

Endogenous formation of morphine in human cells

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Morphine is a plant (opium poppy)-derived alkaloid and one of the strongest known analgesic compounds. Studies from several laboratories have suggested that animal and human tissue or fluids contain trace amounts of morphine. Its origin in mammals has been believed to be of dietary origin. Here, we address the question of whether morphine is of endogenous origin or derived from exogenous sources. Benzyloisoquinoline alkaloids present in human neuroblastoma cells (SH-SY5Y) and human pancreas carcinoma cells (DAN-G) were identified by GC/tandem MS (MS/MS) as norlaudanosoline (DAN-G), reticuline (DAN-G and SH-SY5Y), and morphine (10 nM, SH-SY5Y). The stereochemistry of reticuline was determined to be 1-(S). Growth of the SH-SY5Y cell line in the presence of $^{18}\text{O}_2$ led to the [^{18}O]-labeled morphine that had the molecular weight 4 mass units higher than if grown in $^{16}\text{O}_2$, indicating the presence of two atoms of ^{18}O per molecule of morphine. Growth of DAN-G cells in an $^{18}\text{O}_2$ atmosphere yielded norlaudanosoline and (S)-reticuline, both labeled at only two of the four oxygen atoms. This result clearly demonstrates that all three alkaloids are of biosynthetic origin and suggests that norlaudanosoline and (S)-reticuline are endogenous precursors of morphine. Feeding of [$\text{ring-}^{13}\text{C}_6$]-tyramine, [$1\text{-}^{13}\text{C}$, $N\text{-}^{13}\text{CH}_3$]- (S)-reticuline and [N- CD_3]-thebaine to the neuroblastoma cells led each to the position-specific labeling of morphine, as established by GC/MS/MS. Without doubt, human cells can produce the alkaloid morphine. The studies presented here serve as a platform for the exploration of the function of "endogenous morphine" in the neurosciences and immunosciences.

Recent studies have shown that morphine, apart from its presence in plants, is also present in animal tissue. "Endogenous morphine" has been isolated and identified by MS in trace amounts from specific organs and fluids of vertebrates, including toad skin (1), bovine brain and adrenal gland (2, 3), rat brain and adrenal gland (3–5), and human heart (6) and urine (7, 8), as well as of invertebrates such as *Mytilus edulis* (9). To our knowledge, conclusive evidence that animal tissue is capable of synthesizing morphine has not been presented. The most widely accepted explanation is that morphine that is detected in human and animal tissue is of dietary origin because morphine has been reported to occur in hay, lettuce, human milk, and cow milk, as well as in commercial rat and rabbit feed (10–12). The question of the origin of endogenous morphine in animals and humans can be answered only by demonstrating that the morphine molecule is biosynthesized *de novo* by mammals from distant precursors. In the plant kingdom, the biosynthesis of morphine has been established starting from two molecules of L-tyrosine (13). Three methylation steps depend on S-adenosyl-L-methionine as cosubstrate. In mammals, L-tyrosine can be formed by hydroxylation of L-phenylalanine. L-Phenylalanine and L-methionine (the source of S-adenosyl-L-methionine) are essential amino acids of dietary origin. Two additional atoms of oxygen are incorporated into each morphine molecule during the transformation of two molecules of L-tyrosine into two molecules of dopamine (or L-dopa) by action of tyrosine hydroxylase, the rate-limiting enzyme in dopamine synthesis. If morphine is formed endogenously in mammals, application of labeled potential precursors (such as $^{18}\text{O}_2$ or ^{13}C -tyrosine) in biosynthetic studies should ultimately label morphine (and any

intermediate in the biosynthetic process as well) in specific positions.

In this study, human and animal cell cultures were used as experimental systems, allowing the application of isotopically labeled potential precursors of morphine in a closed system under sterile conditions. Although previous studies have already shown that one cell line, the rat adrenal medullary pheochromocytoma cell line PC-12, contains trace amounts of morphine (14), it could have been derived from serum-bound morphine (dietary origin) or even from laboratory contamination. To achieve maximum sensitivity, we subjected the alkaloids isolated from the cell cultures to different derivatization methods before analysis by GC coupled to ion-trap tandem MS (MS/MS).

Materials and Methods

Chemicals. All reference alkaloids were obtained from the departmental collection (Biocenter, Martin Luther University Halle-Wittenberg). We purchased $^{18}\text{O}_2$ from Euriso-Top (Saint-Aubin, France). [$\text{ring-}^{13}\text{C}_6$]-L-tyrosine and [$\text{ring-}^{13}\text{C}_6$]-tyramine were synthesized, as described in detail in ref. 15. T. Tanahashi (Kobe Pharmaceutical University, Kobe, Japan) provided the [$1\text{-}^{13}\text{C}$, $N\text{-}^{13}\text{CH}_3$]- (S)-reticuline. Methyl- d_3 -iodide (CD_3I) was obtained from Sigma. N-Methyl-N-trimethylsilyl (TMS)-trifluoroacetamide (MSTFA) was obtained from CS Chromatographie Service (Langerwehe, Germany). Trifluoroacetic anhydride (TFAA) and acetic anhydride were purchased from Sigma. Recombinant berberine bridge enzyme was a gift from T. M. Kutchan (Leibniz Institute of Plant Biochemistry).

Synthesis of [N- CD_3]-Thebaine. A solution of northebaine (1.0 g, 3.4 mmol) in acetonitrile (20 ml) was stirred in the presence of CD_3I (0.45 g, 3.1 mmol) for 48 h at room temperature. The reaction mixture was taken to dryness under high vacuum, and the residue was further purified by column chromatography, Silica gel 60 (solvent system: toluene/acetone/ethanol/ NH_4OH ; 45:45:7:3, vol/vol/vol/vol). [N- CD_3]-thebaine (750 mg) was obtained in 71% yield (mass spectra of m/z 315 [$\text{M}+\text{H}$] $^+$ and 61 [$\text{CH}_3\text{CHN}(\text{H})\text{CD}_3$] $^+$), and isotopic distribution of ^2H -labeled thebaine was as follows: 0.1% [$^2\text{H}_0$]/0.6% [$^2\text{H}_2$]/99.0% [$^2\text{H}_3$]/0.3% [$^2\text{H}_5$].

Media and Cell Lines. All media, sera, and gentamicin were obtained from Invitrogen. DAN-G, SH-SY5Y, and the other cell lines were purchased from the German Collection of Microorganism and Cell Cultures (DSMZ; Braunschweig, Germany) or were obtained from the Biocenter collection. Sf9 cells were supplied by T. M. Kutchan. Each cell line was cultured in 175-cm 2 flasks at 37°C with 5% CO_2 for 5 days in its respective medium containing 0.01% (wt/vol) gentamicin. The medium was replaced every other day. Cells that had reached $\approx 75\%$ confluency ($\approx 2 \times 10^7$ cells) were collected after trypsin treatment, pelleted (5 min, 800 \times g), and washed with PBS.

Abbreviations: Ace, acetyl; TFA, trifluoroacetyl; TMS, trimethylsilyl; MS/MS, tandem MS.

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Sample Preparation and Derivatization. To avoid oxidation of alkaloids under the alkaline conditions normally used during the isolation procedure, the cell pellet ($\approx 3 \times 10^9$ cells) was homogenized in 1 M HCl (3 ml), extracted with CHCl_3 /isopropanol (9:1, vol/vol) (5 ml, 5 min of mixing at room temperature), and centrifuged (15 min, $2,500 \times g$). The acidic aqueous phase was adjusted to pH 5 with 2 M KOH and then loaded onto a C18-Sep-Pak Plus cartridge (Waters) that had been conditioned with 2×1 ml of methanol, 2×1 ml of water at a flow rate of 1 ml/min, and then 2×1 ml of 0.05% trifluoroacetyl (TFA) at a flow rate of 5 ml/min. Subsequently, the cartridge was washed with 2×1 ml of water. The alkaloids were eluted with 7 ml of a water/acetonitrile/trifluoroacetic acid mixture (89.95:10:0.05, vol/vol/vol) (14), yielding fraction 1, which contained norlaudanosoline and morphine, and subsequently with 7 ml of water/acetonitrile/trifluoroacetic acid (79.95:20:0.05, vol/vol/vol), yielding fraction 2, which contained reticuline. The morphine-containing fraction was dried under high vacuum and converted to the TMS derivative by addition of 20 μl of TMS-trifluoroacetamide (MSTFA) and heating to 70°C for 30 min. Conversion to the respective TFA derivative was achieved by adding 75 μl of trifluoroacetic anhydride (TFAA) and heating of the mixture to 80°C in 15 μl of acetonitrile for 60 min. Norlaudanosoline- and reticuline-containing fractions were prepared as TFA derivatives by addition of 75 μl of TFAA and heating to 80°C in 15 μl of acetonitrile for 60 min. The acetyl (Ace) derivatives were prepared by addition of 75 μl of acetic anhydride and heating to 80°C in 15 μl of pyridine for 60 min. Derivatization reagents were removed by a stream of N_2 , samples were dissolved in 5 μl of methanol and centrifuged, and 1 μl of the supernatant was analyzed by GC/MS/MS. The TMS derivatives were concentrated to 5 μl and centrifuged, and 1 μl of the supernatant was straight analyzed by GC/MS/MS.

Application Experiment with $^{18}\text{O}_2$. DAN-G or SH-SY5Y cells were cultured in 175-cm² flasks as described above. The 5-day-old cultured cells were treated with trypsin and transferred to a 2-liter Fernbach flask. Each Fernbach flask containing 100 ml of medium was inoculated with 1.5×10^6 cells. After incubation for 24 h at 37°C, the medium was renewed and the cells were incubated for an additional 4 h. The culture flasks were then purged three times with nitrogen gas. Under low vacuum, artificial air containing 10.5% $^{18}\text{O}_2$, 10.5% $^{16}\text{O}_2$, 10% CO_2 , and 69% N_2 was filled into the culture flask, which was then incubated at 37°C for another 48 h. The used medium was then replaced in close system by oxygen-free fresh medium, and the cultured cells were continuously incubated at 37°C in artificial air. After a culture period of 7 days, the cells were treated with trypsin, centrifuged at $800 \times g$ for 5 min, and washed twice with PBS. The cell pellet was then extracted, and the alkaloids were treated as described above.

Feeding Experiment with [ring- $^{13}\text{C}_6$]-Tyramine, [ring- $^{13}\text{C}_6$]-Tyrosine, [N- CD_3]-Thebaine, and [1- ^{13}C , N- $^{13}\text{CH}_3$]-Reticuline. We transferred 3×10^5 DAN-G or SH-SY5Y cells to 175-cm² flasks and incubated them in 25 ml of medium at 37°C for 24 h. After attachment of the cells, the medium was removed and fresh medium containing a labeled precursor at a concentration of 40 μM was added. The cells were incubated for another 6 days, and the medium was replaced every 2 days with fresh medium containing the labeled precursor in question at a concentration of 40 μM . After 7 days of culture, the cells were harvested and processed as described above.

MS. GC/MS/MS analyzes were performed on a Polaris Q Ion-Trap MS/MS (Finnigan) in electron-ionization (EI) mode (70 eV). GC was performed on an FS-Supreme-5 capillary column (60 m, 0.25-mm i.d., 0.25- μm film thickness; CS-

Chromatographic Service) in splitless mode, carrier gas helium at a constant flow rate of 1 ml/min. The initial injector temperature of 65°C was increased after injection by 14.5°C per sec to 300°C. The oven temperature was programmed as follows. For TFA-morphine, the initial oven temperature of 150°C was held for 1 min, increased by 15°C per min to 250°C and held for 1 min, increased to 290°C by 10°C per min and held for 1 min, increased to 300°C by 10°C per min and held for 1 min, and finally increased to 320°C by 40°C per min and held for 5 min. For TFA-reticuline, the conditions were as described above. For TFA- and Ace-norlaudanosoline, the initial oven temperature of 150°C was held for 1 min, increased by 15°C per min to 250°C and held for 1 min, increased to 290°C by 10°C per min and held for 1 min, increased to 300°C by 10°C per min and held for 10 min, and finally increased to 320°C by 40°C per min and held for 5 min. For, TMS-morphine, the initial oven temperature of 150°C was held for 1 min and then increased by 15°C per min to 300°C and held for 20 min. The following MS conditions were used: ion-source temperature, 200°C; MS transfer-line temperature, 300°C.

The MS/MS spectra were performed by using $[\text{M}]^+$ ions as parent ions for TFA-morphine (m/z 477), TMS-morphine (m/z 429), TFA-reticuline (m/z 521), Ace-norlaudanosoline (m/z 497), and a fragment of isoquinoline moiety of TFA-norlaudanosoline (m/z 452).

The positive-ion electrospray/selected-reaction monitoring (liquid chromatography/electrospray ionization/selected-reaction monitoring) data were obtained with a TSQ 7000 instrument (electrospray voltage, 4.5 kV; heated-capillary temperature, 220°C; sheath gas, nitrogen; Finnigan-MAT, San Jose, CA) coupled with a Surveyor Micro liquid-chromatography system equipped with an RP18 column (5 μm , 1×100 mm; Ultrasep, SEPSERV, Berlin). For the HPLC, a gradient system was used, starting from $\text{H}_2\text{O}/\text{CH}_3\text{CN}$ (85:15, vol/vol) (each containing 0.2% HOAc) to 10:90 (vol/vol) within 15 min, followed by a 15-min isocratic period (flow rate, 50 $\mu\text{l}\cdot\text{min}^{-1}$). The selected reactions were recorded during the HPLC run with the following conditions: reaction of m/z 328 to 178 for (S)-scoulerine, reaction of m/z 330 to 180 for [1- ^2H , ^{13}C]- (S)-scoulerine (collision energy, 30 eV), and reaction of m/z 330 to 192 for reticuline (collision energy, 25 eV). The collision gas was argon, and the collision pressure was 1.8×10^{-3} torr (1 torr = 133 Pa).

Results and Discussion

Occurrence of Morphine and Other Benzyloisoquinoline Alkaloids in Cell Cultures. A random search for a cell line producing morphine was performed by analyzing 12 commercially available human and animal cell cultures. Cell extracts were subjected to derivatization methods and analyzed by GC/MS/MS. The analytical methods used for TMS-morphine and TFA-morphine had a precision of 80% and 76%, respectively. The limit of detection of TMS- and TFA-derivatized morphine was 0.1 and 0.5 ng/ μl , respectively. Trypsin, sera, and other medium components and used media were analyzed by GC/MS/MS, which showed that they did not contain free morphine or its alkaloidal precursors before or after cultivation of the cells.

The initial screen of different cell lines identified three benzyloisoquinoline alkaloids (Table 1) by enrichment and by the fragmentation observed in the MS/MS product ion spectra being identical to those obtained with reference compounds. Morphine (identified as a TMS derivative) was shown to be present (m/z 429 $[\text{M}]^+$; Fig. 1) in the human neuroblastoma cell line SH-SY5Y. Also, it was found in the human cell lines HaCaT, HepG2, and JEG-3, as well as the rat cell line PC-12 (identified as a TFA derivative; m/z 477 $[\text{M}]^+$ and 476 $[\text{M}-\text{H}]^+$; Fig. 2A). Reticuline (identified as TFA derivative; m/z 521 $[\text{M}]^+$ and 520 $[\text{M}-\text{H}]^+$, see Fig. 5 A and B, which is published as supporting information on the PNAS web site) occurred in the human

Table 1. Isoquinoline alkaloids present in human and animal cell lines

Cell line	Cell type	Medium	Isoquinoline alkaloids, pmol per 10 ⁹ cells		
			Norlaudanosoline	Reticuline	Morphine
DAN-G	Human pancreas carcinoma	90% RPMI medium 1640/10% FBS	14	9	ND
HaCaT	Human keratinocyte	90% MEM/10% FBS	ND	ND	4
HepG2	Human hepatocellular carcinoma	90% RPMI medium 1640/10% FBS	ND	ND	2
JEG-3	Human choriocarcinoma	90% Ham's F12/10% FBS	ND	ND	1
SH-SY5Y	Human neuroblastoma	80% DMEM/20% FBS	ND	6	15
PC-12	Rat adrenal pheochromocytoma	80% DMEM/10% FBS/10% HS	ND	ND	10

Not detected (ND) refers to an amount below the limit of detection (norlaudanosoline, 0.5 pmol; reticuline, 0.5 pmol; and morphine, 0.05 pmol). All media contain 0.01% (wt/vol) gentamicin. HS, horse serum; DMEM, low-glucose DMEM.

DAN-G and SH-SY5Y cell lines. Norlaudanosoline (tetrahydro-papaveroline) (identified as Ace derivative; m/z 497 $[M]^+$, 496 $[M-H]^+$, and 454 $[M-COCH_3]^+$) was present only in DAN-G cells (see Fig. 6 A and B, which is published as supporting information on the PNAS web site). Neither morphine, reticuline, nor norlaudanosoline were detectable in Caco-2 (human colon adenocarcinoma), JAR (human placenta choriocarcinoma), Jurkat (human T cell leukemia), U-937 (human histiocytic lymphoma), Sf9 (ovarian tissue of *Spodoptera frugiperda*), or CHO-K1 (hamster ovary) cells. The human neuroblastoma cell line SH-SY5Y and the human pancreas carcinoma cell line DAN-G were chosen for further biosynthetic experiments because SH-SY5Y showed good growth characteristics and the stable presence of both morphine and reticuline, and DAN-G contained both norlaudanosoline and reticuline. The amount of morphine obtained from the SH-SY5Y cell line was ≈ 15 fmol per 1×10^6 cells; this amount corresponds to a concentration of ≈ 10 nM, based on packed cell volume.

Application Experiment with ¹⁸O₂. The MS/MS spectrum of morphine isolated from the SH-SY5Y cell line grown in an enriched ¹⁸O₂ atmosphere showed the $[M]^+$ ion (TFA derivative) at m/z 481 (Fig. 2B). This ion is 4 mass units higher than the $[M]^+$ ion of the metabolite obtained from cells grown in a regular ¹⁶O₂ atmosphere at m/z 477 $[M]^+$. This result clearly indicated the presence of two atoms of ¹⁸O per molecule of morphine (m/z 481 vs. 477). The degree of isotopic labeling (i.e., the percentage of ¹⁸O₂-morphine of the total amount of morphine) of the ¹⁸O₂-morphine formed under this condition was 22%. No morphine species containing three atoms of ¹⁸O (m/z 483 $[M]^+$) could be detected (signal to noise ratio, ≥ 2), which rules out any participation of L-phenylalanine in this biosynthetic route. Because the oxygen atom attached to the C-3 position of morphine is derived from the oxygen atom at the C-4 position of L-tyrosine, the labeled ¹⁸O-atoms must reside both in the epoxide bridge between C-4 and C-5 and in the C-6 hydroxyl. Both oxygen atoms from the supplied ¹⁸O₂ are incorporated into the C-3 position of L-tyrosine and/or tyramine, yielding DOPA and/or dopamine. This experiment demonstrates that human neuroblastoma cells are capable of synthesizing morphine *de novo* from distant precursors, such as L-tyrosine (Fig. 3A).

In addition to morphine, reticuline, an established precursor of morphine in the poppy plant (16, 17), was formed by the SH-SY5Y cell line grown in an ¹⁸O₂ atmosphere. Ion-trap MS/MS analysis of the isolated material (TFA derivative) yielded peaks of m/z 525 $[M]^+$ and 524 $[M-H]^+$ (see Fig. 5C), showing, as in the case of morphine, the incorporation of two ¹⁸O-atoms into one molecule of reticuline (degree of isotopic labeling, 22%). The MS/MS spectra of the isoquinoline moiety (parent ion, m/z 288) and benzyl moiety (parent ion, m/z 233) of TFA-reticuline showed that both parts of the reticuline molecule were labeled with one atom of ¹⁸O (m/z 288 vs. 290 and 233 vs. 235).

The pancreatic cell line DAN-G was cultured in an ¹⁸O₂-atmosphere as described above for SH-SY5Y cells yielding labeled reticuline (identified as TFA derivative: m/z 525 $[M]^+$ and 524 $[M-H]^+$) and labeled norlaudanosoline (identified as Ace derivative; m/z 501 $[M]^+$, 500 $[M-H]^+$, and 458 $[M-COCH_3]^+$; see Fig. 6C). The degree of isotopic labeling was 25% and 38%, respectively. Both alkaloids showed an incorporation of two atoms of ¹⁸O per molecule, the isoquinoline and benzyl moiety each being labeled with one atom of ¹⁸O. These findings indicate that reticuline and norlaudanosoline are synthesized along the same pathway as morphine and are most likely precursors of this analgesic alkaloid in human cell lines.

Feeding Experiment with $[ring-^{13}C_6]$ -L-Tyrosine and $[ring-^{13}C_6]$ -Tyramine. L-tyrosine and tyramine have long been proven to be biosynthetic precursors of morphine in the opium poppy plant (18, 19). However, attempts to achieve an incorporation of $[ring-^{13}C_6]$ -L-tyrosine into morphine synthesized by human cell

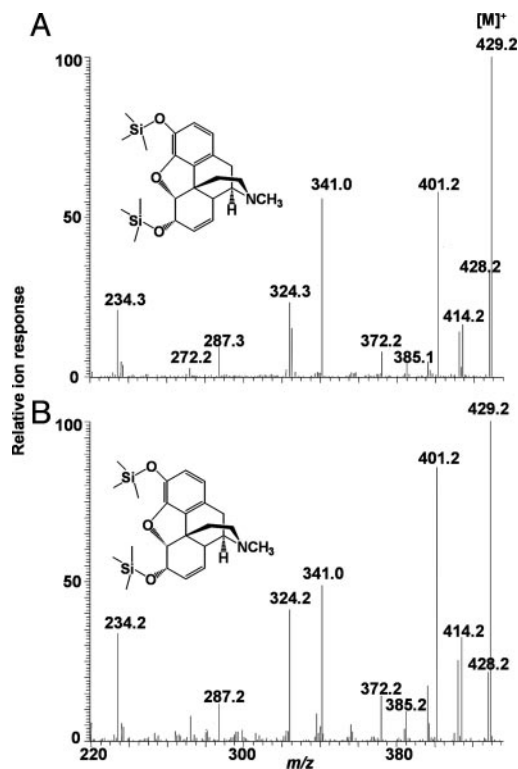


Fig. 1. MS/MS product ion spectra of TMS-morphine. (A) The m/z 429 ion ($[M]^+$ of reference TMS-morphine; plant-derived). (B) The m/z 429 ion ($[M]^+$ of TMS-morphine isolated from the SH-SY5Y human neuroblastoma cell line).

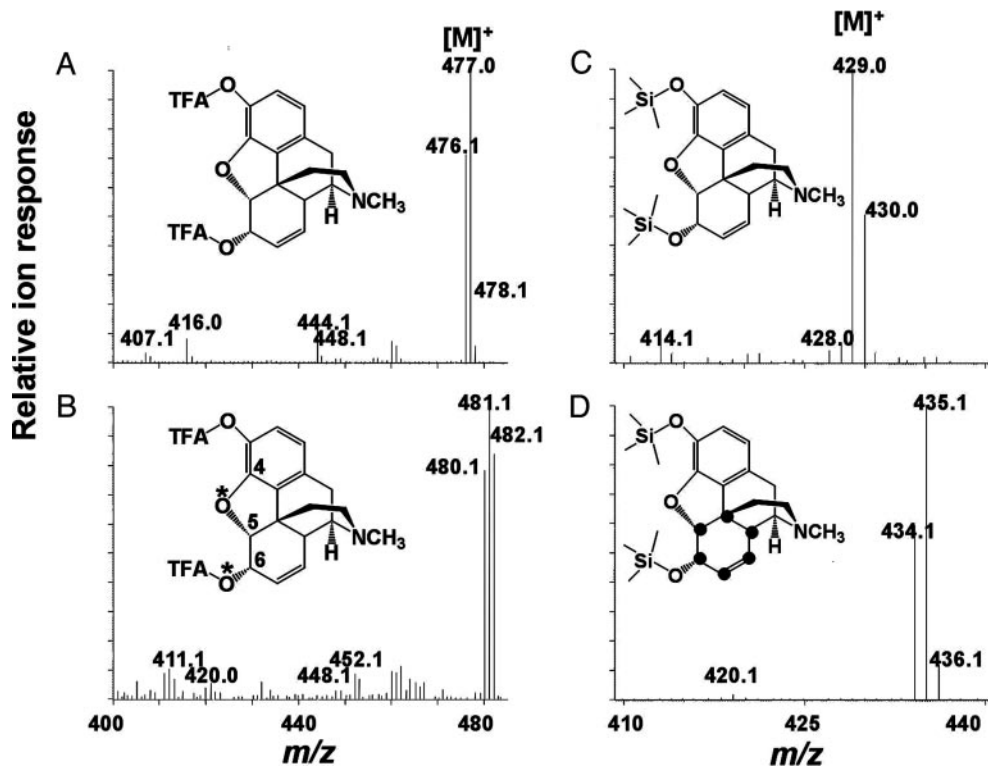


Fig. 2. MS/MS product ion spectra of morphine derivatives. (A) The m/z 477 ion ($[M]^+$ of TFA-morphine isolated from SH-SY5Y cell line cultured in the presence of $^{16}\text{O}_2$). (B) The m/z 481 ion ($[M]^+$ of TFA- $^{18}\text{O}_2$]-morphine labeled with two atoms of ^{18}O per morphine molecule that was isolated from SH-SY5Y cell line cultured in the presence of $^{18}\text{O}_2$). (C) The m/z 429 ion ($[M]^+$ of TMS-morphine isolated from SH-SY5Y cell line). (D) The m/z 435 ion ($[M]^+$ of TMS- $^{13}\text{C}_6$]-morphine isolated from SH-SY5Y cell line cultured in the presence of $40\ \mu\text{M}$ [$\text{ring-}^{13}\text{C}_6$]-tyramine).

lines failed, probably because the medium of the SH-SY5Y cell line contained appreciable amounts of unlabeled L-tyrosine ($400\ \mu\text{M}$) and L-phenylalanine ($400\ \mu\text{M}$), which lead to extensive dilution of this labeled potential precursor. Therefore, [$\text{ring-}^{13}\text{C}_6$]-tyramine, a metabolite of L-tyrosine *en route* to dopamine, was added to the medium. After a growth period of 7 days, SH-SY5Y cells were extracted, and morphine and reticuline were isolated, subjected to derivatization, and analyzed by GC/MS/MS. The ion-trap MS/MS spectrum of morphine (TMS derivative) from control SH-SY5Y cells showed a molecular ion peak at m/z 429 $[M]^+$; morphine obtained from cells that were supplied with [$\text{ring-}^{13}\text{C}_6$]-tyramine had the molecular ion peak that was 6 mass units higher at m/z 435 $[M]^+$ (Fig. 2 C and D). This increase of 6 mass units of the molecular ion corresponds to the incorporation of one molecule of [$\text{ring-}^{13}\text{C}_6$]-tyramine into one molecule of morphine. The degree of isotopic labeling was 2%. Based on GC-MS analysis in the selected-ion monitoring (SIM) mode, the ion chromatograms (Fig. 4) showed that [$\text{ring-}^{13}\text{C}_6$]-tyramine labeled exclusively the cyclohexene ring of morphine (Fig. 3A), which corresponds biogenetically to the aromatic ring of the isoquinoline moiety of this alkaloid. This labeling pattern in the human cell line exactly follows the one found (19) in the poppy plant.

Reticuline isolated from SH-SY5Y cells from the same experiment showed an MS/MS spectrum of TFA derivatives of control vs. labeled reticuline with peaks at m/z 521 vs. 527 $[M]^+$ and 520 vs. 526 $[M-H]^+$ (see Fig. 5D), again indicating an increase of 6 mass units (degree of isotopic labeling of 5%). The MS/MS spectrum of norlaudanoline (Ace derivative) from control DAN-G cells showed a molecular ion peak at m/z 497 $[M]^+$; norlaudanoline obtained from DAN-G cells that were cultured in the presence of $40\ \mu\text{M}$ [$\text{ring-}^{13}\text{C}_6$]-tyramine had the molecular ion peak that was 6 mass units higher at m/z 503 $[M]^+$

(see Fig. 6D). These experiments demonstrate again the incorporation of only one molecule of labeled tyramine (via dopamine) into the isoquinoline alkaloid morphine produced by human cell lines, exactly as has been found for the morphine biosynthesis in opium poppy (19). The fragmentation pattern of the TFA-derivatives showed that [$\text{ring-}^{13}\text{C}_6$]-tyramine was incorporated exclusively into the isoquinoline moiety of reticuline and norlaudanoline (TFA derivative of the respective isoquinoline moiety: m/z 452 $[M]^+$ and 458 $[M]^+$) (see Fig. 7A and B, which is published as supporting information on the PNAS web site), whereas the benzyl moiety of these molecules is devoid of a ^{13}C -label. The benzyl moiety of these mammalian alkaloids is most likely formed from a $\text{C}_6\text{-C}_2$ aldehyde, such as 3,4-dihydroxyphenylacetaldehyde, which is ultimately derived from L-tyrosine (20).

Stereochemistry of Reticuline and Morphine. One of the central questions in the biosynthesis of morphinan alkaloids in the opium poppy was the configuration at C-1 of the tetrahydrobenzylisoquinoline precursor molecules (21, 22). Whereas in the poppy plant the firmly established precursors of the intermediate reticuline all have an (*S*) configuration at C-1, morphine and the morphinan precursors possess (*R*) configuration at the corresponding carbon atom C-9. (*S*)-reticuline formed in the poppy plant is converted by means of 1,2-dehydroreticuline to (*R*)-reticuline, which in turn is then transformed into morphine (15, 21, 23, 24). Therefore, it was necessary to determine the stereochemical configuration of reticuline produced by and accumulated in the human cell lines DAN-G and SH-SY5Y. Making use of the fact that the berberine bridge enzyme (International Union of Biochemistry and Molecular Biology database no. EC 1.21.3.3) (25, 26) converts (*S*)-reticuline quantitatively and stereoselectively to (*S*)-scoulerine, whereas (*R*)-reticuline is not

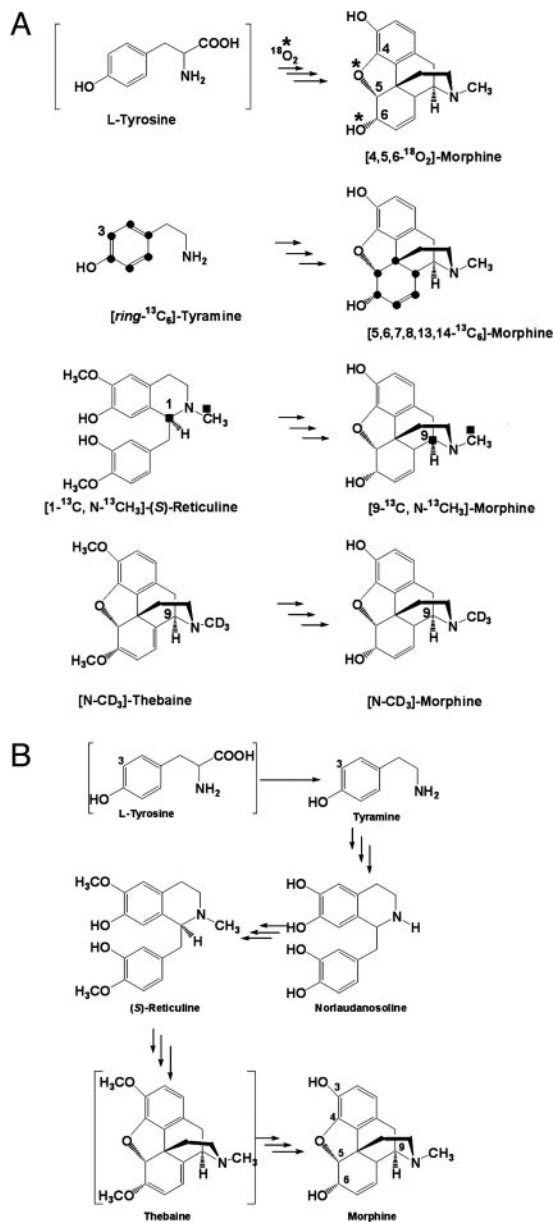


Fig. 3. Precursor studies in human neuroblastoma cell culture. (A) Labeling pattern in morphine molecules produced by SH-SY5Y cells after supplying $^{18}O_2$ (asterisk denotes ^{18}O), [ring- $^{13}C_6$]-tyramine (circle denotes ^{13}C), [1- ^{13}C , N- $^{13}CH_3$]-S-reticuline (square denotes ^{13}C), or [N- CD_3]-thebaine (D denotes 2H) in the culture medium. (B) Predicted intermediates in the pathway from L-tyrosine to morphine in SH-SY5Y cells.

oxidized, allowed the identification of the unknown stereochemistry of unlabeled reticuline produced by DAN-G and SH-SY5Y cell lines, as well as the identification of [ring- $^{13}C_6$]-reticuline obtained from DAN-G and SH-SY5Y human cell lines after application of [ring- $^{13}C_6$]-tyramine. They both possessed an (*S*) configuration, as shown by liquid chromatography/MS/MS in the selected-reaction monitoring (SRM) mode (see Fig. 8, which is published as supporting information on the PNAS web site). Reticuline of unknown stereochemistry has been isolated recently from rat brain (27), but its biogenetic origin was not clarified.

To investigate whether morphine biosynthesis in the human cell lines follows the biosynthetic route already established for the poppy plant (13), the late biosynthetic intermediate thebaine,

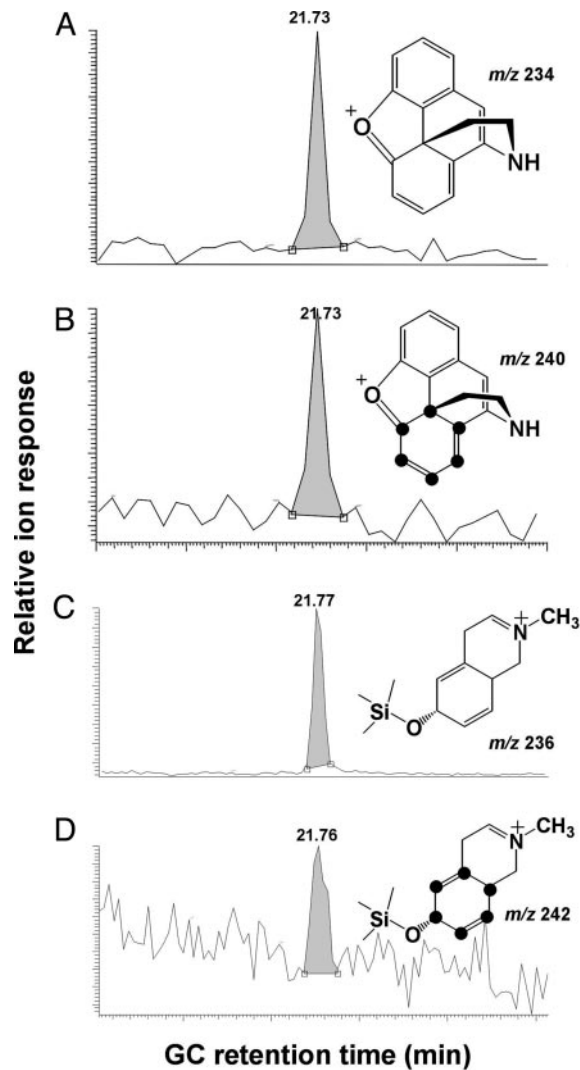


Fig. 4. GC/MS chromatograms obtained from selected-ion monitoring (SIM) mode analysis. (A) The selected ion at m/z 234 represents the proposed fragment of reference TMS-morphine, which includes all five rings. (B) The selected ion at m/z 240, representing the proposed fragment of TMS- $^{13}C_6$ -morphine, which includes all five rings. (C) The selected ion at m/z 236, which represents the proposed fragment of isoquinoline-derived rings of reference TMS-morphine. (D) The selected ion at m/z 242, which represents the proposed fragment of isoquinoline-derived rings of TMS- $^{13}C_6$ -morphine.

labeled as an [N- CD_3] derivative, was supplied to the SH-SY5Y cell line. The morphine formed by the cells in this experiment was isolated and subjected as the TMS derivative to GC/MS/MS analysis. The alkaloid was clearly labeled, as shown by a comparison with cell-derived, unlabeled morphine; m/z 429 vs. 432 [M] $^+$, 414 vs. 417 [M- CH_3] $^+$ and 359 vs. 359 [M- CH_3 -CH(D) $_3$ NHCH $_2$ CH $_2$] $^+$ (see Fig. 9A and B, which is published as supporting information on the PNAS web site). The labeled morphine showed an increase of 3 mass units compared with the unlabeled alkaloid (m/z 429 vs. 432 [M] $^+$), and the m/z 359 ion indicated the correct position of the N- CD_3 group. The degree of isotopic labeling was 10%. Thebaine is clearly a precursor of morphine in this human cell line. Moreover, this biochemical transformation of thebaine to morphine also establishes the correct (*R*) configuration at C-9 of the morphine molecule produced by these human cells. Because the asymmetric center at C-9 in morphine, which is produced from thebaine, has an (*R*) configuration, and because (*S*)-reticuline, which is accumulated

in this cell line, is a potential precursor to morphine, a change of configuration from (*S*)-reticuline to (*R*)-reticuline must occur during the biosynthetic process.

To prove this assumption, an experiment was designed to investigate the incorporation of [$1-^{13}\text{C}$, $N-^{13}\text{CH}_3$]-(*S*)-reticuline into morphine in the SH-SY5Y cell line. The MS/MS spectrum of the TMS derivative of isolated labeled morphine showed an $[\text{M}]^+$ at m/z 431, clearly indicating a mass increase of 2 units compared with the unlabeled molecular ion (see Fig. 9A and C). The degree of isotopic labeling was 24%. This experiment demonstrates that (*S*)-reticuline *en route* to morphine undergoes a change in configuration to (*R*)-reticuline as was observed and well studied in the poppy plant (15, 21–24). Moreover, it suggests an intriguing biochemical analogy to the formation of this analgesic alkaloid in the plant kingdom.

In summary, we have unequivocally shown that morphine is present in human cells at a nanomolar range, based on packed cell volume. A dietary origin or a contamination can be ruled out, demonstrating that morphine is of biosynthetic origin not only in *Papaver* plant but also in human and animal cells. In analogy to the biosynthesis in the plant kingdom (13), we assume that morphine is formed in humans also by a multistep biosynthetic route, starting from the carbon skeleton of the amino acid L-tyrosine and the methyl group of L-methionine. Intermediates

en route to morphine in mammalian cells have been experimentally proven to be norlaudanosoline, (*S*)-reticuline, and thebaine (Fig. 3B). There can no longer be doubt that the human organism is capable of synthesizing one of the most potent analgesic compounds known, although its function is still a matter of speculation. However, there are studies suggesting a general immune, vascular, and nervous role of morphine in mammals. Endogenous opiates may even constitute a new class of signaling molecules. The identification of the genes and enzymes responsible for morphine biosynthesis in humans and animals will possibly indicate how the pathway arose in the animal kingdom and may provide new pharmacological targets for the modulation of pain, immune responses (28, 29), and cell death (30).

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