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A highly toxic morphine-3-glucuronide derivative

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Abstract—By the coupling of octylamine to the uronic acid function of morphine-3-glucuronide (M3G) a new glycoconjugate (morphine-3-octylglucuronamide, M3GOAM) was prepared. When assayed in both rats and mice up to ng/kg (ip) doses none of the animals survived. The aliphatic octyl chain may be the lethal factor since a closely related derivative (M3GNH2), was not toxic and showed similar opioid antagonist properties than naloxone.

Owing to its wide and long standing use, the effects of morphine are well characterized. However, the mechanisms by which morphine produces these effects are not clear. In recent years the potential role of morphine metabolites in such mechanisms has also been addressed.

After administration in man, the major fate of morphine is glucuronidation.[1] Indeed, 70% of a morphine dose is normally converted into morphine glycoconjugates. Among these, by far the most abundant, is morphine-3-glucuronide (M3G) accounting for a 45–55% of the converted morphine, while a small proportion of 10–15% corresponds to morphine-6-glucuronide (M6G).[2,3]

Morphine glucuronides are probably one of the best examples showing that glucuronidation not always terminates the pharmacological activity of medicinal drugs.[4] It is well documented that M6G is a potent agonist of a μ-opioid receptor subtype.[5] Antinociception caused by M6G has been reported in animal models[6–8] and also analgesia has been observed in humans.[9,10] On the other hand, M3G shows nor neuroexcitatory nor opioid effects in man[11,12] but some studies have reported to possibly antagonize the effects of both morphine and M6G when assayed in rats.[13,14] However, such claims of functional antagonism are in contradiction with other reported findings.[15–17]

Controversial hypothesis and results do also concern blood–brain barrier (BBB) penetration by morphine glucuronides. Thus, while molecular modelling studies[18,19] provide support for the observation that these glycoconjugates apparently cross the BBB more easily than anticipated, other investigations of transport across the BBB[20,21] are consistent with their high polarity and poor BBB diffusion.

With the aim of contributing to a better understanding of the endogenous mechanisms describing opioid activity mediated by morphine glucuronides, we have conducted structure–activity relationship studies on morphine glucuronides to better ascertain the role of the sugar molecule on opioid activity.[22] Focusing on M3G and in line with a preceeding study of 3-substituted synthetic opioid antagonists of nalmefene and naltrexone glycoconjugates,[23] we have synthesized a series of M3G analogues by replacing the glucuronic acid moiety on M3G by simple monosaccharide units (i.e., glucose, galactose). After biological in vivo testing of the compounds biological responses were not different from those of M3G (see below).

These studies also aimed at possibly improve M3G diffusion across the BBB by enhancing its hydrophobicity.

Keywords: Morphine glucuronides; M3G; Opioid antagonists; Alkyl glycosides.
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Thus, following our own previously described strategy for opioid peptides[24] we have synthesized a lipophilic M3G analogue (Fig. 1). The simplest route was to form an amide bond between the carboxylic acid group on M3G and a primary lineal alkyl amine. In doing so, octyl amine was chosen as the shortest but yet hydrophobic amine and the uronium reagent, HBTU, because avoided the use of hydroxyl protecting groups[25] Accordingly, the whole procedure for M3GOAM synthesis started following a one-pot reaction method to prepare M3G. This glycosidation reaction departed from morphine and methyl (2,3,4-tri-*O*-acetyl-α-D-glucopyranosyl bromide) uronate as substrates and used LiOH as promotor and base for the removal of acetyl protecting groups.[26] In a second step, the coupling reaction between the glucuronide M3G and octylamine was accomplished by means of the uronium salt HBTU [2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate].[27] Similarly, the new analogue morphine-3-glucuronamide (M3GNH2) was prepared from morphine and the corresponding acetylated glucuronamide bromide derivative also using the LiOH method. The morphine glucuronide derivatives were purified by column chromatography and crystallization. Their purity was assessed by RP-HPLC and their structural characterization[28] performed by ^{1}H and ^{13}C NMR and ESI-MS.

The potential analgesic properties of M3GNH2 and M3GOAM, were studied on the tail flick and paw pressure behavioural tests[29] using as reference compounds morphine and both commercial and our own synthesized M3G.

1. Biological activity of M3GOAM

Knowing that an ip dose of 5 mg/kg of morphine normally produces 65% of analgesia in rats, the initial assay of M3GOAM was conducted at this same dose in a group of $n=10$. The observation that the animals died after 10 min let us supose that the dose was too excessive. In a second series of experiments a 0.1 mg/kg dose was assayed in a group of $n=35$. All animals seemed to present 100% of analgesia in both thermal and mechanical tests but only survived between 35 and 45 min after treatment. Looking for a dose that was analgesic but not toxic a third set of experiments including lower doses and control groups as to work blindly was performed. Table 1 shows that the morphine and saline groups presented the expected biological activity but M3GOAM treated animals died within the next 30 min.

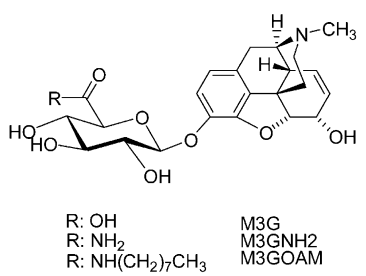

Figure 1. Chemical structures of morphine glycoconjugates: M3G, M3GNH2 and M3GOAM.

It is remarkable that the toxic effect persisted even at a such low dosis of 1 ng/kg. The effect first appeared as flaccidity of both fore and hind paws followed by profound sleep and abrupt cardiorespiratory stop. These results were confirmed by a 10 day study of the behaviour of 10 groups of three animals each one receiving either 1 ng/kg of M3GOAM, 5 mg/kg of morphine or saline. Everyday one group was examined and similar death rate and biological effects could be observed. Experiments were also conducted to also confirm that the toxic effect was neither due to a particular batch of product or lot of animals. Results with a new batch of product matched previous observations in a group of $n=5$ rats receiving 1ng/kg of M3GOAM and a control group ($n=4$) of morphine. In addition, to investigate if the time period between administration and death was always constant a group of $n=12$ was inoculated with a 1 ng/kg dosis. The survival times recorded for $n=5$ were less than 30 min, a group of $n=4$ did not lasted 45 min and the rest ($n=3$) not survived 90 min.

To get some insight about the toxicity mechanism the experiments outlined in Table 2 involving the use of the opiod antagonist naloxone were performed. We wanted to check if by prior receptor ocupancy or by displacement of receptor bound M3GOAM, naloxone could either prevent or revert the toxic effect.

None of the experiments with naloxone provided improvement in survival times and allowed us to conclude that opioid receptors seem not to participate in the toxicity mechanism of M3GOAM.

Table 1. Effect of M3GOAM doses containing decreasing amounts of the compound

Compd	Dose (mg/kg)	*n*	Effect
M3GOAM	5	8	Death
	2.5	4	Death
	1	6	Death
	0.5	6	Death
	0.05	6	Death
	0.005	3	Death
	(1 ng/kg)	6	Death
Morphine	5	5	60–70%[a]
Saline		12	Pain

[a] Values of analgesia.

Table 2. Experiments attempting to revert the toxic effect by pre- or post-administration of the opioid antagonist naloxone[a]

First dose	Second dose	Interval (min)	*n*
Naloxone	M3GOAM	10	5
Naloxone	M3GOAM	15	5
Naloxone	M3GOAM	20	5
M3GOAM	Naloxone	10	5
M3GOAM	Naloxone	15	5
M3GOAM	Naloxone	20	5
M3GOAM	Naloxone	30	5

[a] Naloxone was assayed at three different doses (1, 5, 10 ng/kg) while M3GOAM at only one (1 ng/kg).

Possible interspecies differences were investigated by administering ip doses of 1 ng/kg of the compound to mice. No analgesia was observed as measured by both tail flick and paw pressure tests. All animals ($n=5$) died, however, survival times (2 h) were longer than with rats. These results were confirmed with a second group ($n=4$) showing survival times on the range of 1 to 4 h.

2.. Biological activity of M3G

Validation of the synthetic methods and the biological tests was carried out by comparing the activity of commercially available M3G and our own prepared product. Because reported activities for M3G are of opioid antagonist, it was first check that the products were devoid of agonist activity by working blindly and using the protocol of Table 3.

The opioid antagonist activity of M3G was assessed against morphine and its potency compared to naloxone. Three doses were studied using the protocol of Table 4.

Morphine was able to elicit analgesia only in the group of $n=15$ which received M3G probing that M3G does not act as opioid antagonist by peripheral administration. However, iv injection of 1 μL of a 5 mg/mL solution of M3G in the IV ventricle in a group of $n=10$ rats and after 20 min of ip dosis of 5 mg/kg of morphine, masked the analgesic effect of morphine. The action lasted for at least 120 min indicating that M3G acts as opioid antagonist only after central administration.

3.. Biological activity of M3GNH2

Three ip doses of 1 mg/kg ($n=10$), 5 mg/kg ($n=8$) and 10 mg/kg ($n=5$) of the M3GNH2 analogue were examined for analgesia by the tail flick and paw pressure tests in rats using morphine, 5 mg/kg ($n=5$) and saline ($n=6$) as reference. No analgesia was observed. On the other hand, ip doses of 5 mg/kg of M3GNH2 administered 10 min after morphine (5 mg/kg) ($n=15$) fully inhibited its analgesic effect in a similar pattern as the same doses of naloxone. Accordingly, M3GNH2 was characterized as a new opioid antagonist acting by peripheral administration. Its lack of toxicity allows us to also conclude that the amide function seems not to be the structural feature responsible for the acute toxicity observed in M3GOAM.

In summary, we have found out that two relatively minor modifications on the M3G molecule induce remarkable biological effects. We here report positive results showing that replacement of the carboxylic acid of M3G by a carboxamide function it is a way to modulate BBB transport properties. Thus, the new molecule acted as opioid antagonist by peripheral administration while its parent compound (M3G) showed opioid antagonist properties only after central administration. On the other hand, we disclose an unprecedented negative finding showing that rising M3G lipophilicity produced a highly toxic compound. The result is more striking because similar strategies (addition of octyl moieties) when applied to other sugar derivatives have provided promising drug candidates.[30] Due to such high toxicity it is, up to now, unclear if the compound is able to induce analgesia. However, since the observation that the lethal effect can not be reverted or prevented by naloxone it may seem to indicate that the toxicity mechanism does not involve interactions at the opioid receptor level. This fact may also seem supported by the lack of significative biological activity differences among the two animal species tested.[31]

Table 3. Opioid activity of in house prepared and commercial M3G

Compd	Dose (mg/kg)	*n*	Effect
Synthetic M3G	1	15	None
	5	20	None
	10	20	None
Commercial M3G	1	15	None
	5	5	None
	10	5	None
Morphine	5	7	60–70%[a]
Saline		20	None

[a] Values of analgesia.

Table 4. Opioid antagonist activity of M3G and naloxone by peripheral administration[a]

First dose	Second dose	Interval (min)	*n*
M3G	Morphine	20	5
Naloxone	Morphine	20	5

[a] In all cases M3G and naloxone were administered at three different doses (1, 5, 30 mg/kg) while morphine at a reference dose of 5 mg/kg.

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27. Synthesis of morphin-3-yl-β-D-octylglucuronamide (M3GOAM). To a solution of 60 mg (0.13 mmol) of M3G, 35 mg (0.26 mmol) of HOBt and 125.2 mg (0.39 mmol) of 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) in 20 mL of DMF, octylamine (23 mL, 0.26 mmol) was added dropwise at room temperature under stirring. The reaction was left to proceed for 20 h. Solvent evaporation and column chromatography of the residue on silica gel (230–400 mesh) using a MeOH/H_2O/ethyl acetate (4:3:8, v:v:v) mixture as eluent, yielded the title compound in 40% yield after crystallization from acetone/water.
28. Physicochemical data for final compounds. **Morphine 3-β-D-glucuronide** (**M3G**): ESI-MS: 462.2 $[M+H^+]$; $[\alpha]_D = -117{,}8$ ($c = 0.5$, H_2O); ^{1}H NMR (300 MHz, D_2O): δ 6.68 (d, 1H, $J = 8.0$ Hz, 2-H), 6.42 (d, 1H, $J = 8.0$ Hz, 1-H), 5.41 (d, 1H, $J = 9.5$ Hz, 7-H), 5.07 (d, 1H, $J = 9.5$ Hz, 8-H), 4.78 (d, 1H, $J = 5.5$ Hz, 5-H), 4.74 (d, 1H, $J = 2.7$ Hz, 1′-H), 4.06 (d, 1H, $J = 9.0$ Hz), 3.67 (m, 1H), 3.54 (m, 1H), 3.40 (dd, 1H, $J = 2.5$, 9.0 Hz, 2′-H), 3.21 (m, 1H), 3.03 (m, 1H), 2.97 (m, 1H), 2.89 (m, 1H), 2.81 (m, 1H), 2.62 (m, 1H), 2.53 (s, 3H, N-CH_3), 1.99 (m, 1H), 1.71 (d, 1H, $J = 14.0$ Hz). ^{13}C NMR (75 MHz, D_2O) δ 176.2, 147.9, 139.9, 133.7, 131.1, 128.3, 127.5, 126.8, 121.2, 118.2, 101.6, 92.2, 77.2, 76.4, 73.8, 72.7, 66.9, 60.7, 43.0, 41.8, 38.7, 33.4, 24.2; HPLC: RT = 21.22 min (LiChrospher 100 RP-18, 5 μm, ACN-water gradient elution: 80% to 70% of water in 5 min and 70% to 20% of water in 10 min). **Morphine 3-β-D-octylglucuronamide** (**M3GOAM**): mp = 89–91 °C (from acetone–water); ESI-MS: 572.4 $[M+H^+]$; $[\alpha]_D = -124.5$ ($c = 1$, MeOH); ^{1}H NMR (200 MHz, DMSO-d_6): δ 6.20 (d, 1H, $J = 8.2$ Hz, 2-H), 5.90 (d, 1H, $J = 8.2$ Hz, 1-H), 5.45 (d, 1H, $J = 15.0$ Hz, 7-H), 5.25 (d, 1H, $J = 14.8$ Hz, 8-H), 5.05 (d, 1H, $J = 3.0$ Hz, 1′-H), 4.70 (d, 1H, $J = 5.5$ Hz, 5-H), 4.15 (m, 1H), 3.71 (m, 1H), 3.42 (m, 1H), 3.05 (m, 3H), 2.30 (s, 3H, N-CH_3), 1.81–2.62 (m, 6H), 1.23–1.35 (m, alkyl). ^{13}C NMR (50 MHz, DMSO-d_6) δ 167.9, 147.3, 138.1, 131.1, 128.7, 118.4, 116.6, 100.6, 91.9, 76.3, 75.5, 73.0, 70.9, 66.2, 57.8, 45.8, 42.7, 42.6, 35.3, 31.2, 28.8, 28.7, 28.6, 26.3, 22.0; HPLC: RT = 12.80 min (LiChrospher 100 RP-18, 5 μm, ACN-water gradient elution, same programme as above). **Morphine 3-β-D-glucuronamide** (**M3GNH2**): ESI-MS: 460.2 $[M+H^+]$; $[\alpha]_D = -52.4$ ($c = 1$, MeOH); ^{1}H NMR (200 MHz, CD_3OD): δ 6.84 (d, 1H, $J = 8.3$ Hz, 2-H), 6.58 (d, 1H, $J = 8.3$ Hz, 1-H), 5.65 (d, 1H, $J = 14.7$ Hz, 7-H), 5.34 (d, 1H, $J = 15$ Hz, 8-H), 4.25 (m, 1H), 4.18 (d, 1H, $J = 9.5$ Hz), 3.90 (m, 1H), 3.53 (m, 2H), 3.31 (m, 1H), 3.17 (m, 1H), 3.08 (m, 1H), 2.77 (m, 2H), 2.55 (s, 3H, N-CH_3), 2.49 (m, 1H), 2.46 (m, 1H), 2.21 (m, 1H), 2.17 (m, 1H), 1.97 (d, 1H, $J = 13.5$ Hz); ^{13}C NMR (50 MHz, CD_3OD) δ 180.4, 174.2, 149.3, 140.3, 134.2, 132.5, 130.5, 128.9, 120.6, 119.3, 103.2, 93.4, 77.2, 75.6, 74.5, 73.2, 67.7, 60.2, 44.2, 42.8, 40.9, 35.8, 24.1; HPLC: RT = 2.19 min (LiChrospher 100 RP-18, 5 μm, ACN-water gradient elution, same programme as above).
29. Pharmacological tests. Animals were housed in a room with controlled temperature at 21 ± 1 °C under 12 h light–dark cycle and given free access to food and water. Both, male Sprague–Dawley rats and Swiss-Webster mice were tested for analgesia after 30 min of the administration of different ip doses of the compounds in 500 mL of saline solutions. The tail flick test, was conducted following described protocols.[32] Mechanical nociceptive thresholds were determined by the paw pressure test on the left hind paw as reported in the literature.[33] Pain thresholds were measured with an analgesemeter. The apparatus was set up to apply a force of 0-1000 g increasing from zero at a rate of 64 g/s. The nociceptive threshold was taken as the point at which the animal vocalized or struggled vigorously.
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