Structural Requirements in the Reaction of Morphine Uridine Diphosphate Glucuronyltransferase with Opioid Substances

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The influence of the 3-hydroxyl and *N*-alkyl groups in the reactivity of narcotic compounds with morphine UDP-glucuronyltransferase was studied. Opioids possessing both, one or none of these groups were tested for inhibition of morphine glucuronidation in rabbit liver microsomal preparations. Compounds with only a 3-hydroxyl group (normorphine) or an *N*-methyl group (codeine, ethylmorphine) were less potent competitive inhibitors than those containing both groups (dextrorphan). Norcodeine, with neither of these groups, had no inhibitory effect. The synthetic narcotics (+)- and (–)-methadone, (–)- α -acetylmethadol and meperidine, with only an *N*-alkyl group, were effective competitive inhibitors. No stereoselectivity of the morphine glucuronyltransferase for opioid isomers was observed, and [methionine]enkephalin does not react with morphine glucuronyltransferase. Differences of pK_a values and water/lipid solubility of narcotics could not explain the effects. Results indicate that the *N*-alkyl group plays a critical role in the interaction of narcotics with the morphine UDP-glucuronyltransferase.

Our laboratory has reported a glucuronyltransferase in microsomal fractions isolated from rat or rabbit liver which is active in morphine glucuronidation. The kinetics of this reaction have been reported for both microsomal and for solubilized and partially purified glucuronyltransferase preparations (Sanchez & Tephly, 1974; del Villar *et al.*, 1975). Similar kinetic values were obtained for both preparations, and narcotic antagonists were competitive inhibitors of morphine glucuronidation (del Villar *et al.*, 1977). We subsequently noted that the *N*-alkyl moiety of morphine and its substituents played an important part in determining either substrate specificity or inhibitory properties of narcotics for the morphine glucuronyltransferase. The present paper deals with the reactivity of morphine or morphinan agonists that have been selected for their substituents at the *N*-alkyl position or the 3-hydroxy position. Also, synthetic narcotic agents were used to reveal the importance of the *N*-alkyl group in the reactivity of these substances with morphine UDP-glucuronyltransferase (morphine-UDP-glucuronate glucuronyltransferase, EC 2.4.1.17) of rabbit hepatic microsomal fractions.

Materials and Methods

Chemicals

UDP-glucuronic acid (ammonium salt), β -glucuronidase (type B₁₀) and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO,

U.S.A.). [*N*-methyl-¹⁴C]Morphine hydrochloride (54mCi/mol) was obtained from Amersham/Searle (Arlington Heights, IL, U.S.A.). Morphine sulphate and codeine phosphate were obtained from Mallinckrodt Chemical Works (St. Louis, MO, U.S.A.). Norcodeine hydrochloride was from Merck and Co. (Rahway, NJ, U.S.A.). Ethylmorphine hydrochloride was obtained from Winthrop Laboratories (New York, NY, U.S.A.). [Methionine]enkephalin was purchased from Beckman (Drug Division, Palo Alto, CA, U.S.A.). (–)-Methadone hydrochloride was a gift from Eli Lilly (Indianapolis, IN, U.S.A.). Dextrorphan tartrate was kindly supplied by Dr. William E. Scott from the Research Department of Hoffman-La Roche Inc. (Nutley, NJ, U.S.A.). Meperidine hydrochloride was a gift from Dr. Fred Nachaud of the Research Department of Winthrop Laboratories. (+)-Methadone, (–)- α -acetylmethadol, (–)- α -acetylmethadol and (–)- α -acetyldinormethadol were kindly supplied by Dr. Ruth E. Billings (Toxicology Center, Iowa City, IA, U.S.A.).

Enzyme preparations

Frozen livers of 24h-starved mature albino rabbits (Pel-Freez, Rogers, AR, U.S.A.) were used. Homogenates and washed microsomal fractions were obtained, as described by Graham & Wood (1973), and suspended in 5.0mM-Tris/HCl buffer (pH8.0)/0.154M-KCl before use. Microsomal protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Determinations in triplicate of UDP-glucuronyl-

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transferase activity with morphine as a substrate were performed as described previously (del Villar *et al.*, 1974), except that the final volume of incubation mixtures was 0.5 ml. All substances added to reaction mixtures for morphine glucuronidation were freely soluble at pH 8.0 and at the concentrations used. Control reactions contained no UDP-glucuronate and reactions were terminated by the addition of 1.0 ml of ice-cold 10% (w/v) trichloroacetic acid. After centrifugation (1000 *g*, 15 min), 1 ml of clear supernatant was taken for the analysis of morphine 3-glucuronide. The rate of morphine 3-*O*-glucuronidation was determined by the radioisotopic technique described previously (del Villar *et al.*, 1974). All the product formed was the 3-*O*-glucuronide (Sanchez & Tephly, 1974). All the morphine detected in the aqueous phase could be extracted into the organic phase after treatment with 2000 units of β -glucuronidase for 16 h. Michaelis constants were determined with an IBM 360 computer with programs for enzyme kinetics written by Cleland (1963, 1967). All inhibition patterns were competitive.

Results

Effect of morphine analogues on the rate of morphine glucuronidation in rabbit hepatic microsomal preparations

Essentially all glucuronide synthesis with morphine occurs at the 3-hydroxy position (Fujimoto & Way, 1958). Therefore the 3-hydroxyl group of the morphine molecule is a major determinant for reactivity of a substance with microsomal morphine UDP-glucuronyltransferase. This was shown by studying the inhibitory properties of morphine analogues on morphine glucuronidation. Structural characteristics of these compounds are presented in Table 1. 3-*O*-Alkyl ethers of morphine, such as codeine and ethylmorphine, were weaker competitive inhibitors of morphine glucuronidation than was dextrorphan, which has a free 3-hydroxyl group (Table 2). However, it was surprising that norcodeine, which differs from codeine only in that it has no *N*-methyl group, was completely inactive as an inhibitor of this reaction. This is an indication that the *N*-alkyl substituent is important for the interaction of opiate substrates with the enzyme.

Normorphine produces an effective competitive inhibition, which was expected since it has a free 3-hydroxyl group. In general, agents with the 3-hydroxyl group, such as the narcotic antagonists naloxone, nalorphine, levorphan and naltrexone, are more potent as inhibitors of the reaction than is normorphine (del Villar *et al.*, 1977). Dextrorphan, which contains a free 3-hydroxyl group and which also possesses an *N*-methyl group, is a more potent competitive inhibitor than is normorphine. Therefore not only is the 3-hydroxyl position a critical one

Table 1. *Structural features of morphine analogues reacting with morphine UDP-glucuronyltransferase*

R ¹	R ²	Compound
-H	-CH ₃	Morphine
-CH ₃	-CH ₃	Codeine
-CH ₂ -CH ₃	-CH ₃	Ethylmorphine
-CH ₃	-H	Norcodeine
-H	-H	Normorphine
-H	-CH ₃	Dextrorphan (<i>o</i> -dextro isomer) Levorphan (<i>o</i> -laevo isomer); no C ₍₄₎ -C ₍₅₎ O bridge, no C ₍₇₎ -C ₍₈₎ double bond

Table 2. *Inhibition constants of certain morphine analogues on morphine glucuronidation in rabbit liver microsomal fractions*

Concentrations of inhibitors were 1.0 mM in morphine glucuronidation reaction mixtures, which contained variable concentrations of morphine sulphate (0.2–2.0 mM), UDP-glucuronic acid (10 mM) [*N*-methyl-¹⁴C]morphine hydrochloride (0.136 μ Ci), Tris/HCl (50 mM), MgCl₂ (5.0 mM) and 1.0 mg of microsomal protein in 0.5 ml reaction volumes. *K*_i values ($\pm$ S.E.M., *n* = 2) were determined with the use of computer programs prepared by Cleland (1963, 1967) for competitive enzyme inhibition.

Inhibitor	<i>K</i> _i (mM)
Dextrorphan	0.17 $\pm$ 0.02
Levorphan	0.25 $\pm$ 0.07
Normorphine	0.38 $\pm$ 0.09
Codeine	0.60 $\pm$ 0.16
Ethylmorphine	1.42 $\pm$ 0.30
Norcodeine	No inhibition

for substrate reactivity with morphine UDP-glucuronyltransferase, but the *N*-alkyl group also seems to be an important determinant in the reactivity of a substance with morphine UDP-glucuronyltransferase. Morphine UDP-glucuronyltransferase is reactive with (+)-isomers of the morphine class, since dextrorphan is as effective as an inhibitor as is levorphan.

Table 3. *Structural features of synthetic narcotic agents reacting with morphine UDP-glucuronyltransferase*

R ¹	R ²	R ³	Compound
-CH ₃	-CH ₃	-CO-C ₂ H ₅	(+)-Methadone
-CH ₃	-CH ₃	-CH(CO ₂ CH ₃)-C ₂ H ₅	(-)-α-Acetylmethadol
-H	-CH ₃	-CH(CO ₂ CH ₃)-C ₂ H ₅	(-)-α-Noracetylmethadol
-H	-H	-CH(CO ₂ CH ₃)-C ₂ H ₅	(-)-α-Dinoracetylmethadol
			Meperidine

Table 4. *Inhibition constants of certain synthetic narcotics on morphine glucuronidation in rabbit liver microsomal fractions*

Conditions were similar to those described in Table 2. K_i values ($\pm$ S.E.M., $n=2$) were determined with the use of computer programs prepared by Cleland (1963, 1967) for competitive enzyme inhibition.

Inhibitor	K_i (mM)
(+)-Methadone	0.09 ± 0.02
(-)-Methadone	0.10 ± 0.02
Pethidine	0.40 ± 0.05
(-)-α-Acetylmethadol	0.12 ± 0.02
(-)-α-Noracetylmethadol	0.28 ± 0.03
(-)-α-Dinoracetylmethadol	0.25 ± 0.07

Effect of synthetic narcotic analgesics on the rate of morphine glucuronide formation in rabbit hepatic microsomal preparations

To study further the influence of the *N*-alkyl group in the interaction of narcotic substances with morphine glucuronyltransferase, a number of synthetic narcotic agents bearing *N*-alkyl groups, but lacking any hydroxyl groups, were tested for their effects on morphine glucuronidation *in vitro*. Structural features of these compounds are shown in Table 3. Bisubstrate kinetic reactions were studied, and inhibition constants obtained in these experiments are summarized in Table 4. The enzyme reacts equally well with (+)- and (-)-methadone, indicating the lack of recognition of stereospecificity by enzyme in agent-enzyme interaction. In the (-)-α-acetylmetha-

dol series the *N*-methylated compound is more potent than the nor- or dinor-demethylated substance.

The pharmacology of an endogenous morphine-like substance, [methionine]enkephalin, a synthetic pentapeptide with analgesic activity equivalent to that of normorphine, has been under intense investigation (Goldstein, 1976). This substance has been reported to interact with the opioid receptor in systems *in vitro* and *in vivo* (Kosterlitz & Waterfield, 1975), effects that are apparently antagonized by naloxone. Since narcotic agonists and antagonists were competitive inhibitors of morphine glucuronidation *in vitro*, we studied the effect of [methionine]-enkephalin on this reaction to determine whether a metabolic interaction exists between the synthetic pentapeptide and the morphine UDP-glucuronyltransferase. In experiments where [methionine]-enkephalin was added up to concentrations of 1.0 mM, no inhibition of morphine glucuronidation was observed in rabbit hepatic microsomal preparations.

Discussion

Previous results from our laboratory have clearly shown the separation of a morphine UDP-glucuronyltransferase activity from a *p*-nitrophenol UDP-glucuronyltransferase activity. These results were obtained from both rat and rabbit hepatic microsomal preparations (Sanchez & Tephly, 1974; del Villar *et al.*, 1975, 1977). The solubilized separated morphine UDP-glucuronyltransferase activity possessed characteristics similar to those that had previously been studied in the intact microsomal

preparation, in that there was no mutual competitive inhibition between morphine and *p*-nitrophenol; there was inhibition of the morphine glucuronyltransferase by morphine 3-glucuronide and by narcotic antagonists, but no effect of these substances on *p*-nitrophenol glucuronidation. *p*-Nitrophenyl glucuronide did not inhibit morphine glucuronidation, nor did morphine glucuronide inhibit *p*-nitrophenol glucuronidation.

Recent studies have shown the usefulness of the binding technique of certain optical isomers of narcotic substances to opioid receptors and its reversal by narcotic agonists or antagonists (Terenius, 1973; Pert & Snyder, 1974). The current study takes advantage of this approach in a similar way, except that functional activity was assessed, namely a glucuronide-synthetic reaction instead of release of binding. The availability of a variety of derivatives of morphine, morphinans and synthetic narcotic analgesics provided an opportunity to study certain structural features of the narcotic agonist or antagonist class of agent and to associate the structural features with reactivity of the given substance and morphine glucuronyltransferase. Since it is well known that the 3-hydroxyl and the *N*-alkyl groups of the morphine structure are important for its pharmacological activity, and since substances were available which possessed the *N*-alkyl moiety without a phenolic group, it was possible to evaluate the importance of these positions in the interaction of a substance with morphine glucuronyltransferase.

The morphine glucuronyltransferase could not distinguish between stereoisomers, since (+)- and (–)-methadone were equally effective as inhibitors, and dextrorphan and levorphan were also equally effective as inhibitors of morphine glucuronidation. Therefore (+)- and (–)-isomers could be used for study interchangeably. Importance of the 3-hydroxy position is implicit in the reaction mechanism, and substitution at this position, as with codeine or ethylmorphine, provided a less effective inhibitory substance than those agents that possess a free 3-hydroxyl group, such as dextrorphan and levorphan. Although normorphine possessed a 3-hydroxyl group, it was surprising that it was less effective as an inhibitor of morphine glucuronidation than was dextrorphan or levorphan. This was the first indication that the *N*-alkyl position was important in the interaction of the substance with the morphine glucuronyltransferase. Norcodeine, a morphine analogue that contains neither an *N*-alkyl nor a free hydroxyl group, was completely unreactive with the morphine glucuronyltransferase. This information shows a key role for the *N*-alkyl position in the binding of a substance with the morphine glucuronyltransferase. In agreement with this concept are the effective competitive-inhibitory properties of the methadone and (–)- α -acetylmethadone class, agents

that possess *N*-alkyl groups but lack hydroxyl moieties. With the (–)- α -acetylmethadol class of compounds the dimethylated compound was more potent than the demethylated substances, an indication that the presence of more than one alkyl group leads to a more effective reactivity between substances and the enzyme. These results suggest that the *N*-alkyl group confers on the synthetic narcotics a high degree of affinity for the active site(s) of UDP-glucuronyltransferase, enabling them to behave as competitive inhibitors of morphine glucuronidation, in spite of the lack of a hydroxyl group. In this type of inhibition the inhibitor may act as alternative substrate for the enzyme or as a 'dead-end' inhibitor. The first possibility would only involve the formation of *N*-glucuronides, a metabolite that has not been shown to be formed from narcotics in reactions *in vitro*. In experiments using pure synthetic morphine 3-*O*-glucuronide as a standard we were only able to identify this metabolite as a product of the reaction of morphine with UDP-glucuronate, in liver microsomal preparations (Sanchez & Tephly, 1974). *N*-Glucuronidation of narcotics *in vitro* would require an *N*-demethylation followed by *N*-hydroxylation reaction, before the conjugation with UDP-glucuronate. The cofactors and optimal conditions for these oxidative steps are not provided in the system for morphine glucuronidation. Therefore the competitive inhibition of morphine glucuronidation by narcotics is more probably a result of a 'dead-end' inhibition. It can be assumed that, in this case, the substituted amine reversibly interacts with sensitive components of the active site of morphine UDP-glucuronyltransferase. In fact, this effect may be specific for morphine binding to the enzyme, since in the reaction mechanism of competitive inhibition of morphine glucuronide formation by naloxone, this compound produced competitive inhibition for morphine (second substrate) and not for UDP-glucuronate (del Villar *et al.*, 1977).

The relative reactivity of substances with the UDP-glucuronyltransferase in hepatic microsomal fractions cannot be attributed to physical-chemical characteristics of these substances used in this study. Although one might argue that different lipid solubilities regulate the reactivity of substances with morphine glucuronyltransferase in hepatic microsomal fractions, there is no evidence to support this view. For example, the relative pK_a values between morphine and normorphine are similar (9.85 and 9.70 respectively) and the relative water solubility appears to play no role, since morphine 3-glucuronide is a potent inhibitor of morphine glucuronyltransferase in hepatic microsomal fractions and possesses much higher water-solubility than does the parent substrate (Kumbler, 1955). In addition, the potent competitive inhibition by nalorphine of morphine glucuronidation is not correlated with its pK_a (7.23). Norco-

deine, which is ineffective as an inhibitor of morphine glucuronyltransferase, has a similar pK_a to codeine, a modest inhibitor of morphine UDP-glucuronyltransferase.

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