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3-O-Acetylmorphine-6-O-Sulfate: A Potent, [illegible]

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[illegible] conjugations at the 3- and 6-position, appear to be due to their altered interactions with opioid receptors.

Morphine derivatives Analgesia Tail-flick test Central administration μ-Receptor δ-Receptor κ-Receptor

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THE OPIATE analgesic morphine, when administered to humans, is converted by the liver into three major metabolites, viz. morphine-3-O-glucuronide (M3G), morphine-6-O-glucuronide (M6G), and morphine-3-O-sulfate (M3S) (9,19). M6G is found in the systemic circulation in concentrations exceeding those of morphine itself, after both parenteral (12,19) and oral administration (12,18). M6G is a very potent μ-receptor agonist (3) with a high affinity for both mu_1 and mu_2 receptors (1,14) and appears to cross the blood–brain barrier in spite of [illegible] the clinical activity of M6G in a group of six cancer patients, it has been concluded that the analgesic effect of administered morphine is due mainly to metabolically formed M6G rather than to morphine itself (11). Interestingly, the related conjugate, morphine-6-O-sulfate (M6S) was shown to exhibit more potent and longer acting analgesia than morphine itself in mice (2). On the other hand, M6S showed comparatively reduced competing potencies toward mu-receptors but enhanced delta-receptor affinity compared to parent compound morphine (10).

In view of the potent analgesia exhibited by the apparent structurally dissimilar 6-O-glucuronide and 6-O-sulfate conjugates of morphine, we have examined the effect of structural [illegible] particular interest was the effect of increasing lipophilicity on activity by esterification of the 3-hydroxy group and the effect of increasing polarity by conversion of M6S to its *N*-methylmorphinium betaine (see Fig. 1). As part of these struc-

[1] [illegible] KY 40546-0236.

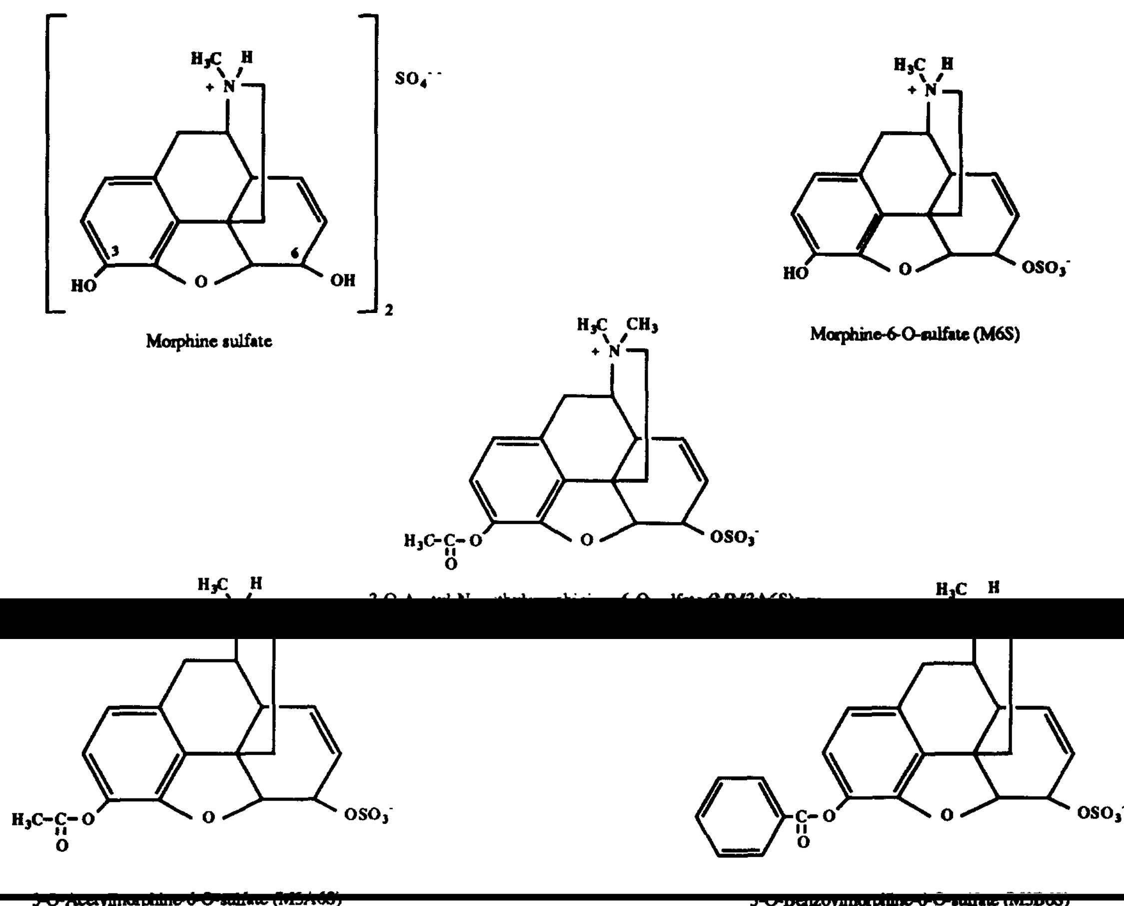

FIG. 1. Structures of morphine and morphine-6-O-sulfate derivatives.

ture–activity studies, we now report on the potent, centrally acting antinociceptive activity of the morphine derivative, 3-

METHOD

Animals and Surgery

The assessment of analgesic response by the tail flick test was carried out on male Sprague–Dawley rats (Harlan Industrial, Indianapolis, IN) weighing 270–310 g at the time of

a 12 L : 12 D cycle for 1 week before use in the studies. Food and water were available ad lib.

the duration of the surgery. A stainless steel guide cannula (Plastic Products, Roanoke, VA) was implanted over the left lateral cerebral ventricle. The rat was positioned in a stereotaxic frame (David Kopf); stereotaxic coordinates were 0.8 mm posterior to (Lang Dental Manufacturing Co., Chicago, IL). A 28-gauge dummy cannula was kept in the implanted cannula, except during intracerebroventricular (ICV) injec-

Antinociceptive Testing

D'Amour and Smith (4). The radiant heat was focused on the tail tip of a male Sprague–Dawley rat, and the heat intensity was adjusted so that a control animal flicked its tail within 1.7–2.6 s of exposure. To prevent tissue damage in drug-

Each rat served as its own control, because the latency to response was measured both before and after drug administra-

$$= \frac{\text{Post-drug Latency} - \text{Base line Latency}}{\text{Cutoff Time (8.0 s)} - \text{Base line Latency}} \times 100$$

Experimental Protocol

On the day of the experiment, rats were placed in a restrainer for 15 min before actual testing began. Four baseline

Scientific Co.) prior to injection (1 ml/kg). Morphine [5 mg/

continued.

For ICV administration, test compounds were dissolved in saline prior to administration. On the day of the experiment, 30 min before drug administration, animals were handled

nula into the lateral ventricle. The injector consisted of a segment of stainless steel tubing connected to a Hamilton Syringe by PE20 tubing. The rats were then placed in restrainers for

of the drug solution (each treatment consisted of 4 μl of solution per rat) was made over 1 min, using a preprogrammed syringe pump (Tracor, Atlas, Houston, TX). The tail-flick procedure was then continued at 3-min intervals for the first 30–45 min and then at longer time intervals (15–30 min) until

doses of morphine were used; low dose morphine 0.236 μg/rat (0.83 pmol μmol/rat as free base), high dose morphine 23.6 μg/rat (83 pmol/rat). Doses of morphine derivatives administered ICV were equimolar to the low dose of morphine and were as follows: M6S (0.22 μg); M3A6S (0.25 μg); M3B6S

movement of a small air bubble over a calibrated distance

was verified by examining the cerebral ventricle after a 4 μl

tized rat.

Receptor Binding

Hartley guinea pigs were decapitated and their brains were quickly removed and weighed. The brains were then homoge-

tration of 6.67 mg original wet weight of tissue per ml, except

receptor binding sites indicated in parentheses: (^{3}H)DAMGO [D-Ala^2,N-Me-Phe^4,Gly ol^5]-enkephalin, (mu), (^{3}H)DPDPE [D-Pen^{2,5}-enkephalin], (delta), (^{3}H)U69,593 (kappa$_1$), (^{3}H)-NalBzoH (kappa$_3$). The guinea pig brain suspension (1.8 ml)

determined by incubating in the presence of 1 μM of the cold unlabeled counterpart of each labeled ligand, except that 10 μM $\pm$ NalBzoH was used for the kappa$_3$ assay. The samples were then filtered through glass fiber filters on a 48-well Brandel cell harvester. The filters were washed three times with 3 ml of buffer. Filters were incubated overnight with 5 ml of scintillation cocktail before counting.

Results have been reported in terms of IC$_{50}$ (concentration

[L]/K_d. The K_d values were obtained by computer analysis of

Guinea pig ileum (GPI) preparation. Male Hartley guinea pigs (350–400 g wt.) were decapitated and their small intestines were removed; about 20 cm of the terminal ileum was discarded. The longitudinal muscle with the myenteric plexus attached was gently separated from the underlying circular muscle by the method of Paton and Vizi (13). The muscle strip was mounted in an 8 ml water-jacketed organ bath containing

mM KH$_2$PO$_4$, 1.2 mM MgSO$_4$ and 11.5 mM glucose. The tissues were kept at 37°C and bubbled with 5% CO$_2$ in oxygen. An initial tension of 0.6–1.0 g was applied to the strips. The muscle strip was stimulated for 60 min before the start of each experiment.

electrode is a ring of 4 mm diameter. The parameters of rectangular stimulation were as follows: supramaximal voltage, 1 ms impulse duration at a rate of 0.1 Hz. A Grass S-88 electrostimulator was used for stimulation. The electrically

from Swiss-Webster mice (30–35 g) were prepared as described

containing 8 ml of magnesium free Krebs solution at 31°C, which was bubbled with a mixture of oxygen and carbon dioxide

paired shocks of 100 ms delay between supramaximal rectangular pulses of 1 ms delay between supramaximal rectangular pulses of 1 ms duration were delivered at a rate of 0.1 Hz. A

Functional Activity

contractions. To determine the site(s) at which the agonists acted, assays were conducted in the presence of site-selective antagonists. A shift of the dose–response curve to the right is indicative of activity at the antagonist bound site. CTAP (100

nM nor-BNI [nor-binaltorphimine] were added to the GPI preparation to selectively block mu (7) and kappa (15) receptors, respectively; 1 nM naltrindole was added to the MVD preparation to selectively block delta receptors (16). This activity was characterized by dose ratios (DR). DR was calculated from the following equations:

$$DR = IC_{50} \text{ in the presence of antagonist}/IC_{50}$$

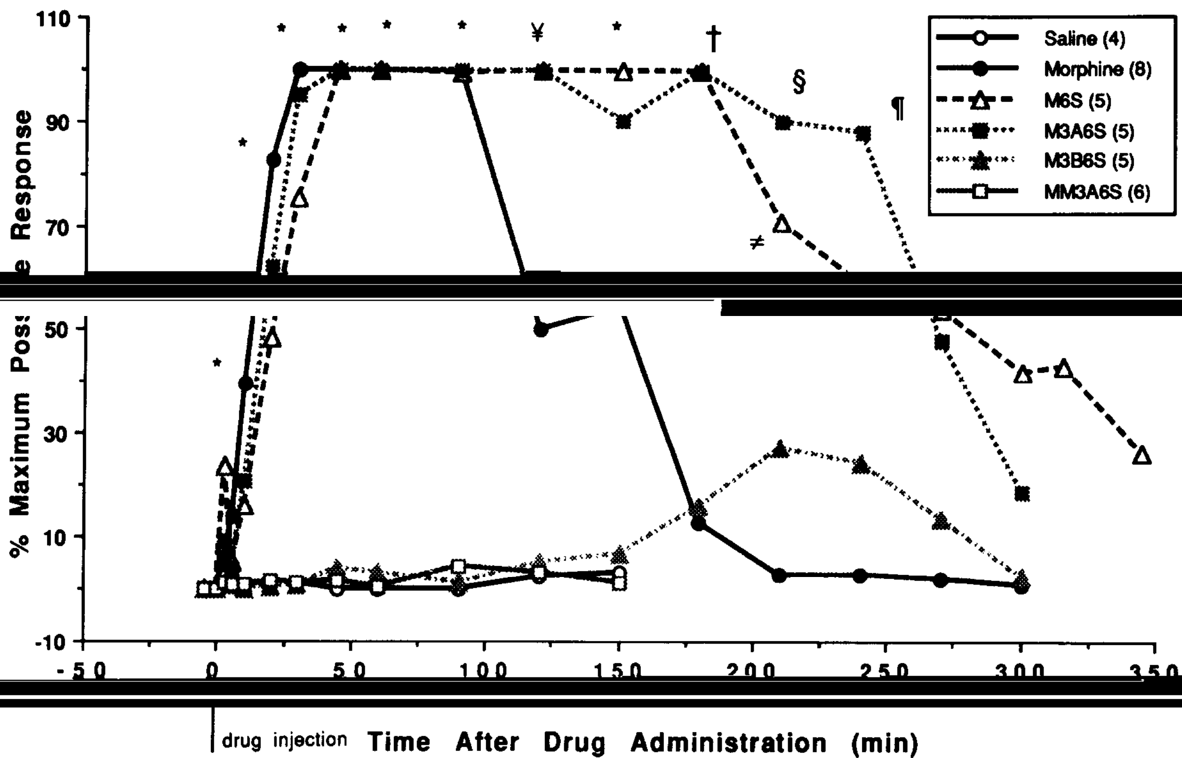

FIG. 2. Antinociceptive response to SC treatment with morphine. Antinociception is shown as the percent of maximum possible
morphine derivatives were as follows: M6S (6.4 mg/kg); M3A6S (7.1 mg/kg); M3B6S (8.2 mg/kg); and MM3A6S (7.4 mg/kg) were administered subcutaneously. Predrug response latencies for saline, morphine, M6S, M3A6S, M3B6S, and MM3A6S were 2.15, 1.96, 2.37, 1.79, 1.95, and 2.11 s, respectively. Analysis of variance revealed a highly significant treatment by time interaction. *Represents a significant difference ($p < 0.005$) of morphine, M6S, and M3A6S from the corresponding response to saline-, M3B6S-, and MM3A6S-treated rats. ¥Represents a significant difference ($p < 0.0006$) of M6S and M3A6S from the
and M3A6S from the corresponding response to morphine- and M3B6S-treated rats. §Represents a significant difference ($p < 0.0007$) of M6S and M3A6S from the corresponding response to morphine. ≠Represents a significant difference ($p < 0.0007$) of M3A6S from the corresponding response to M3B6S treated rats. ¶Represents a significant difference ($p < 0.004$) of morphine, M6S, and M3A6S from the corresponding response to morphine treated rats. Comparisons were made using two-way repeated measure ANOVA followed by Tukey's test. Numbers in parentheses represent the number of rats per group.

Chemicals

The compounds utilized in this study were morphine sul-

zoylmorphine-6-O-sulfate (M3B6S), and 3-O-acetyl-*N*-methylmorphinium-6-O-sulfate (MM3A6S). The sulfate esters were synthesized in our laboratories and their preparation is reported elsewhere (Crooks et al., manuscript in preparation).

Equithesin was composed of chloral hydrate (2.13 g, Sigma Chemical Co., St. Louis, MO), magnesium sulfate (1.07 g, Fisher Scientific Co., Fairlawn, NJ), propylene glycol (14.1 ml, Fisher Scientific Co.), ethanol (3.8 ml, Curtin Matheson,

barbital (7.5 ml, 64.8 mg/ml, Butler Co., Columbus, OH).

Statistical Analysis

number of rats. Two-way repeated measure with interaction ANOVA followed by Tukey's post hoc test for comparisons was utilized for analysis between groups treated with different drugs and tested for analgesia.

Antinociceptive Activity of Morphine-6-O-Sulfate Derivatives After Subcutaneous Injection

The antinociceptive activities of M6S, M3A6S, M3B6S,

method following SC injection, are illustrated in Fig. 2. The onset of morphine (5 mg/kg, SC) analgesia started within 10 min of administration; maximal analgesic response was achieved within 30 min and was maintained for 60–80 min.

lished reports (5,8). An equimolar dose of M6S produced a maximal analgesic effect similar to morphine within 30 min

of action and maximal analgesic effect, which was maintained for 4 h. Interestingly, M3B6S at an equimolar dose, showed

only a gradual onset of weak analgesia over 100 min, which reached a maximum equivalent to only 27% of maximal analgesia over 4 h. The dipolar betaine, MM3A6S, was devoid of [illegible] dose.

Antinociceptive Activity of Morphine-6-O-Sulfate Derivatives After Intracerebroventricular Administration

The above five compounds were also evaluated for analgesic activity by the tail flick assay after administration by intracerebroventricular injection. These results are shown in Fig. 3. Two doses of morphine were initially employed. The lower dose of morphine (0.23 μg/4 μl/rat) failed to produce an analgesic effect. The higher dose (23.6 μg/4 μl/rat) produced a rapid analgesic response (6 min after ICV injection), and maximal analgesia was achieved within 10 min of administration and maintained for an additional [illegible] of M6S at a molar dose equivalent to the lower dose of morphine produced a maximal analgesic effect, within 10 min, that was maintained for 70 min. M3A6S administered at [illegible] maximal analgesia being established at 10 min and lasting for an additional 90 min. A similar dose of either M3B6A or MM3A6S afforded no analgesic response over the 100 min [illegible]

Receptor Binding

The results of binding studies using guinea pig brain homogenates are presented in Table 1. M6S and M3A6S displayed a greater affinity than that of morphine to mu and $kappa_3$ receptors. Both compounds were even more mu-selective than DAMGO, a mu-preferring peptide. M6S also had greater affinity than that of morphine to delta receptors. Both M6S and M3A6S bound kappa1 sites weakly in guinea pig brain homogenate. In addition, M3A6S showed a better ability to discriminate between mu and delta sites than morphine [illegible] morphine, respectively). In contrast, the K_i of the nonanalgesic compounds M3B6S and MM3A6S were 15× and 14× [illegible]

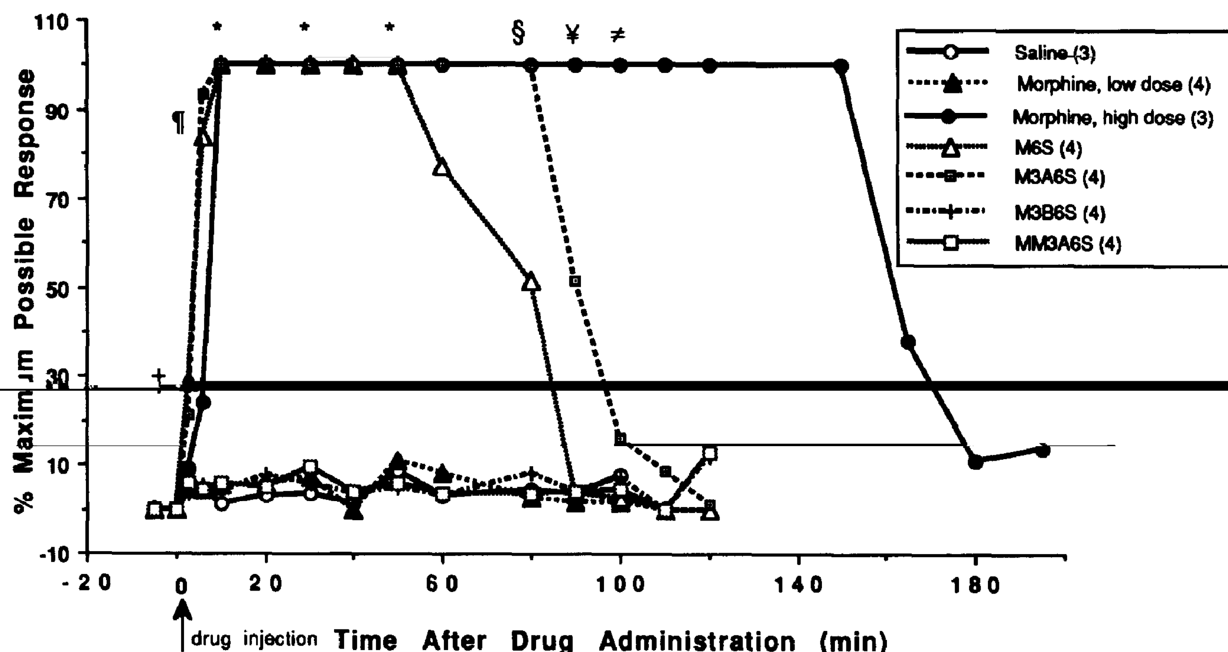

FIG. 3. Antinociceptive response to treatment with saline vehicle, morphine, and morphine derivatives. For intracerebroventricular drug administration, two doses of morphine were used: low dose morphine [illegible] μmol/rat as free base) and high dose of morphine (23.6 μg/rat, 83 pmol/rat). Doses of morphine derivatives administered intracerebroventricularly were equimolar to the low dose of morphine and were as follows: M6S (0.22 μg); M3A6S (0.25 μg); M3B6S (0.29 μg); and MM3A6S (0.26 μg). Antinociception is shown as the percent of maximum [illegible] M6S, M3A6S, M3B6S, and MM3A6S were 2.05, 2.06, 1.69, 1.92, 2.02, 2.06, and 1.82 s, respectively. Analysis of variance revealed a highly significant treatment by time interaction. †Represents a significant difference ($p < 0.009$) of M6S from the corresponding response to saline-, M3B6S-, and MM3A6S-treated rats. ¶Represents a significant difference ($p < 0.0001$) of M6S and M3A6S from the corresponding response to morphine high dose-, saline-, M3B6S-, MM3A6S-, and morphine low dose-treated rats. *Represents a significant difference ($p < 0.0001$) of morphine high dose, M6S, and M3A6S from the corresponding response to MM3A6S, morphine low dose, M3B6S, and [illegible] corresponding response to M3B6S, saline, MM3A6S and morphine low dose. ¥Represents a significant difference ($p < 0.001$) of morphine high dose from the corresponding response to M3B6S-, MM3A6S-, saline-, M6S-, and morphine low dose-treated rats. ≠Represents a significant difference ($p < 0.004$) of morphine I from the corresponding response to MM3A6S, M3B6S, M3A6S, M6S, and saline treated rats. Comparisons were made using two-way repeated measure ANOVA followed by Tukey's test. Numbers in parentheses represent the number of rats per group.

TABLE 1

INHIBITORY EFFECT (K_i) OPIOIDS ON THE BINDING OF TRITIATED LIGANDS TO MU, DELTA, [illegible]

Cold Ligand	K_i (nM)			
	[^{3}H]DAMGO mu	[^{3}H]DPDPE delta	[^{3}H]U69593 kappa-1	[^{3}H]NalBzoH kappa-3
DAMGO	1.1 ± 0.2	180.4 ± 16.0	1,841.3 ± 22	26.9 ± 0.9
DPDPE	> 10,000	2.8 ± 0.4	> 10,000	> 10,000
U69593	692.0 ± 97.0	1,358.0 ± 118.0	0.7 ± 0.1	1,099.0 ± 0.2
NalBzoH	0.2 ± 0.0	1.4 ± 0.1	0.4 ± 0.1	0.3 ± 0.07
Morphine	2.0 ± 0.3	50.0 ± 0.6	33.9 ± 9.0	13.9 ± 1.7
M6S	0.9 ± 0.0	18.0 ± 0.4	1,192.0 ± 697.0	6.3 ± 0.3
M3A6S	0.8 ± 0.0	307.8 (1)	1,165.0 ± 185.0	5.5 ± 1.2
M3B6S	36.9 ± 1.3	599.5 ± 150.0	> 10,000	513.3 ± 15.0
MM3A6S	34.2 ± 12	841.8 ± 189.0	> 10,000	400.1 ± 58.0

[illegible]

In Vitro Functional Assays (Guinea Pig Ileum and Mouse Vas [illegible]

The effects of opioids standards and the synthetic deriva- [illegible] guinea pig ileum (enriched in mu sites) and mouse vas deferens [illegible] electrically induced twitches of GPI. M3A6S showed the highest potency followed by M6S > M3B6S. MM3A6S showed no activity on GPI. All four compounds displayed weak activity in MVD assay. Based on their activities in both tissues, [illegible]

[illegible]

The data from this study clearly show that esterification of the 3-hydroxyl group of M6S with an acetyl group affords a morphine derivative that has enhanced duration of action as M6S when administered via the SC route in the rat. Both M6S [illegible] an equimolar dose of morphine given SC (Fig. 2). More importantly, when given ICV, M3A6S enhanced duration of ac- [illegible] and M3A6S possessed greater activity than morphine via this [illegible] extremely weak antinociceptive activity via the SC route, and was essentially devoid of activity when administered ICV at doses equimolar to morphine used in this study. We attribute this result to metabolic factors, and propose that the activities [illegible] enzymatic cleavage by esterases to M6S. Thus, the acetyl [illegible] relatively resistant to esterolysis. Therefore, one may regard M3A6S as a prodrug form of M6S. However, both compounds M6S and M3A6S showed high affinity binding to mu

TABLE 2

INHIBITION OF ELECTRICALLY EVOKED TWITCHES IN GUINEA PIG ILEUM AND MOUSE VAS DEFERENS PREPARATION

Compound	Guniea Pig Ileum			Mouse Vas Deferens	
	IC_{50} (nM)	DR With* CTAP	DR With* Nor-BNI	IC_{50} (nM)	DR With* Naltrindole
DAMGO	8.3 ± 2.0 (13)	5.0 ± 0.3 (4)	1.7 ± 0.1 (6)	177.6 ± 134.0 (7)	0.9 ± 0.1 (4)
DPDPE	4,130.0 ± 870.0 (6)	5.8 ± 2.6 (3)	1.5 ± 0.3 (4)	4.11.0 ± 1.3 (80)	53.1 ± 17.6 (3)
U69593	1.7 ± 0.6 (12)	0.7 ± 0.1 (4)	363.0 ± 97.0 (7)	208.3 ± 139.0 (8)	0.4 ± 0.1 (4)
Morphine	24.8 ± 2.4 (4)	4.3 ± 0.1 (2)	1.1 ± 0.1 (2)	2,131.0 ± 904.0 (4)	0.9 ± 0.4 (4)
M6S	67.0 ± 12.1 (4)	5.0 ± 2.2 (2)	0.9 ± 0.1 (2)	No IC_{50} could be determined	
M3A6S	26.0 ± 5.3 (4)	5.4 ± 0.2 (2)	1.4 ± 0.1 (2)	No IC_{50} could be determined (Max. inhibition 30%)	
M3B6S	657.5 ± 130.0 (4)	3.9 ± 0.3 (2)	1.3 ± 0.3 (2)	No IC_{50} could be determined (max. inhibition 29%)	
MM3A6S	No activity	–	–	No IC_{50} could be determined (max. inhibition 20%)	

The number of observations are given in parentheses.

*CTAP (100 nM) and 20 nM nor-BNI were added to the GPI preparation to selectively block mu and kappa receptors, respectively. Naltrindole (1 nM) was added to the MVD preparation to selectively block delta receptors.

Dose Ratio (DR) = IC_{50} in the presence of antagonist/IC_{50} in the absence of antagonist.

opioid receptors in our in vitro binding assay using guinea pig brain homogenate.

The observation that both M6S and M3A6S are active with a rapid onset of antinociception after ICV and SC administration, strongly suggests that these morphine derivatives, like the 6-O-glucuronide conjugates, are capable of penetrating the blood–brain barrier after peripheral administration, in spite of their zwitterionic nature at physiological pH. How- [illegible]

Addition of a sulfate group to the 6-position of morphine, [illegible] receptors (2.2 fold greater than morphine) in our receptor binding assay. Furthermore, M3A6S showed better ability to [illegible] or even DAMGO, a mu-preferring peptide, in receptor binding assay using guinea pig brain homogenate. This selectivity has also been supported by its high potency in GPI (enriched in mu-sites) and decreased potency in the MVD (enriched in delta-sites) preparations.

Increasing the polarity of the M3A6S molecule by quaternization of the tertiary amino group to form *N*-methylmorphinium-6-O-sulfate betaine (MM3A6S) abolished all antinociceptive activity following peripheral or central administration at doses equimolar to morphine used in this study. It seems that this dipolar derivative is unable to interact with CNS opioid receptors because it is inactive via the ICV route. In addition, it lacks activity in both GPI and MVD assays and shows weak binding affinity to opioid receptors in our recep- [illegible]

These results indicate that lipophilicity alone is not a deter- [illegible] interactions with opioid receptors.

This work was supported in part by the Tobacco and Health Research Institute, University of Kentucky. The in vitro bioassay results for opiate receptor binding and functional activity testing were provided by the National Institute on Drug Abuse (NIDA), Medication Development Division Contract No. 271-89-8159 awarded to SRI International (Menlo Park, CA).

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