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An (*R*)-specific *N*-methyltransferase involved in human morphine biosynthesis

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Abstract

The biosynthesis of morphine, a stereochemically complex alkaloid, has been shown to occur in plants and animals. A search in the human genome for methyltransferases capable of catalyzing the *N*-methylation of benzyloquinoline alkaloids, as biosynthetic precursors of morphine, yielded two enzymes, PNMT (E.C. 2.1.1.28) and NMT (E.C. 2.1.1.49). Introduction of an N-terminal poly-histidine tag enabled purification of both proteins by immobilized metal affinity chromatography. Recombinant PNMT and NMT were characterized for their catalytic activity towards four benzyloquinolines: tetrahydropapaveroline (THP), 6-*O*-methyl-THP, 4'-*O*-methyl-THP and norreticuline. Human PNMT accepted none of the offered alkaloids and was only active with its established substrate, phenylethanolamine. The second enzyme, human NMT, converted all four benzyloquinolines, however, with a strict preference for (*R*)-configured morphine precursors. Determination of kinetic parameters of NMT for the four (*R*)-configured benzyloquinoline alkaloids by LC-MS/MS revealed (*R*)-norreticuline to be the best substrate with an even higher catalytic activity as compared to the previously reported natural substrate tryptamine. In addition, isolation of the morphine precursor salutaridine from urine of mice injected (i.p.) with (*R*)-THP provides new evidence that the initial steps of morphine biosynthesis in mammals occur stereochemically and sequentially differently than in plants and suggests an involvement of the herein characterized (*R*)-specific NMT.

Keywords

Human *N*-methyltransferase; Benzyloquinoline alkaloids; Morphine biosynthesis; Urinary excretion; LC-MS analysis

Introduction

Methyltransferases are ubiquitous enzymes with wide ranging functional roles. Among others, they are involved in the synthesis of small molecules such as antibiotics in microorganisms [1], neurotransmitters in animals [2] or alkaloids in plants [3]. The alkaloid morphine, a highly chemically condensed benzyloquinoline (BIQ) with five asymmetric

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centers (Fig. 1), requires up to three methylations that all occur *in planta* exclusively on (*S*)-configured substrates. It is notable however, that only the 9-(*R*)-configured morphine is highly pharmacologically active, while the 9-(*S*)-configured morphine is without biological activity [4].

In the biosynthetic formation of morphine, it has to take into consideration that morphine and its close precursors are (*R*)-configured at carbon atom 9. In plants, the vast multitude of isoquinoline alkaloids found are (*S*)-configured [5–7], except for very rare alkaloid members that are converted from (*S*) to (*R*) during their biosynthetic course [8]. This conversion takes place as shown in Fig. 2. (*S*)-Reticuline is dehydrogenated to form the quaternary tetrahydrobenzylisoquinoline alkaloid 1,2-dehydroreticuline [8,9] and subsequently stereoselectively reduced at the expense of NADPH to yield (*R*)-reticuline, an irreversible reaction [10].

Morphine occurs in substantial amounts only in one single plant species, in *Papaver somniferum* the opium poppy, while in certain mammals it seems to occur widespread but in very small quantities [11–15]. Recently, the capability of living animals to form morphine *de novo* was elucidated [16]. The biosynthesis of morphine in mammals has been suggested to start with tetrahydropapaveroline (THP), a simple BIQ alkaloid, yielding the first morphinan alkaloid salutaridine by four enzymatic reactions: two *O*-methylation reactions at C6, C4', one *N*-methylation and one phenol coupling reaction. However, it is not known if these initial steps occur with (*S*)-configured substrates as has been shown for the plant pathway.

It has been found in mammals that catechol *O*-methyltransferase methylates simple BIQ alkaloids predominantly at the hydroxyl group at position C6 [17]. Cashaw et al. [18] described also the detection of 4'-*O*-methyl-THP after intracerebroventricular administration of THP into rats. We reported recently on two human cytochrome P450 (CYP) enzymes, CYP 2D6 and CYP 3A4, as catalysts of the oxidative *C*-*C* phenol-coupling reaction of (*R*)-reticuline to salutaridine [19]. A mammalian enzyme capable of catalyzing the *N*-methylation of BIQ alkaloids to yield (*R*)-reticuline has not yet been reported. The present study was thus directed towards the identification and characterization of such a human *N*-methyltransferase.

The *N*-methylation of tetrahydroisoquinoline alkaloids, which are structurally similar to BIQ alkaloids, has been previously shown for the synthesis of *N*-methyl-(*R*)-salsolinol from (*R*)-salsolinol in humans [20–23]. In addition, two rabbit and bovine amine methyltransferases, A and B, have been isolated [24,25]. Distinct pH optima and catalytic activities were determined for both isoforms and upon comparison, isoform A was characterized by a basic pH optimum with a greater catalytic activity for tetrahydroisoquinoline alkaloids [24,25]. Information on the encoding gene sequences and on catalytic properties towards BIQ alkaloids of these *N*-methyltransferases, however, were missing.

Our search in the database of the human genome resulted in two promising candidates for an enzyme involved in the *N*-methylation of BIQ alkaloids: phenylethanolamine *N*-methyltransferase (EC 2.1.1.28, PNMT) and indolethylamine *N*-methyltransferase (EC 2.1.1.49, NMT). While PNMT is involved in the final step of the biosynthesis of the neurotransmitter adrenaline from noradrenaline [2,26,27], a functional role for NMT has not yet been assigned [28,29]. Both human *N*-methyltransferase-encoding cDNAs, *PNMT* and *NMT*, were heterologously expressed in *Escherichia coli* in order to determine the catalytic activity of the recombinant enzymes with BIQ alkaloids.

Material and methods

Alkaloids

Alkaloidal substrates, except (*R*)- and (*S*)-THP, were from our laboratory collection. (*R*)- and (*S*)-THP were synthesized according to [30] from (*R*)- and (*S*)-norreticuline, respectively. The reaction product obtained from [30] was dried for further purification under a stream of nitrogen, resuspended in 50% methanol containing 0.2% acetic acid and subjected to HPLC equipped with a L-7100 HPLC pump, L-7200 autosampler and L-7450 diode array detector (Merck Hitachi). Separation of (400 μ l) samples was achieved using a Hibar Pre-Packed column RT250-25 (Merck, 7 μ m, LiChrosorb RP-18). The mobile phase total flow was set to 8 ml/min with binary gradient elution, using 0.1% TFA (solvent A) and acetonitrile (solvent B). The gradient started with 10% B and was increased to 30% B over 60 min. Fractions containing THP were pooled, concentrated, shock-frozen in liquid nitrogen and lyophilized. THP trifluoroacetate was obtained in 77% yield and purified further *via* thin layer chromatography (TLC) in butanol/water/acetic acid (4:1:1). The UV-absorbing band corresponding to THP was eluted with methanol and filtered through a 0.2 μ m filter to remove remaining silica residue. After excess methanol was evaporated, THP was reconstituted in 24% HBr and subsequently lyophilized. THP hydrobromide was obtained in 64% yield.

Cloning into pET28a and heterologous expression of his-tagged PNMT and NMT cDNA

Stab cultures of PNMT and NMT were obtained from the American Type Culture Collection (clone MGC-26382) and Deutsches Ressourcenzentrum für Genomforschung (clone BC106902.1), respectively. Plasmid DNA was prepared according to the protocol of QIAprep Spin Miniprep Kit (Qiagen). A standard PCR-reaction was used to introduce *Nde*I and *Bam*HI restriction sites for PNMT or *Nhe*I and *Xho*I restriction sites for NMT at the 5'- and 3'-end of each cDNA inserted into pCR BluntII-TOPO. The corresponding primers are shown in Table 1. The cDNA for PNMT or NMT was inserted into pET28a (Novagen) followed by transformation into *E. coli* Plus KJ (Expression Technologies Inc). This cloning strategy resulted in recombinant proteins containing a His-tag sequence located at the N-terminus of the complete amino acid sequence of PNMT (Met1-Leu282, N-terminal His-tag sequence MGSSHHHHHHSSGLVPRGSHM, 33 kDa) and of NMT (Met1-Phe263, N-terminal His-tag sequence MGSSHHHHHHSSGLVPRGSHMAS, 31 kDa). Expression of cDNA was induced by adding isopropyl β -D-1-thiogalactopyranoside (final concentration 1 mM). After incubation for 16 h at 28°C, the one-liter-culture was centrifuged and the bacterial pellet was resuspended in 50 mM Tris/HCl, pH 7.5, containing 500 mM NaCl, 10% (v/v) glycerol, 2.5 mM imidazole and 5 mM β -mercaptoethanol (His-tag buffer). Addition of 1 mg/ml lysozyme was followed by a 40 min-incubation at 4°C. Cells were disrupted by sonication and centrifuged. The supernatant was incubated with Talon resin (Clontech) for 40 min at 4°C. Unbound protein was discarded, protein bound to the resin was transferred to a disposable gravity column (Clontech) and left to settle for 30 min. Bound protein was eluted with His-tag buffer containing 150 mM imidazole. Eluted His-tag protein was desalted on a PD10 column (GE Healthcare) with 20 mM Tris/HCl pH 7.5 containing 150 mM NaCl, 10% (v/v) glycerol and 5 mM β -mercaptoethanol.

Thrombin digestion of His-tagged protein

All steps were conducted at 4°C. Digestion was started by adding 0.5 μ l of thrombin solution (Sigma Aldrich, 1 unit/ml) to 1 mg of His-tagged protein (1/6400 the amount of NMT by weight, [31]). The reaction mixture was transferred into the dialysis membrane (VISKING Typ 1 7/8 ss, Roth, cat. 5358.1, molecular weight cut-off of 14000 kDa) that had been previously washed with 1 l MilliQ water for 2 $\times$ 30 min. After dialysis for 2 $\times$ 12 h in 1 l of 20 mM Tris/HCl pH 7.5 containing 150 mM NaCl, 10% (v/v) glycerol and 5 mM β -

mercaptoethanol, the dialyzed sample was transferred into an Eppendorf tube and a resin mixture consisting of 100 μ l Talon (Clontech) and 80 μ l Benzamidine Sepharose (GE Healthcare) was added to the dialyzed sample. After an incubation time of 20 min, the mixture was centrifuged at $700 \times g$ for 5 min and thrombin-digested protein was recovered in the supernatant. After thrombin digestion, the N-terminal fragments GSHM and GSHMAS remained at PNMT and NMT, respectively.

Enzyme assays

PNMT enzyme assays utilizing 5 μ M [14 C-methyl]-S-adenosyl-L-methionine ([14 C-methyl]-SAM) (0.025 μ Ci, 50000 cpm) were conducted according to [32] in a total volume of 100 μ l containing 100 mM potassium phosphate buffer (pH 8.0), 62.5 μ M PNMT and 200 μ M phenylethanolamine or BIQ alkaloids. NMT enzyme assays utilizing 5 μ M [14 C-methyl]-SAM (0.025 μ Ci, 50000 cpm) were conducted according to [24] in a total volume of 100 μ l containing 50 mM Tris/HCl (pH 8.5), 62.5 μ M NMT and 2 mM tryptamine or BIQ alkaloids. Reaction mixtures were incubated for 2 h at 37 $^{\circ}$ C. For identification of products, reaction mixtures were separated by TLC either in butanol/acetic acid/water, 4:1:1, v/v/v, or chloroform/ethylacetate/ethanol/ NH_4OH , 6:1:2:1, v/v/v/v. Radioactive bands were detected by phosphorimager with a Typhoon 9410 (Molecular Dynamics). Standard reactions for kinetic data analysis for NMT were conducted in a total volume of 100 μ l containing 50 mM phosphate buffer (pH 7.4), 62.5 μ M NMT, 5–50 μ M SAM and 250–2500 μ M BIQ alkaloids. Reactions were incubated for 10 min at 37 $^{\circ}$ C, terminated by addition of 10 μ l 10% trichloroacetic acid and subjected to LC-MS/MS. Kinetic parameters were estimated by non-linear regression with GraphPad Prism. For pH profile analysis enzymatic assays were conducted as described above in a reaction mixture containing 50 mM of the following buffer: sodium citrate pH (4–6), potassium phosphate (pH 6–8), Tris/HCl (pH 8–9) and glycine/NaOH (pH 9–10.5).

LC-MS/MS analysis

The system consisted of a CTC Pal autosampler (LEAP Technologies), a Shimadzu LC-20AD liquid chromatograph and a 4000 QTRAP mass spectrometer (Applied Biosystems). The following TIS source parameters were used: CUR 30, CAD high, IS 5000, TEM 500, EP 10, CXP 17, dwell time 50 ms. Chiral separation of (10 μ l) samples was achieved by using a Chiral-CBH HPLC column (Chromtech, 100 $\times$ 4.0 mm) combined with a Chiral-CBH guard cartridge (Chromtech, 10 $\times$ 4 mm). The mobile phase total flow was set to 0.9 ml/min with isocratic elution using 5% acetonitrile, 10 mM ammonium acetate pH 5.5. Between each run the column was regenerated with 5% acetonitrile, 10 mM ammonium acetate pH 5.5 containing 50 μ M EDTA (with diverter set to waste). Separation of standard reactions for kinetic data analysis was achieved by using an Eclipse XDB-C18 HPLC column (Agilent, 2.1 $\times$ 150 mm, 3.5 μ m) combined with a microfilter unit (Sigma, 1/16 OD tubing). The mobile phase total flow was set to 0.3 ml/min with binary gradient elution, using 0.1% formic acid (solvent A) and acetonitrile (solvent B). The gradient started with 95% A for 2 min and was increased to 100% B over 6 min. Elution was continued for 4 min at 100% B followed by a 5-min equilibration with starting conditions. Compound specific parameters for the LC-MS/MS analysis of BIQ alkaloids are shown in Table 2.

Injection (i.p.) of (R)-THP into mice and urine analysis

Administration of (R)-THP*HBr was conducted as previously described [16]. Briefly, a sterile solution containing (R)-THP*HBr was injected (i.p.) into each mouse every 24 h for 4 days resulting in a total of 6.1 μ mol injected alkaloid per mouse. Urine obtained from the mice during the 4 days of the injection (i.p.) experiment was combined, subjected to solid phase extraction with Bond Elut Certify (Varian) and analyzed by high resolution LC-MS (HR-LC-MS) with an LTQ-Orbitrap Spectrometer (Thermo Fisher, USA).

Results

Heterologous expression of PNMT and NMT

Comparison of the deduced amino acid sequence of PNMT revealed 100% identity to the published sequence [33]. For NMT, however, three amino acids were found to differ, which can be explained by single nucleotide polymorphisms leading to DNA variations [28]. *PNMT* and *NMT* were functionally expressed in *E.coli* and both His-tagged proteins were purified to electrophoretic homogeneity by Talon affinity chromatography (Fig. 3).

Enzyme activity of PNMT and NMT with benzyloquinoline alkaloids

Enzyme activity was first analyzed by radio-TLC. Enzyme assays of His-tagged PNMT and NMT with phenylethanolamine and tryptamine, respectively, in the presence of [^{14}C -methyl]-SAM confirmed the activity of both enzymes. Specific activities were determined as 17.1 pmol/min/mg protein for PNMT and 13.7 pmol/min/mg protein for NMT in a 2 h reaction. The active enzymes were subsequently incubated under the same conditions with the following BIQ alkaloids: THP, 6-*O*-methyl-THP, 4'-*O*-methyl-THP and norreticuline. Each of the four BIQ alkaloids were provided as either the (*R*)- or (*S*)-configured enantiomer resulting in a total of eight different alkaloidal structures that were offered as substrate (Fig. 4). The chiral purity of these eight different BIQ alkaloids was verified by chiral LC-MS/MS analysis.

While PNMT was inactive with any of the eight enantiomerically pure BIQ alkaloids, the second human *N*-methyltransferase, NMT, was highly active with a specific activity of 0.5–5.6 pmol/min/mg protein depending on the BIQ alkaloid substrate (Table 3). However, NMT activity was strictly limited to (*R*)-configured BIQ alkaloids. This stereospecificity has never been observed for NMT and was consistent with all four (*R*)-configured BIQ alkaloids offered as substrate. The same incubations were subsequently repeated with both recombinant proteins missing the polyhistidine extension to exclude a possible interference of the tag with enzyme activity. PNMT without a His-tag lacked activity for BIQ alkaloids while NMT without a His-tag showed an absolute (*R*)-specificity for BIQ alkaloids with a specific activity of 7.7 pmol/min/mg protein for (*R*)-norreticuline compared to 13.8 pmol/min/mg protein for tryptamine in a 2 h reaction.

Catalytic properties of NMT as determined by LC-MS/MS

LC-MS/MS in multiple reaction monitoring (MRM) mode was chosen to further analyze with greater sensitivity and accuracy the pH profile for NMT. The clear benefit of the MRM mode is the high selectivity for the analyte of interest resulting in a reduction of interference with isobaric masses and an increase of the signal-to-noise ratio. The limit of detection (LOD) was defined as greater than 10 times the signal-to-noise ratio of the analyte peak. An LOD of 71 fmol was determined for the reaction products laudanosoline, 6-*O*-methyllaudanosoline, 4'-*O*-methyllaudanosoline and reticuline. The LC-MS/MS analysis of incubation mixtures with (*R*)-norreticuline as substrate revealed a basic pH optimum of pH 8.5–9 for NMT (Fig. 5).

Kinetic parameters were derived by non-linear regression and are summarized in Table 4. Catalytic efficiencies for each substrate, calculated from the estimated Michaelis-Menten parameters (K_m and k_{cat}), indicate that (*R*)-norreticuline followed by (*R*)-4'-*O*-methyl-THP are the preferred substrates for human NMT. In addition, K_m and k_{cat} for the co-substrate SAM were determined in incubations with (*R*)-norreticuline as substrate. An apparent K_m of 6.9 μM and k_{cat} of 1.1 nmol/min/mg were estimated by non-linear regression for the co-substrate SAM in incubations with (*R*)-norreticuline as substrate. These values are similar to those reported by Thompson et al. [28] for SAM in incubations with tryptamine as substrate.

Conversion of (R)-THP in vivo

Since NMT exclusively accepts (*R*)-configured BIQ alkaloids, the possibility of a potential precursor function of (*R*)-THP in mammalian morphine biosynthesis was explored. Provided that an injection (i.p.) of racemic THP yields the morphine precursor salutaridine [16], injection of (*R*)-THP should likewise result in the urinary excretion of the phenol-coupled product if the initial steps of the biosynthesis of morphine in animals involves (*R*)-configured intermediates. Indeed, after injection (i.p.) of (*R*)-THP, the presence of the morphinan alkaloid salutaridine in a concentration of 3 pmol/ml urine (0.0001 % of the administered dose) was unequivocally confirmed by retention time and HR-MS upon comparison with a salutaridine standard (Fig. 6). The morphine precursor was not found in control urine obtained from untreated animals. The presence of salutaridine in the urine of mice injected (i.p.) with (*R*)-THP correlates with the stereospecific preference of human NMT.

Discussion

Methylation is a common reaction occurring during the biosynthesis of a variety of small molecules. The formation of morphine in mammals requires the incorporation of three methyl groups to produce the BIQ alkaloid (*R*)-reticuline. In search for a human enzyme capable of catalyzing the *N*-methylation of BIQ alkaloids, we found two *N*-methyltransferases, NMT and PNMT, in the human genome and characterized them for their catalytic properties towards (*R*)- or (*S*)-configured BIQ alkaloids. We determined that none of the potential mammalian morphine precursors were accepted by human PNMT. However, the second enzyme, human NMT, was capable of *N*-methylating (*R*)-configured BIQ alkaloids.

(*R*)-Specific *N*-methylation of tetrahydroisoquinolines, which are structurally closely related to BIQ alkaloids, was previously described in humans [20–23]. Two pH optima were found for this enzyme activity at pH 7.0 and 8.5, but only the activity of the neutral *N*-methyltransferase raised interest due to its suggested involvement in the pathogenesis of Parkinson's disease [20,22,23]. Since information on the sequence encoding this *N*-methyltransferase was missing, it was not clear whether the herein described enzyme is identical to previously reported *N*-methyltransferases [23]. However, the neutral pH optimum and a molecular weight of 35 kDa for the enzyme isolated from the cytosolic fraction of human brain gray matter [34] suggests that an isoform has been characterized by Naoi and co-workers that is different from the herein described *N*-methyltransferase.

Another bovine *N*-methyltransferase exhibiting a basic pH optimum was isolated and tested with (*R*)- and (*S*)-configured tetrahydroisoquinoline alkaloids as substrates. This methyltransferase appeared to be isoform A of bovine NMT, however, information on the encoding sequence was not provided. The enzyme was shown to be active with both enantiomers of tetrahydroisoquinoline alkaloids [25]. Reduced stereospecificity was determined for the bovine isoform in such that (*R*)-isosalsoline was methylated three times faster than the (*S*)-enantiomer. The activity for (*R*)-salsolidine, however, was about 30 x lower than that for (*S*)-salsolidine (Fig. 7). If this bovine isoform A is indeed an ortholog of NMT, our results suggest that an addition of a benzyl ring to the tetrahydroisoquinoline molecule may lead to a shift of enzyme activity from relaxed to strict stereospecificity. A change in catalytic activity due to larger substrate size is also indicated in enzymatic studies with the rabbit ortholog showing that the more bulky substrate α -*N*-methyltryptamine is preferred over tryptamine [29].

We determined for NMT and the best BIQ substrate, (*R*)-norreticuline, an apparent K_m of 0.9 mM, which is three times lower than the one described for tryptamine, the previously

characterized substrate of NMT [28]. This newly observed characteristic of an enzyme, for which a role in human physiology has not yet been assigned, to accept only one enantiomer of a precursor of mammalian morphine biosynthesis may suggest a new *in vivo* function of NMT. We investigated this hypothesis further by injecting (i.p.) (*R*)-THP into mice with subsequent urine analysis by HR-LC-MS. In a previous study, salutaridine was found in urine of mice after injection (i.p.) of racemic, deuterium labeled THP [16]. We confirm now that salutaridine is also excreted by mice injected (i.p.) with the enantiomer (*R*)-THP. This finding provides conclusive evidence that the living animal is capable of forming morphine precursors from (*R*)-configured BIQ alkaloids and suggests that the initial steps as well as the enzymatic sequence yielding (*R*)-reticuline may proceed stereochemically differently than in the plant pathway.

The results for the *in vivo* conversion of (*R*)-THP in animals match with the newly found strict preference of human NMT for (*R*)-configured BIQ alkaloids thus allowing us to predict a possible involvement of the enzyme in human morphine biosynthesis. In addition, it suggests that all three methylation steps in mammalian morphine biosynthesis yielding reticuline might proceed with (*R*)-configured substrates. Considering that (*R*)-norreticuline is the best substrate for human NMT, the two *O*-methylation at C6 and C4' are very likely to occur before the *N*-methylation. In mammals, the methylation of (*R*)-norreticuline to (*R*)-reticuline could therefore be the final methylation step occurring before the phenol coupling of (*R*)-reticuline to salutaridine, which is then further converted to morphine (Fig. 8).

A high activity and stereospecificity of human NMT for BIQ alkaloids has been shown in the present study while the second *N*-methyltransferase investigated, human PNMT, was completely inactive. This is the first time that an (*R*)-specific enzymatic activity is reported for human NMT for which a role in morphine biosynthesis has been indirectly proven herein by the detection of the morphine precursor salutaridine in urine of mice injected (i.p.) with (*R*)-THP. Future studies involving X-ray crystallography of NMT in complex with BIQ substrates as well as NMT knockout mice will shed light on enzyme-substrate interactions and further aid our understanding of the involvement of NMT in morphine biosynthesis.

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Abbreviations used

BIQ	benzylisoquinoline
CYP	cytochrome P450
NMT	amine <i>N</i> -methyltransferase
PNMT	phenylethanolamine <i>N</i> -methyltransferase
THP	tetrahydropapaveroline

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Fig. 1.
Molecular structure and absolute configuration of morphine.

Fig. 2. (S)-Reticuline is converted to (R)-reticuline *via* formation of 1,2-dehydroreticuline during the biosynthesis of morphine in plants.

Fig. 3.
SDS-PAGE of His-tagged human PNMT and human NMT produced in *E. coli*.

THBIQ	R ₁	R ₂
THP	OH	OH
6- <i>O</i> -methyl-THP	CH ₃	OH
4- <i>O</i> -methyl-THP	OH	CH ₃
Norreticuline	CH ₃	CH ₃

Fig. 4. (*R*)- and (*S*)-BIQ alkaloids tested as possible substrates for human PNMT and NMT. Enzyme activity was analyzed in reaction mixtures containing 200–2000 μ M BIQ alkaloids incubated with 5 μ M [¹⁴C-methyl]-SAM (0.025 μ Ci, 50000 cpm) and 52.5 μ M PNMT in 100 mM potassium phosphate buffer (pH 8.0) or 62.5 μ M NMT in 50 mM Tris/HCl (pH 8.5) for 2 h at 37 °C.

Fig. 5. pH dependence for the methylation of (*R*)-norreticuline by human NMT. Enzyme activity was analyzed in 100 mM buffer containing 1 mM (*R*)-norreticuline, 50 μ M SAM and 62.5 μ M NMT for 10 min at 37 $^{\circ}$ C. Following buffer systems were used: sodium citrate (pH 4–6), potassium phosphate (pH 6–8), Tris/HCl (pH 8–9) and glycine/NaOH (pH 9–10.5).

Fig. 6.

Salutaridine is detected in urine of mice injected (i.p.) with (R)-THP (6.1 μ mol (R)-THP per mouse in 4 days). (A) Full scan mass spectrum of salutaridine standard. (B) High resolution mass spectrum of salutaridine standard. (C) Full scan mass spectrum of salutaridine detected in urine of mice injected (i.p.) with (R)-THP. (D) High resolution mass spectrum of salutaridine detected in urine of mice injected (i.p.) with (R)-THP.

Fig. 7.
Structures of tetrahydroisoquinolines accepted as substrates by a bovine isoform of NMT [25].

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Table 1

Primers used for the cloning of PNMT and NMT into pET28a. This cloning strategy resulted in PNMT and NMT recombinant protein containing a His-tag sequence located at the N-terminus of the complete amino acid sequence of PNMT (Met1-Leu282, His-tag sequence MGSSHHHHHHSSGLVPRGSHM) and NMT (Met1-Phe263, His-tag sequence MGSSHHHHHHSSGLVPRGSHMAS).

Primer designation	Primer sequence
PNMT <i>Nde</i> I 5'-restriction site	5'-GGGCGC <i>CATATG</i> ATG AGC GGC GCA GAC CGT A-3'
PNMT <i>Bam</i> HI 3'-restriction site	5'-CGC <i>GGATCC</i> CAGAAGGTTGGGCTG-3'
NMT <i>Nhe</i> I 5'-restriction site	5'-GGGGCTAGCATGAAGGGTGGCTTCACTG-3'
NMT <i>Xho</i> I 3'-restriction site	5'-GGGGCTAGCATGAAGGGTGGCTTCACTG-3'

Table 2

Compound specific parameters for the LC-MS/MS analysis of BIQ alkaloids.

Analyte	Collision energy (V)	Declustering potential (V)	Quantifier MRM Transition	Qualifier MRM transition
THP	30	70	288 → 164	288 → 123
laudanosoline	26	40	302 → 178	302 → 123
4'- <i>O</i> -methyl-THP	33	35	302 → 164	302 → 285
4'- <i>O</i> -methyllaudanosoline	30	40	316 → 178	316 → 123
6- <i>O</i> -methyl-THP	30	40	302 → 178	302 → 285
6- <i>O</i> -methyllaudanosoline	30	40	316 → 192	316 → 123
norreticuline	30	45	316 → 178	316 → 299
reticuline	35	45	330 → 299	330 → 192

Table 3

Enzyme activity of 62.5 μ M human PNMT or human NMT with 2 mM BIQ alkaloids and 5 μ M [14 C-methyl]-SAM (0.025 μ Ci, 50000 cpm) incubated for 2 h at 37°C. Human NMT is active exclusively with (*R*)-configured BIQ alkaloids, whereas no product formation could be observed for human PNMT.

BIQ tested as substrate	specific activity of PNMT (EC 2.1.1.28), (pmol/min/ mg protein)	specific activity of NMT (EC 2.1.1.49), (pmol/min/ mg protein)
(<i>R</i>)-6- <i>O</i> -Methyl-THP	Nd	0.5
(<i>R</i>)-THP	Nd	1.1
(<i>R</i>)-4'- <i>O</i> -Methyl-THP	Nd	3.0
(<i>R</i>)-Norreticuline	Nd	5.6
(<i>S</i>)-6- <i>O</i> -Methyl-THP	Nd	Nd
(<i>S</i>)-THP	Nd	Nd
(<i>S</i>)-4'- <i>O</i> -Methyl-THP	Nd	Nd
(<i>S</i>)-Norreticuline	Nd	Nd

nd, not detected (below a limit of detection of 71 fmol for the reaction product)

Table 4

Kinetic parameters of NMT for the substrates (*R*)-norreticuline, (*R*)-4'-*O*-methyl-THP, (*R*)-6-*O*-methyl-THP and (*R*)-THP. Enzyme activity of 62.5 μ M human NMT was determined in 10 min incubations at 37°C with 250–2500 μ M BIQ alkaloids and 50 μ M SAM.

substrate/co-substrate	K_m (mM)	k_{cat} (nmol/min/mg)	k_{cat}/K_m (mM ⁻¹ s ⁻¹)
(<i>R</i>)-norreticuline	0.9 $\pm$ 0.1	3.0 $\pm$ 0.1	0.056
(<i>R</i>)-4'- <i>O</i> -Methyl-THP	1.6 $\pm$ 0.2	1.8 $\pm$ 0.1	0.019
(<i>R</i>)-6- <i>O</i> -Methyl-THP	10.1 $\pm$ 3.6	1.4 $\pm$ 0.4	0.002
(<i>R</i>)-THP	7.5 $\pm$ 3.1	0.7 $\pm$ 0.2	0.002