Finnzymes), gels scanned with Typhoon 9400 Variable Mode Imager (GE Healthcare) and quantitated with QuantityOne software (BioRad).

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SUPPLEMENTAL TABLE 1. Metabolism of TETA (1 mM) and SpmTrien (100 μM) in HEPG2 cells (24h).

The HEPG2 cells were incubated with 1 mM TETA or 100 μM SpmTrien for 24 h, and samples analyzed for polyamines and metabolites.

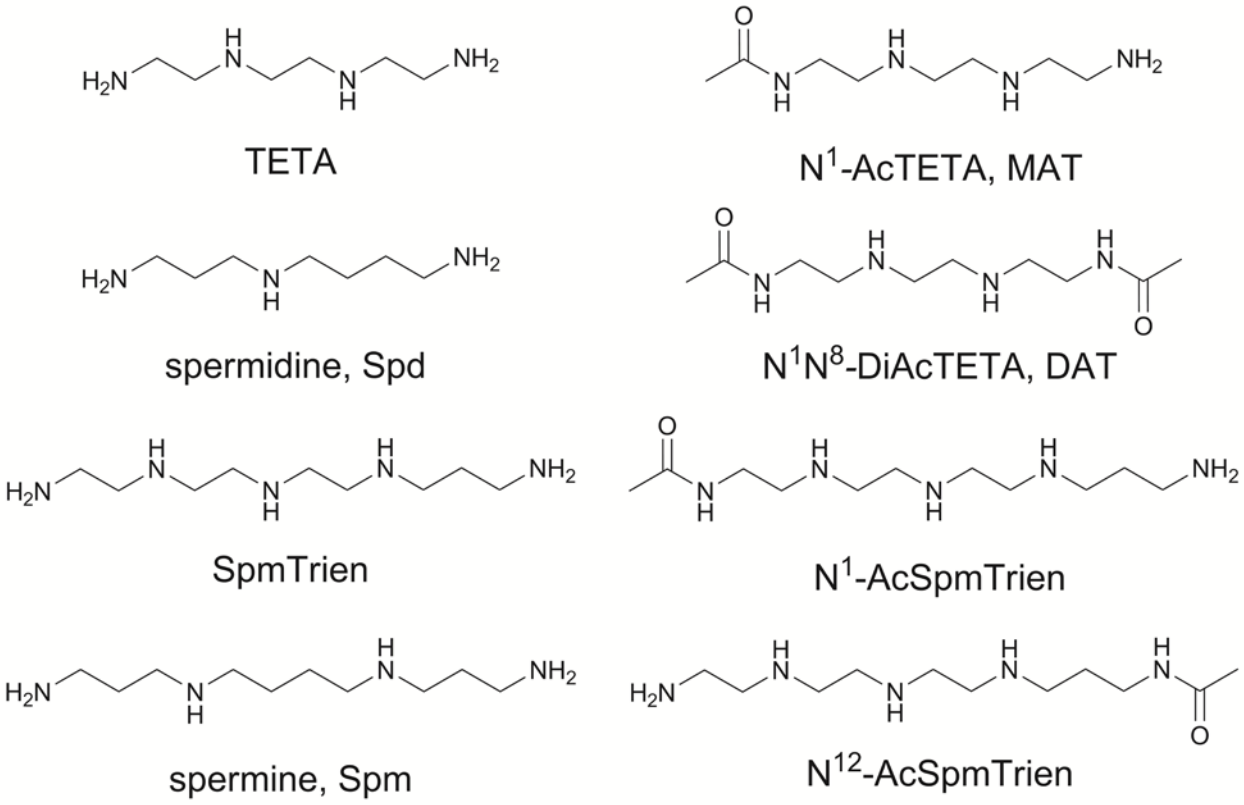
Treatment	**Put**	**Spd**	**Spm**	**N^1-AcSpd**	**N^1-AcSpm**	**TETA**	**MAT**	**SpmTrien**	**N^1-Ac-SpmTrien**[a]
	(pmol/μg DNA)								
Control	16 ± 1	185 ± 12	211 ± 10	33 ± 2	4 ± 0				
TETA	16 ± 3	122 ± 3***	323 ± 40***	63 ± 3***	16 ± 2***	85 ± 11	71 ± 17		
SpmTrien	27 ± 6***	131 ± 28***	119 ± 29***	106 ± 22***	18 ± 4***	8 ± 2	42 ± 7	203 ± 41	6 ± 0

[a] N^{12}-AcSpmTrien was not detectable.

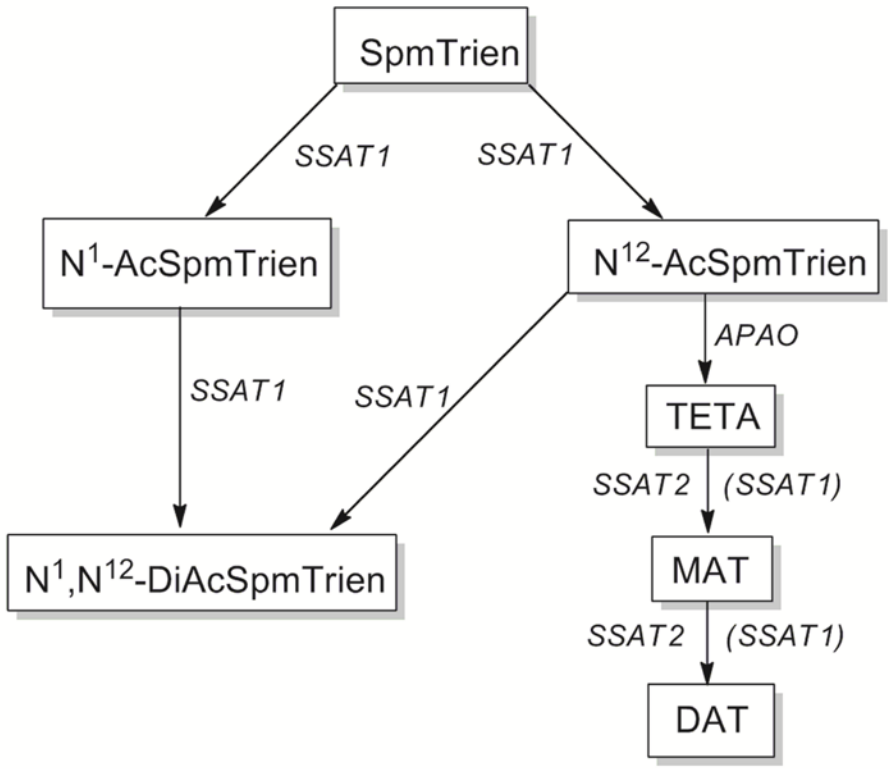
Data are means ± SD, n=3.

***, statistical significance $p<0.001$.

SUPPLEMENTAL FIGURES



Supplemental Fig. 1. Structures of TETA, SpmTrien, their N-acetylated metabolites and the natural polyamines spermidine and spermine.



Supplemental Fig. 2. Suggested metabolic pathways of TETA and SpmTrien in hepatic cells.