How human neuroblastoma cells make morphine

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Contributed by Meinhart H. Zenk, April 20, 2005

Recently, our laboratory demonstrated that human neuroblastoma cells (SH-SY5Y) are capable of synthesizing morphine, the major active metabolite of opium poppy. Now our experiments are further substantiated by extending the biochemical studies to the entire morphine pathway in this human cell line. L-[1,2,3-$^{13}C_3$]- and [*ring*-2′,5′,6′-$^{2}H_3$]dopa showed high isotopic enrichment and incorporation in both the isoquinoline and the benzyl moiety of the endogenous morphine. [2,2-$^{2}H_2$]Dopamine, however, was exclusively incorporated only into the isoquinoline moiety. Neither the trioxygenated (*R,S*)-[1,3-$^{13}C_2$]norcoclaurine, the precursor of morphine in the poppy plant, nor (*R*)-[1,3,4-$^{2}H_3$]norlaudanosoline showed incorporation into endogenous morphine. However, (*S*)-[1,3,4-$^{2}H_3$]norlaudanosoline furnished a good isotopic enrichment and the loss of a single deuterium atom at the C-9 position of the morphine molecule, indicating that the change of configuration from (*S*)- to (*R*)-reticuline occurs via the intermediacy of 1,2-dehydroreticuline. Additional feeding experiments with potential morphinan precursors demonstrated substantial incorporation of [7-^{2}H]salutaridinol, but not 7-[7-^{2}H]episalutaridinol, and [7-^{2}H,*N*-$^{2}C^{2}H_3$]oripavine, and [6-^{2}H]codeine into morphine. Human morphine biosynthesis involves at least 19 chemical steps. For the most part, it is a reflection of the biosynthesis in opium poppy; however, there is a fundamental difference in the formation of the key intermediate (*S*)-reticuline: it proceeds via the tetraoxygenated initial isoquinoline alkaloid (*S*)-norlaudanosoline, whereas the plant morphine biosynthesis proceeds via the trioxygenated (*S*)-norcoclaurine. Following the plant biosynthetic pathway, (*S*)-reticuline undergoes a change of configuration at C-1 during its transformation to salutaridinol and thebaine. From thebaine, there is a bifurcate pathway leading to morphine proceeding via codeine or oripavine, in both plants and mammals.

endogenous morphine | mammalian morphine | morphine biosynthesis | SH-SY5Y cells

The presence of opiate receptors in mammalian tissue has stimulated the search for endogenous ligands, leading to the discovery of opioid peptides. Recent studies, however, also provided evidence for the presence of morphine alkaloids in animals. Trace amounts have been detected in various tissues of mammals and lower animals and have been identified by MS or even NMR (summarized in ref. 1). However, the origin of the endogenous morphine has been a matter of controversy, because morphine also was found in hay, lettuce, human milk, and cow's milk as well as in commercial rat and rabbit feed (2–4). Even laboratory contamination with morphine could not be excluded.

To clarify the origin of morphine in mammalian tissues, classical biochemical studies as used in plants can be performed (5). Morphine's biosynthetic pathway in *Papaver somniferum* was elucidated by the administration of isotopically labeled putative precursors to intact plants and cell cultures, isolation of the labeled products, and subsequent determination of the labeling pattern. Based on these experiments, most of the enzymes of the morphine biosynthesis were discovered and purified, and subsequently some of the corresponding genes were cloned (summarized in ref. 6). The current picture of the morphine biosynthesis in plants, involving 19 steps, is nearly complete. This knowledge of the morphine biosynthesis in plants and the methodology of its elucidation were now applied to investigate the origin and the biosynthetic pathway of morphine found in mammalian cells. In brief, heavy isotope-labeled precursors (e.g., with ^{13}C, ^{2}H, and ^{18}O labels) are administered to human cell cultures and incubated for a certain period. Subsequently, the desired products are isolated, and their structure, isotope content, and labeling pattern, which has to be nonrandom, are elucidated by spectroscopic methods. The method of choice is ion-selective tandem MS (MS/MS), because it provides the highest sensitivity and specificity. Regarding the interpretation of the obtained quantitative incorporation data, the physicochemical characteristics of the applied substrates have to be taken into consideration, because they influence cell permeability and transport. Also, tracer dilution plays a role if the applied substrate is present endogenously or in the cell culture medium.

The human cell lines SH-SY5Y and DAN-G have been identified as suitable systems, because they both produce benzylisoquinoline alkaloids (1) and can be cultured for a sufficient period, on average up to 7 days. In previous studies, by using the least invasive isotopic precursor of morphine, ^{18}O, nonrandom, position-specific incorporation of two atoms of ^{18}O into one molecule of morphine produced by the living, metabolizing neuroblastoma cells (at a concentration of 10 nM) could be demonstrated in our laboratory. Moreover, the potential precursor alkaloids norlaudanosoline (tetrahydropapaveroline) and (*S*)-reticuline, which were also produced by these cells, were shown to be labeled with two atoms of ^{18}O (1). In a separate set of experiments, application of [*ring*-$^{13}C_6$]tyramine, (*S*)-[1-^{13}C,*N*-$^{13}CH_3$]reticuline, and [*N*-$C^{2}H_3$]thebaine to SH-SY5Y cells yielded specific incorporation into endogenous morphine (1). With these experiments, we unequivocally proved that human neuroblastoma cells are capable of synthesizing morphine.

Here, we present additional evidence for endogenous morphine biosynthesis focusing on the elucidation of intermediates of the biosynthetic pathway between the two C_6-C_2 units derived from L-tyrosine and the final product, morphine. The biosynthetic route, which had been established in the poppy plant (summarized in ref. 6), built the basis for the tracer experiments with human neuroblastoma and pancreas carcinoma cells (1) by using heavy isotope-labeled potential precursors.

Materials and Methods

Chemicals. All solvents were of either reagent grade (used for synthesis) or chromatographic grade (used for sample preparation and analysis) and were purchased from ACROS Organics (Geel, Belgium). Alkaloids used as standards were obtained either from the department collection or by synthesis. Nitro[^{13}C]methane (99%) and [2,2-$^{2}H_2$]dopamine (98%) were purchased from Eurisо-Top (Saint-Aubin, France). [$^{2}H_3$]Methyl iodide, deuterium chloride (35% in $^{2}H_2O$; wt/wt), and L-[*ring*-2′,5′,6′-$^{2}H_3$]dopa were from Sigma-Aldrich. *N*-methyl-*N*-trimethylsilyl trifluoroacetamide (MSTFA) was obtained from CS Chromatographie Service (Langerwehe, Germany).

Synthesis of Isotope-Labeled Potential Precursors. The synthesis of L-[1,2,3-$^{13}C_3$]dopa, (*R,S*)-[1,3-$^{13}C_2$]norcoclaurine, (*R,S*)-[1,3-$^{13}C_2$, 6-O-$^{13}CH_3$]coclaurine, (*R*)- and (*S*)-[1,3,4-$^{2}H_3$]norlaudanosoline,

Abbreviations: TMS, trimethylsilyl; MS/MS, tandem MS; $[M]^{\bullet+}$, molecular ion.

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Table 1. Feeding experiments with heavy isotope-labeled precursors to human neuroblastoma (SH-SY5Y) cells

Substrate applied to SH-SY5Y cells				Isolated labeled morphine from SH-SY5Y cells		
Substrate	% isotopic enrichment	Position of labeling	Applied concentration, μM	m/z* of $[M]^{\bullet+}$	Position of labeling in morphine molecule	% isotopic enrichment
L-dopa	95	[1,2,3-$^{13}C_3$]	20	433	C-9, C-10, C-15, C-16	56
L-dopa	98	[*ring*-2′,5′,6′-$^{2}H_3$]	20	432	[1,2,8$^{2-2}H_3$]†	40
Dopamine	98	[2,2-$^{2}H_2$]	20	431	[15,15-$^{2}H_2$]	22
(*R,S*)-norcoclaurine	95	[1,3-$^{13}C_2$]	20	431	ND	<0.5‡
(*R,S*)-coclaurine	99	[1,3-$^{13}C_2$,6-*O*-$^{13}CH_3$]	40	431	ND	<0.5‡
(*R*)-norlaudanosoline	81	[1,3,4-$^{2}H_3$]	20	432	ND	<0.5‡
(*S*)-norlaudanosoline	85	[1,3,4-$^{2}H_3$]	20	431	[15,16-$^{2}H_2$]	15
(*R*)-reticuline	99	[*N*-$C^{2}H_3$]	20	432	[*N*-$C^{2}H_3$]	25
(*S*)-reticuline	98	[*N*-$C^{2}H_3$]	20	432	[*N*-$C^{2}H_3$]	23
1,2-dehydroreticulinium ion	96	[*N*-$C^{2}H_3$]	20	432	[*N*-$C^{2}H_3$]	<0.5‡
Salutaridinol	94	[7-^{2}H]	20	430	[7-^{2}H]†	15
7-Episalutaridinol	95	[7-^{2}H]	20	430	ND	<0.5‡
Oripavine	69	[7-^{2}H,*N*-$C^{2}H_3$]	20	433	[7-^{2}H,*N*-$C^{2}H_3$]	12
Codeine	99	[6-^{2}H]	20	430	[6-^{2}H]†	21

$[M]^{\bullet+}$, molecular ion; ND, below the limit of detection (0.05 pmol).
*TMS-morphine derivative (m/z 429 $[M]^{\bullet+}$ unlabeled morphine).
†Theoretically labeled position.
‡An isotopic enrichment calculated from an amount of labeled morphine at the limit of detection.

(*R*)- and (*S*)-[*N*-$C^{2}H_3$]reticuline, [*N*-$C^{2}H_3$]1,2-dehydroreticulinium ion, [7-^{2}H]salutaridinol, 7-[7-^{2}H]episalutaridinol, [7-^{2}H,*N*-$C^{2}H_3$]oripavine, and [6-^{2}H]codeine is described in detail in *Supporting Text*, which is published as supporting information on the PNAS web site.

Enzymes. *S*-adenosylmethionine synthetase from *Escherichia coli* DM 25 pkA8 was kindly provided by George D. Markham (National Institutes of Health, Bethesda, MD). His-tagged *N*-methyltransferase from *E. coli* and pig liver microsomes was prepared in our laboratory. The clone of the recombinant *N*-methyltransferase enzyme in *E. coli* was supplied by T. M. Kutchan (Leibniz Institute of Plant Biochemistry, Halle, Germany).

Medium and Cell Lines. All culture media, sera, and gentamycin were obtained from Invitrogen. The human neuroblastoma SH-SY5Y cell line (ACC 209) was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The cells were cultured in 175-cm^2 flasks (Sarstedt) in Dulbecco–Vogt-modified Eagle's medium (Invitrogen) supplemented with 20% (vol/vol) FBS and 0.01% gentamycin (wt/vol) for 5 days. The flasks were incubated at 37°C in a humidified atmosphere (relative humidity >95%) of 5% CO_2 in air. The medium was renewed every other day. Cells that had reached ≈75% confluency (≈2×10^7 cells) were collected after brief exposure to trypsin/EDTA (0.05% trypsin, 0.02% EDTA, Sigma-Aldrich), pelleted (5 min, 800 × *g*), and washed with PBS. The cells were either directly processed or shock-frozen in liquid nitrogen and subsequently stored at −80°C for up to 7 days.

Sample Isolation and Derivatization. The cell pellet (≈3×10^9 cells) was resuspended in 3 ml of 1 M HCl, subjected to ultrasonic treatment (Sonifier 250, Branson), extracted with $CHCl_3$/isopropyl alcohol (9:1, vol/vol; 5 ml, 5 min of mixing at room temperature), and centrifuged (2,500 × *g*, 4°C, 15 min). 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 two 1-ml portions of methanol, two 1-ml portions of water at a flow rate of 1 ml/min, and then with two 1-ml portions of 0.05% trifluoroacetic acid at a flow rate of 5 ml/min. Subsequently, the cartridge was washed twice with 1 ml of water. Morphine was eluted with 7 ml of a mixture of water/acetonitrile/trifluoroacetic acid (89.95:10:0.05, vol/vol/vol). Eluted fractions were dried by using a high-vacuum evaporator (Bachofer, Reutlingen, Germany) and the isolated products were converted to the TMS derivatives by the addition of 20 μl of *N*-methyl-*N*-TMS-trifluoroacetamide and heating to 70°C for 30 min in an oil bath. The solution was concentrated to 5 μl under a gentle stream of nitrogen gas and centrifuged. One microliter of the supernatant was immediately analyzed by GC/MS/MS.

Feeding Experiments. We transferred 3×10^5 SH-SY5Y cells to 175-cm^2 flasks and cultured them in 25 ml of medium at 37°C for 24 h. After attachment of the cells, the medium was removed and replaced by fresh medium containing a labeled precursor at the concentration shown in Table 1. The cells were incubated for another 6 days, replacing the medium every 48 h with fresh medium containing the labeled precursor in question. After 7 days of culture, the cells were harvested and processed as described above.

GC/MS/MS. The GC/MS/MS analysis was performed on a Polaris Q^e Ion-Trap MS/MS (Finnigan-MAT, San Jose, CA) in electron-ionization mode (70 eV); ion source temperature, 200°C and MS transfer-line temperature, 300°C. GC analysis was performed with a FS-Supreme-5 capillary column (60 m, 0.25-mm i.d., 0.25-μm film thickness; CS Chromatographie Service) in splitless mode, with carrier gas helium at a constant flow rate of 1 ml/min. After sample injection, the initial injector temperature of 65°C was increased by 14.5°C/sec to 300°C. The initial oven temperature of 150°C was held for 1 min and then increased by 15°C/min to 300°C and held for 20

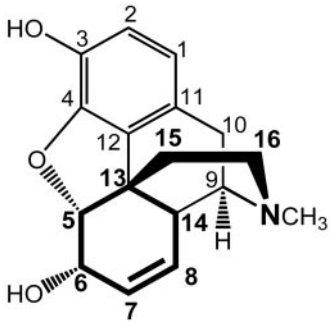

Fig. 1. Structure of the alkaloid morphine (the isoquinoline moiety is shown by bold lines and the nitrogen atom).

min. The MS/MS spectra were obtained by using molecular ions ($[M]^{\bullet+}$) as parent ions (TMS derivative of unlabeled morphine; m/z 429 $[M]^{\bullet+}$).

Results and Discussion

The Pathway to (*S*)-Reticuline. Numerous tracer experiments in plants have established that the morphine skeleton is derived from two molecules of L-tyrosine (for review, see ref. 5). From these early experimental results, it was inferred that (*S*)-norlaudanosoline, formed by means of condensation of dopamine and 3,4-dihydroxyphenylacetaldehyde, both derived from L-dopa, would be the first benzylisoquinoline intermediate (7). This suggestion was in agreement with early theoretical predictions (8, 9). However, the unequal distribution of radioactivity between the two labeled centers in L-[^{14}C]tyrosine-derived thebaine (5) and the nonrandom incorporation of $^{14}CO_2$ into morphine (10) could not be explained by the exclusive utilization of L-dopa as precursors, but rather required two C_6-C_2 units differing from each other in their biosynthetic origin. Indeed, reinvestigation of the early precursors of the morphine skeleton revealed that not the assumed tetraoxygenated (*S*)-norlaudanosoline, but rather the trioxygenated (*S*)-norcoclaurine, is the true precursor of the morphinan-type alkaloids in poppy plants (11–14). This finding also is in agreement with the hitherto inexplicable fact, that L-tyrosine labels both moieties of the alkaloids of the morphinan family, whereas L-dopa, dopamine, or tyramine label exclusively the isoquinoline part (that is, carbon atoms C-5–C-8 and C-13–C-16 and the nitrogen atom; Fig. 1) of these alkaloids in plants.

The revelation of the trioxygenated (*S*)-norcoclaurine as the correct entry metabolite of the tetrahydrobenzylisoquinoline pathway also led to the reinvestigation of the reticuline pathway in plants, (*S*)-reticuline being the central intermediate of the biosynthesis of >2,500 isoquinoline alkaloids in the plant kingdom including morphine and its congeners (14). The sequence of the (*S*)-reticuline biosynthetic pathway in plants is now shown to be (*S*)-configured norcoclaurine, coclaurine, *N*-methylcoclaurine, 3′-hydroxy-*N*-methylcoclaurine, and reticuline (14). Whereas the trioxygenated norcoclaurine has been isolated frequently from plants, including poppy, the tetraoxygenated norlaudanosoline has never been found as a natural plant product. However, in mammals, norlaudanosoline has been detected on few occasions: in rat striatum (15), human brain (16), a human pancreatic cell line (1), and humans treated with various pharmacological agents, for instance alcohol and L-dopa (17). In agreement with these findings, we have proven the endogenous origin of norlaudanosoline in human cell cultures by our previous experiments showing position specific labeling with ^{18}O and norlaudanosoline being part of the (*S*)-reticuline pathway in DAN-G cells (1).

Both in the poppy plant and in human neuroblastoma cells it could be demonstrated that labeled tyramine was incorporated exclusively into the isoquinoline moiety of morphine (1), albeit with low isotopic enrichment. In a similar application experiment, the neuroblastoma cells were cultured in the presence of 20 μM [2,2-$^2H_2$]dopamine, resulting in a 22% isotopic enrichment of this precursor in morphine. According to the characteristic MS fragmentation pattern of morphine, as investigated in our laboratory (18, 19), the dopamine molecule resided exclusively in the cyclohexene ring of morphine together with C-15, C-16, and the nitrogen atom (Table 1). The isolated morphine was also clearly labeled, as shown by a comparison of cellular derived unlabeled morphine vs. [15,15-$^2H_2$]morphine from this feeding experiment; m/z 429 vs. 431 $[M]^{\bullet+}$, 359 vs. 359 $[M - CH\text{-}CH_3NCH_2C^2H_2]^+$ (Fig. 2). When this experiment was repeated with L-[1,2,3-$^{13}C_3$]dopa as substrate, both parts of the morphine molecule, the benzyl and the isoquinoline unit, were labeled by the C_6-C_2 units derived from this amino acid (m/z 433 $[M]^{\bullet+}$, 361 $[M - CH\text{-}CH_3N^{13}CH_2{}^{13}CH_2]^+$; Fig. 3 *B* and *C*). The isotopic enrichment was 56%. Moreover, ring-labeled L-[*ring*-2′,5′,6′-$^2H_3$]dopa gave an isotopic enrichment of 40% of the

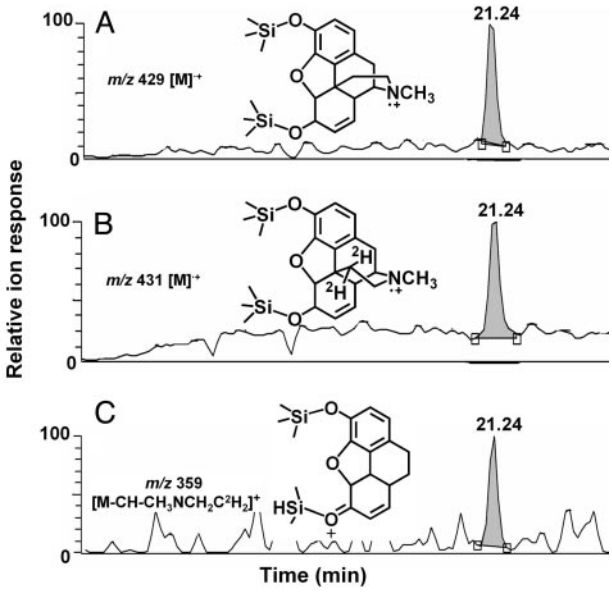

Fig. 2. GC/MS/MS chromatograms of morphine derivatives. (*A*) The m/z 429 $[M]^{\bullet+}$ of TMS-morphine isolated from SH-SY5Y cells cultured under standard conditions without isotope-labeled precursor. (*B*) The m/z 431 $[M]^{\bullet+}$ of TMS-[15,15-$^2H_2$]morphine isolated from SH-SY5Y cells cultured in the presence of 20 μM [2,2-$^2H_2$]dopamine. (*C*) The m/z 359 $[M - H\text{-}CH_3NCH_2C^2H_2]^+$ of TMS-[15,15-$^2H_2$]morphine isolated from SH-SY5Y cells cultured in the presence of 20 μM [2,2-$^2H_2$]dopamine. This fragment ion showed loss of two ^2H atoms at position C-15 of the morphine molecule.

morphine produced by the human cells, with the positions 1, 2, and 8 of the alkaloid retaining the deuterium atoms, whereas deuterium atoms in positions 5, 12, and 14 were lost during the biosynthetic transformation process (m/z 432 $[M]^{\bullet+}$, 362 $[M - CH\text{-}CH_3NCH_2CH_2]^+$; Fig. 3 *D* and *E*). This application experiment provided strong evidence that the pathways to (*S*)-reticuline in plants and mammals are highly different. This finding was further supported when the application of the isotope-labeled precursors of morphine in poppies, the trioxygenated norcoclaurine and coclaurine, to human neuroblastoma cells did not lead to incorporation in morphine (detection limit, level of isotopic enrichment of <0.5%), whereas feeding experiments with [1,3,4-$^2H_3$]norlaudanosoline showed 15% isotopic enrichment of the (*S*)-enantiomer but no incorporation of the (*R*)-enantiomer (Table 1). Moreover, these results showed that the methyltransferases catalyzing the transformation of (*S*)-norlaudanosoline to (*S*)-reticuline are highly stereoselective enzymes, because they did not accept the (*R*)-configured enantiomer.

The bulk of the now-available evidence is consistent with the idea that the initial steps of morphine biosynthesis in mammals involve a conversion of two molecules of L-tyrosine to L-dopa, one molecule being decarboxylated to dopamine, whereas the other molecule may be transformed to 3,4-dihydroxyphenylpyruvate, most likely by transamination, and subsequently decarboxylated to 3,4-dihydroxyphenylacetaldehyde or a similar reaction sequence. The product of an enzyme-catalyzed Pictet–Spengler-type reaction of dopamine and the corresponding aldehyde is thus the tetraoxygenated norlaudanosoline, formed stereospecifically as the (*S*)-enantiomer, which is subsequently transformed to (*S*)-reticuline by two O- and one N-methylation step. The sequence of these methylation steps, their underlying enzymes, and stereochemistry require further investigation. In view of these and our previous results (1), the former assumption that two molecules of dopamine, one molecule being transformed to 3,4-dihydroxyphenylacetaldehyde by a monoamine oxidase, can furnish norlaudanosoline does not seem to be correct for the *in vivo* situation. We clearly demonstrated

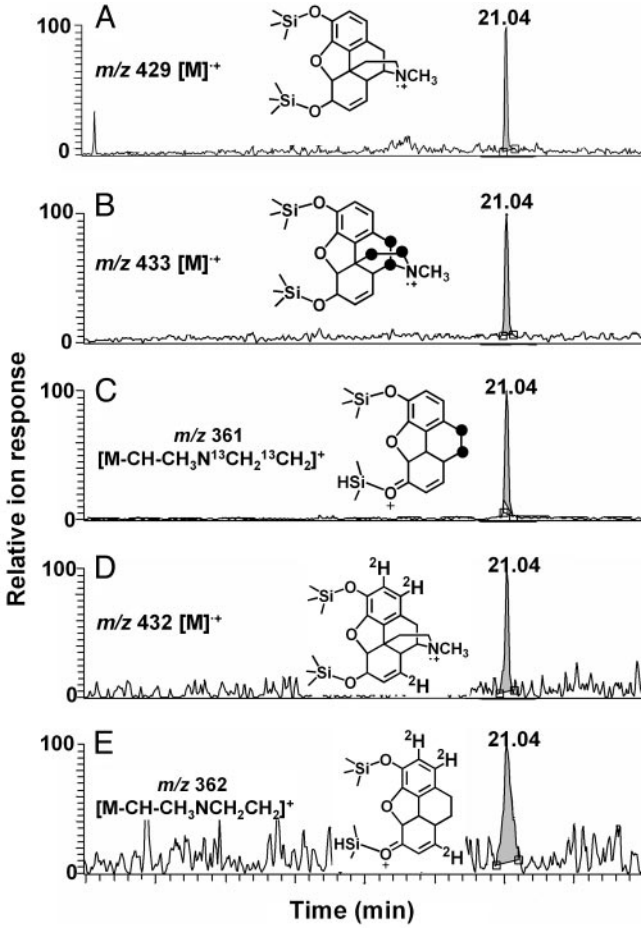

Fig. 3. GC/MS/MS chromatograms of morphine derivatives. (*A*) The *m/z* 429 $[M]^{\bullet+}$ of TMS-morphine isolated from SH-SY5Y cells cultured under standard conditions without isotope-labeled precursor. (*B*) The *m/z* 433 $[M]^{\bullet+}$ of TMS-[9,10,15,16-$^{13}C_4$]morphine isolated from SH-SY5Y cells cultured in the presence of 20 μM L-[1,2,3-$^{13}C_3$]dopa (^{13}C-enriched carbon atoms are indicated by ●). (*C*) The *m/z* 361 $[M - H\text{-}CH_3N^{13}CH_2{}^{13}CH_2]^+$ of TMS-[9,10,15,16-$^{13}C_4$]morphine isolated from SH-SY5Y cells cultured in the presence of 20 μM L-[1,2,3-$^{13}C_3$]dopa. This fragment ion showed loss of two ^{13}C atoms at positions C-15 and C-16 of the morphine molecule and retention of two ^{13}C atoms at positions C-9 and C-10 (^{13}C-enriched carbon atoms are indicated by ●). (*D*) The *m/z* 432 $[M]^{\bullet+}$ of TMS-[1,2,8-$^2H_3$]morphine isolated from SH-SY5Y cells cultured in the presence of 20 μM L-[*ring*-2′,5′,6′-$^2H_3$]dopa. (*E*) The *m/z* 362 $[M - H\text{-}CH_3NCH_2CH_2]^+$ of TMS-[1,2,8-$^2H_3$]morphine isolated from SH-SY5Y cells cultured in the presence of 20 μM L-[*ring*-2′,5′,6′-$^2H_3$]dopa. This fragment ion showed retention of all three ^{2}H atoms at positions C-1, C-2, and C-8.

that the benzyl moiety of morphine is labeled by neither tyramine nor dopamine (Fig. 2). The original observation of this reaction (summarized in refs. 17 and 20) was obviously because of chosen cell-free conditions. Dopamine can be transformed to 3,4-dihydroxyphenylacetaldehyde *in vitro*, which then forms racemic norlaudanosoline with excess dopamine. This reaction sequence does not occur *in vivo* (ref. 1 and as shown here).

Epimerization of Reticuline. (*S*)-reticuline is the central intermediate of isoquinoline alkaloid biosynthesis in higher plants. Morphine, however, belongs to the (9*R*)-series of the morphinan alkaloids. In plants, both (*S*)- and (*R*)-reticuline are well established precursors of morphine. Furthermore, it was shown that the C-1 hydrogen atom of (*S*)-reticuline is completely lost, whereas that one atom of (*R*)-reticuline is fully preserved during incorporation of these precursors into thebaine in *P. somniferum* plants (21). This inversion of configuration was most plausibly explained by the intermediate formation of the 1,2-dehydroreticulinium ion from (*S*)-reticuline, followed by stereospecific reduction to yield the desired (*R*)-configured enantiomer. This hypothesis was verified by feeding (22, 23) and enzymatic (24, 25) experiments with that quaternary alkaloid in poppy plants.

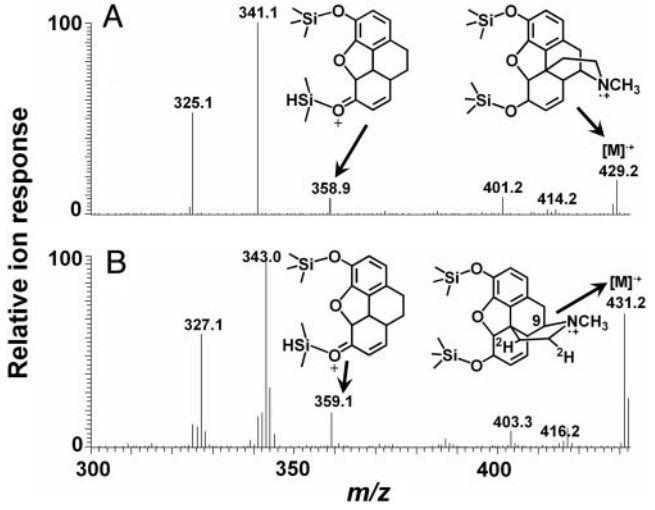

Fig. 4. MS/MS product ion spectra of morphine derivatives. (*A*) The *m/z* 429 $[M]^{\bullet+}$ ion of TMS-morphine isolated from SH-SY5Y cells cultured under standard conditions without isotope-labeled precursor. (*B*) The *m/z* 431 $[M]^{\bullet+}$ of TMS-[15,16-$^2H_2$]morphine isolated from SH-SY5Y cells cultured in the presence of 20 μM (*S*)-[1,3,4-$^2H_3$]norlaudanosoline. This product ion spectrum showed that the C-9 position, originating from C-1 of (*S*)-[1,3,4-$^2H_3$]norlaudanosoline, of the mammalian morphine did not carry a ^{2}H label, demonstrating a loss of one deuterium atom during mammalian morphine biosynthesis.

As in the poppy system, both enantiomers, (*S*)- and (*R*)-[*N*-C^2H_3]reticuline, were equally well incorporated into morphine in cultured human neuroblastoma cells, showing 23% and 25% isotopic enrichment, respectively. The mass spectra of the morphine formed from (*S*)- and (*R*)-[*N*-C^2H_3]reticuline showed fragments at *m/z* 432 $[M]^{\bullet+}$, 417 $[M - CH_3]^+$, and 359 $[M - CH\text{-}C^2H_3NCH_2CH_2]^+$ (Table 1 and Fig. 6, which is published as supporting information on the PNAS web site). In contrast, only the (*S*)-configured enantiomer of norlaudanosoline served as a precursor of morphine in these cells, the (*R*)-enantiomer exhibiting an isotopic enrichment of <0.5%, which is below the detection limit. Morphine that had been isolated from SH-SYSY cells cultured in the presence of (*S*)-[1,3,4-$^2H_3$]norlaudanosoline for 7 days was clearly labeled, as shown by a comparison of the mass spectra of cell-derived, unlabeled morphine vs. [15,16-$^2H_2$]morphine from the feeding experiment with labeled (*S*)-norlaudanosoline. GC/MS/MS analysis was performed with the respective TMS derivatives of morphine; *m/z* 429 vs. 431 $[M]^{\bullet+}$, 414 vs. 416 $[M - CH_3]^+$, 359 vs. 359 $[M - CH\text{-}CH_3NCH(D)CH(D)]^+$ (Fig. 4). The mass spectra also provided evidence that the deuterium atom located at position C-1 of (*S*)-norlaudanosoline corresponding to the C-9 position of the morphine molecule is lost during the enzymatic conversion to morphine. Based on these experiments, we suggest that a change of configuration from (*S*) to (*R*) during morphine biosynthesis in mammals takes place at the level of reticuline proceeding via the 1,2-dehydroreticulinium ion. Attempts to further test this hypothesis by feeding 20 μM [*N*-C^2H_3]1,2-dehydroreticuline to the human neuroblastoma cells and isolating labeled morphine failed, most likely because the quaternary alkaloid could not permeate the cell membrane. Nevertheless, the results presented here strongly indicate that the inversion of configuration of (*S*)- to (*R*)-reticuline in human neuroblastoma cells, the latter alkaloid being necessary to yield the (9*R*)-morphine, takes

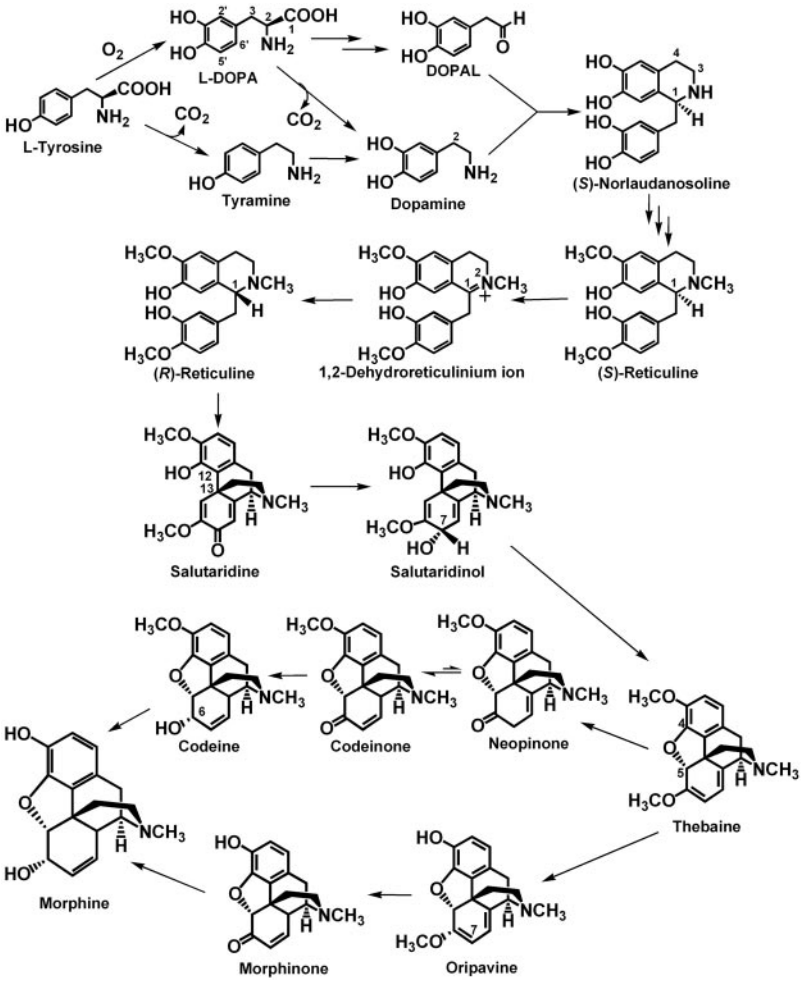

Fig. 5. An abbreviated biosynthetic pathway leading from L-tyrosine to morphine showing the initial isoquinoline alkaloid, (*S*)-norlaudanosoline, the change of configuration from (*S*)- to (*R*)-reticuline and the bifurcate pathway from thebaine to morphine.

place biochemically exactly in the same way as proven for the *Papaver* plant (24, 25).

The Morphinan Pathway. A crucial step in morphine biosynthesis is the C12–C13 oxidative phenol coupling of (*R*)-reticuline that generates the morphinan skeleton (26). Crude rat liver homogenates were shown to transform racemic reticuline to a compound that was most likely salutaridine (27). Later, a cytochrome P450 enzyme catalyzing the conversion of (*R*)-reticuline to salutaridine in the presence of cytochrome *c* reductase, NADPH, and O_2 was purified to homogeneity from pig liver and shown to be a highly regiospecific and stereospecific enzyme (28).

Focusing on steps succeeding the formation of salutaridine, we applied chemically synthesized and stereochemically pure 7-deuterated salutaridinol diastereomers separately to the human neuroblastoma cells at concentrations of 20 μM under standard conditions. Subsequently, the morphine formed by the cells was isolated and subjected as a TMS derivative to GC/MS (selected ion monitoring mode). However, morphine obtained from control SH-SY5Y cells (grown under standard conditions without isotope-labeled compound) showed not only the $[M]^{\bullet+}$ peak at m/z 429 $[M]^{\bullet+}$ but also the naturally occurring ^{13}C isotope peak at 1 mass unit higher (m/z 430 $[M]^{\bullet+}$), which would have interfered with the analysis of the [7-^{2}H]morphine (m/z 430 $[M]^{\bullet+}$). Accordingly, the isotopic enrichment of [7-^{2}H]morphine was analyzed by subtracting the selected ion monitoring mode signal of the natural ^{13}C-isotopic abundance from the selected ion monitoring mode signal at m/z 430 $[M]^{\bullet+}$ of the 7-^{2}H-labeled morphine obtained from SH-SY5Y supplied with [7-^{2}H]salutaridinol or 7-[7-^{2}H]episalutaridinol. The concentration of the naturally occurring ^{13}C isotope ion of reference morphine (m/z 430 $[M]^{\bullet+}$) was determined to be 18% ($\pm$1%; $n = 5$) of the $[M]^{\bullet+}$ (m/z 429 $[M]^{\bullet+}$). The concentration of m/z 430 $[M]^{\bullet+}$ representing both the natural ^{13}C-isotope ion and the [7-^{2}H]morphine $[M]^{\bullet+}$ was determined to be 33% of the $[M]^{\bullet+}$ of unlabeled morphine (m/z 429 $[M]^{\bullet+}$). Therefore, the isotopic enrichment of [7-^{2}H]morphine formed by SH-SY5Y cells that were supplied with [7-^{2}H]salutaridinol was calculated to be 15%. In contrast, the morphine obtained from the 7-[7-^{2}H]episalutaridinol feeding experiment was not significantly labeled, because the amount of m/z 430 $[M]^{\bullet+}$ representing both the naturally occurring ^{13}C-isotope ion of TMS-morphine and the $[M]^{\bullet+}$ of TMS-[7-^{2}H]morphine was only 17.8% of the $[M]^{\bullet+}$ of unlabeled morphine. As in the morphine biosynthesis in plants (29), these results indicate that salutaridinol, but not its 7-epimer, is the biological active intermediate and is stereo- and position-selectively transformed into morphine in human cells.

Ring closure to form the ether linkage during the transformation of salutaridinol to thebaine is the result of a nucleophilic attack of the phenol group on the dienol system and subsequent displacement of the hydroxyl (S_N2'-elimination). In *Papaver* plants, an additional reaction is used to improve the nature of the leaving group, which is accomplished by means of acetylation by a highly substrate-specific and stereospecific enzyme, acetyl-CoA: salutaridinol-7-*O*-acetyltransferase (30). The subsequent ring closure seems to occur without enzyme participation at pH values between 8 and 9 in the cytosol of the plant cell. We could show in our laboratory that this acetylation reaction also occurs in mammalian systems. Cell-free preparations of rat liver catalyzed the acetylation

of salutaridinol but not 7-epi-salutaridinol (data not shown). In light of these findings, it is very likely that the formation of thebaine from salutaridinol follows the same mechanism in human cells that has been proven for opium poppy (30).

Previously, we had shown that thebaine applied to neuroblastoma cells was transformed to morphine with 10% isotopic enrichment (1), establishing thebaine as a precursor in the morphine pathway. Thus, it also was demonstrated that the morphine produced by these human cells possesses the correct (*R*)-configuration at position C-9 of the molecule.

To complete the biosynthetic pathway leading to morphine, [6-^{2}H]codeine was applied to the SH-SY5Y cells. Morphine obtained from control SH-SY5Y cells (cultured under standard conditions without isotope-labeled compound) showed both the $[M]^{\bullet+}$ peak at m/z 429 $[M]^{\bullet+}$ and the naturally occurring ^{13}C-isotope peak at m/z 430 $[M]^{\bullet+}$, which again interfered with the analysis of monodeuterated [6-^{2}H]morphine (m/z 430 $[M]^{\bullet+}$). Therefore, the isotopic enrichment of [6-^{2}H]morphine was analyzed by GC/MS with selected ion monitoring mode, as described for the determination of the isotopic enrichment of [7-^{2}H]morphine obtained from the feeding experiment with [7-^{2}H]salutaridinol and SH-SY5Y cells. According to this method, the isotopic enrichment was calculated to be 21%, which clearly demonstrates that codeine can be transformed into morphine by these human cells.

Intriguingly, plants are capable of not only demethylating thebaine at the enol ether position (C-6) leading into the codeine branch toward morphine but also of demethylating first the phenol ether (at position C-3) yielding oripavine (31). This capability also was detected in rat liver and brain microsomes, which transformed thebaine in the presence of NADPH simultaneously into codeine and oripavine (32). Therefore, we investigated whether human cells also are able to generate morphine from oripavine. Doubly labeled [7-^{2}H,*N*-C^{2}H$_3$]oripavine was applied to the human neuroblastoma cell line; the morphine formed in these cells was isolated, subjected to TMS derivatization, and further analyzed by GC/MS/MS. The labeled morphine afforded an $[M]^{\bullet+}$ at m/z 433; the isotopic enrichment was 12% (see Fig. 7, which is published as supporting information on the PNAS web site). These results confirm that both codeine and oripavine are intermediates of morphine biosynthesis in human cells. This finding, in turn, implies that thebaine can serve as a substrate for two different competing cytochrome P450-dependent methyl ether cleaving enzymes acting either first at position C-3 or C-6, exactly as in the poppy plant. Which branch of the bifurcated pathway is dominant in human cells remains to be established.

On the basis of these and our previous results (1) of classical precursor feeding experiments, we are now capable of providing a detailed picture of the pathway to morphine in human cells starting from two molecules of tyrosine (Fig. 5). L-tyrosine is hydroxylated to yield L-dopa, which can undergo either decarboxylation to dopamine or a possible deamination to 3,4-dihydroxyphenylpyruvate, which, in turn, is assumed to be decarboxylated to 3,4-dihydroxyphenylacetaldehyde. The product of the stereospecific enzymatic condensation of dopamine and the aldehyde, resembling a Pictet–Spengler-type reaction, is (*S*)-norlaudanosoline [(*S*)-tetrahydropapaveroline]. This first tetraoxygenated isoquinoline alkaloid of the morphine pathway in mammals is subject to three methylation reactions involving *S*-adenosylmethionine as a methyl group donor to yield (*S*)-reticuline (33, 34). The sequence of methylation reactions remains to be established. The (*R*)-configured enantiomer of norlaudanosoline is biologically inactive. The necessary change of configuration to furnish the biological active (9*R*)-configuration of (−) morphine is accomplished at the level of (*S*)-reticuline by means of a stereoselective oxidation–reduction process and the intermediate 1,2-dehydroreticulinium ion. (*R*)-reticuline is the substrate for the intramolecular C12–C13 phenol oxidative coupling, catalyzed by a cytochrome P450-dependent enzyme (28), to yield salutaridine. Salutaridinol is formed from salutaridine by stereospecific enzymatic reduction of the carbonyl group. Ring closure to form the ether linkage in thebaine is the result of a nucleophilic substitution reaction. This cyclization step requires the acetylation of salutaridinol by an acetyl-CoA transferase to improve the nature of the leaving hydroxyl. Thebaine can undergo either an enol–ether (C-6) or phenol–ether (C-3) demethylation reaction to form codeine or oripavine, respectively. Both codeine and oripavine serve as substrates in a final demethylation step to produce the (−)morphine.

We have unequivocally shown that human neuroblastoma cells are able to synthesize morphine. The metabolic route starting from L-tyrosine involving at least 19 chemical steps shares remarkable similarities with the morphine biosynthesis in opium poppy. In the future, the identification of the respective genes and enzymes in humans and animals will provide information on the evolution of this pathway in the animal kingdom. The function of endogenous morphine is still a matter of discussion. In contrast to plants, where morphine is a highly specific secondary metabolite providing protection against herbivores, presumptions indicate that morphine in animals and humans may play a role as a general regulator and/or transmitter. Once these functions are identified, the genes and enzymes of morphine biosynthesis may become attractive targets for the modulation of pain, immune response, cell death, and behavioral phenomena.

We thank Dr. J. Schmidt (Leibniz Institute of Plant Biochemistry, Halle, Germany) for liquid chromatography MS/MS verification of the synthetic alkaloids. Chotima Boettcher (née Poeaknapo) thanks the German Academic Exchange Service (Deutscher Akademischer Austauschdienst, Bonn) for a scholarship. This work was supported by grants from the Deutsche Forschungsgemeinschaft (Bonn).

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