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Opioids and Efflux Transporters. Part 2: P-Glycoprotein Substrate Activity of 3- and 6-Substituted Morphine Analogs

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Abstract

Continuing our studies investigating opioids with reduced P-glycoprotein (P-gp) substrate activity, a series of known 3- and 6-hydroxy, -methoxy, and -desoxymorphine analogs was synthesized and analyzed for P-gp substrate activity and opioid binding affinity. 6-Desoxymorphine (**7**) showed high affinity for opioid receptors and did not induce P-gp-mediated ATP hydrolysis. Additionally, **7** demonstrated morphine-like antinociceptive potency in mice, indicating this compound as an ideal lead to further evaluate the role of P-gp in opioid analgesic tolerance development.

Introduction

Morphine (**1**) is the drug of choice for the treatment of chronic, severe pain and exerts its analgesic actions primarily through stimulation of central opioid receptors in the brain and spinal cord.¹ In addition to providing analgesia and euphoria, stimulation of peripheral μ opioid receptors in the GI tract is primarily responsible for the development of constipation, which accompanies opioid therapy.² The persistent use of morphine results in the rapid development of analgesic tolerance, prompting the need for increasing doses to provide equivalent analgesia.³ Clinically, increasing the dose in response to analgesic tolerance causes compounded side effects (nausea, constipation), drug—drug interactions, and an overall diminished quality of care.

Several mechanisms involved in opioid central tolerance include receptor oligomerization⁴ and cross-talk⁵ and disruption of cellular signaling pathways.⁶ It is unclear how these mechanisms contribute to the differential rate of tolerance development between central and peripheral opioid receptors,⁷ however, prompting the investigation of alternate mechanisms that may play a role in this differential tolerance.

There is a growing body of evidence which identifies efflux transporters in the blood-brain barrier (BBB) as contributors to opioid tolerance. Specifically, P-glycoprotein (P-gp) has been implicated, as morphine and various opioids are P-gp substrates,^{8–11} P-gp is up-regulated in morphine-¹² and oxycodone¹³-tolerant animals, and this up-regulation has a

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^aAbbreviations: BBB, blood-brain barrier; P-gp, P-glycoprotein; ABC, ATP binding cassette; TMS-CHN₂, trimethylsilyl diazomethane; SAR, structure–activity relationships.

significant physiological effect on the brain clearance of paclitaxel, a P-gp substrate.¹³ P-gp, a member of the ATP binding cassette (ABC) family of transporters, is expressed at various tissues throughout the body, notably the BBB.¹⁴ The effect of P-gp up-regulation at the BBB on opioids is to restrict their exposure to the brain, limiting their interaction with central opioid receptors and reducing their analgesic effect. A more thorough investigation of the role of P-gp in the development of central tolerance requires opioids with diminished P-gp substrate activity.

The development of opioids with diminished P-gp substrate activity involves combining structure–activity relationships (SAR) of opioid agonists with SAR of P-gp substrates. Modifications which affect antinociceptive potency have been well-characterized,¹⁵ however, few specific SAR studies exist for P-gp substrates. Generally, hydrogen bond donating and accepting groups have been identified as putative enhancers of P-gp activity for a variety of substrates.¹⁶ Indeed, earlier studies with opioids have shown that masking the hydrogen bond donating 3-phenol of morphine with a methyl ether (codeine, **2**) results in a reduction of active efflux transport.¹⁷ However, such modification results in a decrease in opioid potency.¹⁵

We have continued our previous work¹⁸ into iteratively modifying morphine toward eliminating P-gp activity without simultaneously causing great reductions in potency. Morphine contains several hydrogen bond donating and accepting groups that can potentially bind P-gp. In particular, the alcohol functions at the 3- and 6-positions could donate and accept hydrogen bonds with P-gp. It is hypothesized that sequentially masking or removing these alcohol functions from morphine will reduce P-gp substrate activity, while maintaining morphine-like opioid potency. Much is known about how these substitutions affect opioid activity, however, very little is known about how morphine interacts with P-gp. To delineate the structural features of morphine responsible for identification by P-gp, a series of known 3- and 6-hydroxy, methoxy, and desoxy morphine derivatives have been synthesized and analyzed for P-gp substrate activity and opioid binding affinity. Selected compounds that exhibit a profile of insignificant P-gp substrate activity and high affinity for opioid receptors have also been analyzed in vivo in antinociception studies to determine their potency relative to morphine.

Results and Discussion

Chemistry

Morphine (**1**) and codeine (**2**) were a generous gift by Mallinckrodt, Inc. (St. Louis, MO). Compounds **3–9** were synthesized by literature methods or by modifications of known procedures (see Supporting Information).^{19–23} The synthesis of the dihydro-3-desoxy-6-methyl ether derivative **6** has not been described and is outlined here. As shown in Scheme 1, the dihydro-3-desoxy derivative **3** was synthesized by first converting the phenolic hydroxyl of **1** to the 3-*O*-(5-phenyltetrazole).¹⁹ Following this procedure, hydrogenolysis of the morphine-3-*O*-tetrazole using 10% Pd/C in acetic acid at 55 psi for 4 days resulted in incomplete removal of the phenol function. Complete hydrogenolysis was achieved following subsequent Pd-catalyzed transfer hydrogenation for 2 days using ammonium formate in refluxing ethanol.²⁰

The formation of the 6-methyl ether derivatives **4–6** is shown in Scheme 2. Starting from the 3-substituted compounds **2** and **3**, secondary alcohols were then methylated using trimethylsilyl diazomethane (TMS-CHN₂) according to a recently developed procedure.²¹ TMS-CHN₂ application (10 equiv/day) using silica gel in dichloromethane at room temperature for 5 days resulted in the 6-methyl ether derivatives **5** and **6**, respectively. Heterocodeine (**4**) was synthesized using this process, after first protecting the phenol with

acetic anhydride as shown in Scheme 2. As shown in Scheme 3, analogs **7–9** were synthesized according to previously described procedures.^{22,23}

P-gp Substrate Activity Studies

P-gp substrate activity was determined using the P-gp-Glo assay, wherein P-gp-mediated ATP hydrolysis is monitored using firefly luciferase.²⁴ Specifically, a significant reduction in firefly luciferase-induced luminescence indicates an increase in ATPase activity mediated by P-gp. Non-P-gp substrates do not significantly alter ATP consumption and, therefore, would not show a significant reduction in luminescence from basal levels. Compounds that act as P-gp inhibitors reduce basal ATPase activity and, therefore, show a significant increase in luminescence. The results of these assays are shown in Figure 1.

Compounds **1**, **2**, **4**, and **9** showed significant reductions in luminescence, indicating an increase in P-gp-mediated ATP consumption. Compounds **3**, **5**, **6**, and **7** were identified as non-P-gp substrates due to a lack of significant ATPase activity. Compound **8** showed a significant decrease in ATPase activity, identifying this compound as a P-gp inhibitor.

In the case of morphine (**1**), the ATP hydrolysis detected in this assay results in significant P-gp-mediated efflux *in vivo*, as shown in previous reports.^{9,10} Codeine (**2**), however, stimulates P-gp-mediated ATP hydrolysis, but previous studies¹⁷ indicate it is not actively effluxed by P-gp. *In vitro* transport assays performed by our group confirm these reports, indicating that codeine should be considered a nontransported P-gp substrate. Nontransported substrates generally exhibit greater passive permeability, thus limiting the effect of P-gp on transport across biological membranes.²⁵

There appeared to be no definitive correlation between specific removal of the 3- or 6-hydroxyl group and P-gp substrate activity. In the case of **3** and **7** (3- and 6-desoxymorphine, respectively), ATPase activity was eliminated for both analogs. Of the desoxycodine analogs, the 3-desoxy analog **6** showed no significant ATPase activity, whereas the 6-desoxy analog **8** significantly inhibited ATPase activity. Interestingly, removal of both 3- and 6-oxygen functions (**9**) resulted in a significant increase in ATP hydrolysis. Of the monodesoxy analogs, **8** was the only exception to the finding that removing a single oxygen function from either the 3- or 6-position results in a loss of P-gp activity.

With the exception of **7**, the presence of phenols resulted in P-gp substrates: morphine (**1**) and heterocodeine (**4**) both significantly increased ATP hydrolysis. In the case of **4**, the 3-phenol appears to be responsible for this increase in ATPase activity, because the rest of the 6-methyl ether series (**5** and **6**) was unable to significantly affect transporter activity.

It is difficult to define specific SAR from the results of this study; however, reducing the hydrogen bonding capability of the 3- and 6-positions of morphine by removing a single oxygen function generally reduces the ability of P-gp to identify these compounds as substrates. Because the synthesis of the 3-desoxy compounds (**3**, **6**, **9**) featured the use of Pd-catalyzed hydrogenolysis, the C₇–C₈ etheno group was reduced for these compounds. This additional structural modification should be taken into consideration when interpreting the results of this P-gp SAR study. This assay identifies compounds **3**, **5**, **6**, and **7** as non-P-gp-substrate ligands, which are therefore promising leads for designing non-P-gp-substrate opioids.

Opioid Receptor Binding Studies

The opioid activity of most compounds in the presented 3- and 6-desoxymorphine series is known; however, to the best of our knowledge, the μ , δ , and κ receptor binding affinities of these derivatives have not been determined. Previous reports¹⁹ have described general opioid binding of 3-substituted morphine analogs in rat brain homogenate; however, these studies do not include affinities at individual opioid receptor subtypes. 6-Desoxymorphine analogs have been synthesized previously, though receptor subtype binding studies were not performed.²² Therefore, it was of interest to determine how these modifications would alter binding affinity and subtype selectivity relative to the parent morphine.²⁶ Binding studies were conducted as described previously.²⁷

As shown in Table 1, removal of the 6-hydroxyl function (**7**, **8**, **9**) had a modest effect on binding affinity at all three opioid receptor subtypes relative to the respective parent compounds (**1**, **2**, **3**). This effect was most noticeable at δ opioid receptors, ranging from an approximately 9-fold increase in binding affinity of the 3-hydroxyl series (δK_i **1**/8 K_i **7** = 8.9) to a 22-fold increase in binding in the 3-desoxy series (δK_i **3**/8 K_i **9** = 21.8). This resulted in a loss of the preference of the parent 6-hydroxyl compounds for μ receptors, seen most noticeably in the loss of receptor selectivity between **1** and **7**. For the 3-phenols, this loss of preferential binding activity is tied to the absence of an oxygen function at the 6-position, as the 6-methyl ether (**4**) showed a similar selectivity profile as **1**.

Alteration of the aromatic 3-substituent played the most significant role in opioid binding affinity. Opioid receptor binding was greatest with free 3-phenols for all 6-substituted analogs (**1**, **4**, **7**) at all receptor subtypes. Removal of this oxygen function and hydrogenation of the 7,8-etheno group resulted in a 70-fold reduction in the binding affinity of morphine (μK_i **3**/ μK_i **1** = 72). The 3-methyl ether series (**2**, **5**, **8**) routinely exhibited the poorest affinity for all receptors, although **6** showed the poorest affinity for δ and κ receptors. For binding affinity in the 3-desoxy series, substitution at the 6-position followed the pattern of 6-desoxy > 6-hydroxy > 6-methoxy for all receptors, an effect which was less noticeable in the 3-methoxy series. Generally, the greatest μ selectivity was seen in the parent 6-hydroxy series (**1**, **2**, **3**) and the least selectivity was seen in the 6-desoxy series (**7**, **8**, **9**). Despite exhibiting lower selectivity, the 3-hydroxyl analog **7** exhibited high (nM) affinity at μ receptors.

Antinociception Studies

Compounds that showed diminished P-gp substrate activity, as well as high μ opioid receptor binding affinity, were considered for further in vivo antinociception studies. Of the nonsubstrates, compounds **3** and **6** have approximately 10- and 50-fold lower binding affinity to μ opioid receptors compared to morphine and were, therefore, not chosen for further study. In addition to showing the poorest opioid binding affinity of all compounds analyzed, **5** would be 3- and 6-*O*-demethylated to heterocodeine (**4**) and morphine (**1**), compounds which are both P-gp substrates. Compound **7** was therefore selected for further analysis, as this compound exhibited the greatest binding affinity of the non-P-gp substrates and would not be prone to demethoxylation in vivo. Tail flick, phenylquinone, and hot plate assays were performed in mice as previously described,²⁸ and results were compared to those previously determined for morphine.²⁹

As shown in Table 2, compound **7** produces potent antinociception, exhibiting greater potency compared to morphine in all assays performed. Compound **7** is approximately 10 times more potent than morphine in the tail flick (0.2 vs 1.92 mg/kg) and phenylquinone (0.03 vs 0.4 mg/kg) assays. These data confirm previous studies³⁰ that describe the high opioid potency of 6-desoxy derivatives of morphine.

Conclusions

Results of in vitro ATPase activity assays show that P-gp substrate activity in the morphine series can be attenuated by masking or removing hydrogen bonding substituents at the 3- and 6-positions. Substitution to the 3-phenol generally reduces opioid receptor binding affinity; however, removal of the 6-hydroxyl moiety greatly increases binding to all three opioid receptor subtypes. Additionally, 6-desoxymorphine analogs exhibit diminished selectivity for binding μ receptors over δ and κ . Antinociception studies performed on 6-desoxymorphine (**7**) show an approximate 10-fold increase in antinociceptive potency compared to morphine. These results indicate that compound **7** is a μ opioid agonist with morphine-like potency and no P-gp substrate activity and is, therefore, an ideal lead compound to elucidate the effect of P-gp on the development of analgesic tolerance of opioids.

Experimental Section

Chemistry

Compounds were hydrogenated using a Parr hydrogenation apparatus, and HCl salts were lyophilized using a VirTis Freezemobile lyophilizer where described. ^1H NMR spectra were obtained using a 500 MHz Varian NMR. Melting points were determined in open capillary tubes using a Mel-Temp melting point apparatus. TLC was performed on silica gel 60 GF plates (Analtech, Inc., Newark, DE). Microanalysis was performed by Atlantic Microlab, Inc. (Norcross, GA). Opioids used in this study were a generous gift from Mallinckrodt, Inc. (St. Louis, MO). Reagents were purchased from Sigma-Aldrich, Inc. Detailed syntheses of known opioids **3–5** and **7–9** are available in Supporting Information.

7,8-Dihydro-3-desoxyheterocodeine (**6**)

Compound **3**^{19,20} (460 mg, 1.70 mmol) was 6-*O*-methylated using silica gel (2.0 g) and TMS-CHN₂ (1.94 g, 10 equiv) daily for 5 days. On days 2–5, 1–2 drops of water were added. On day 5, the reaction was quenched with water, and the organic layer was separated. The aqueous layer was extracted with CH₂Cl₂, and the combined organic layers were dried (Na₂SO₄) and evaporated under reduced pressure. Column chromatography (silica gel, 5% MeOH/CH₂Cl₂), followed by precipitation of the oxalate salt from acetone, resulted in 13.2 mg (2.1%). Anal. (C₂₀H₂₅NO₆ · 0.1H₂O) C, H, N; mp 190–192 °C; ^1H NMR (500 MHz, CDCl₃) δ 7.05 (t, J = 8.0 Hz, 1H), 6.64 (d, J = 8.0 Hz, 2H), 4.68 (d, J = 4.50 Hz, 1H), 3.47 (m, 1H), 3.38 (s, 3H), 3.09 (dd, J = 6.27 and 2.85 Hz, 1H), 3.02 (d, J = 18.82 Hz, 1H), 2.52 (dd, J = 12.54 and 5.13 Hz, 1H), 2.40–2.46 (m, 1H), 2.41 (s, 3H), 2.29 (dd, J = 12.58 and 3.99 Hz, 1H), 2.20–2.26 (m, 1H), 1.90 (td, J = 12.58 and 1.13 Hz, 1H), 1.72 (m, 1H), 1.50–1.60 (m, 2H), 1.30 (m, 1H), 1.08 (m, 1H); m/z : 286 (M + H)⁺.

P-gp-Glo Assay

In a 96-well plate, recombinant human P-gp were incubated with P-gp-Glo assay buffer (20 μL), verapamil (200 μM), sodium orthovanadate (100 μM), or 200 μM of test compound (**1–9**). Each compound was loaded in four individual wells. The reaction was initiated by addition of MgATP (10 mM) and quenched 40 min later by addition of 50 μL of firefly luciferase reaction mixture (ATP detection reagent), which initiated an ATP-dependent luminescence reaction. Signals were measured 60 min later using an Lmax luminometer (Molecular Devices Corporation, Sunnyvale, CA).

Opioid Binding

Binding assays were performed as described,²⁷ using [³H]diprenorphine in membranes from C₆ rat glioma cells expressing recombinant μ or δ receptors and CHO cells expressing the recombinant κ receptors.

Antinociception Studies

Antinociception was determined using the tail flick (TF), *p*-phenylquinone (PPQ), and hot plate (HP) assays in mice as described previously by the Drug Evaluation Committee (DEC) of the College on Problems of Drug Dependence (CPDD).²⁸

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Figure 1.

P-gp-mediated ATP consumption in the presence of 200 μ M of test compound using the P-gp-Glo assay system. Nontreated (NT), vanadate (P-gp inhibitor), and verapamil (P-gp substrate) are included as controls. Data are presented as means $\pm$ SEM * $p < 0.05$ (t -test).

Scheme 1. Synthesis of Dihydro-3-desoxymorphine (**3**)^a^a Reagents and conditions: (a) 5-Cl-1-phenyl-1*H*-tetrazole, K₂CO₃, DMF, RT, 24 h; (b) H₂, Pd/C, HOAc, 55 psi, 4d; NH₄HCO₂, Pd/C, EtOH, reflux, 2 d.

Scheme 2. Synthesis of 6-Methoxy Analogs (**4–6**)^a

^a Reagents and conditions: (a) Ac_2O , NaHCO_3 (aq), RT, 5 min; (b) TMS-CHN_2 , SiO_2 , CH_2Cl_2 , H_2O , RT, 5d; (c) NaOH , MeOH , reflux, 8 h.

Scheme 3. Synthesis of 6-Desoxy Analogs (7-9)^a

^a Reagents and conditions: (a) Ts-Cl, pyridine, 0 °C, 5 h; (b) LiAlH₄, THF, reflux, 3 h; (c) BBr₃, CHCl₃, 0 °C, 30 min; (d) 5-Cl-1-phenyl-1 *H*-tetrazole, K₂CO₃, DMF, RT, 24 h; (e) H₂, Pd/C, HOAc, 55 psi, 4 d; NH₄HCO₂, Pd/C, EtOH, reflux, 2 d.

Opioid Receptor Binding Affinity and Selectivity to Cloned Receptors Transfected into C₆ Rat Glioma Cells (μ , δ) and Chinese Hamster Ovary (CHO) Cells (κ)

Table 1

compd	3-R	6-R'	7,8	K_i^a (nM)				selectivity	
				μ	δ	κ		δ/μ	κ/μ
1 ^b	OH	OH	CH=CH	1.70 $\pm$ 0.50	104.57 $\pm$ 27.18	65.5 $\pm$ 22.6		61	38
2 ^b	OMe	OH	CH=CH	727 $\pm$ 128	52207 $\pm$ 25421	25411 $\pm$ 10015		72	35
3	H	OH	CH ₂ -CH ₂	122 $\pm$ 37	5250 $\pm$ 2450	4910 $\pm$ 320		43	40
4	OH	OMe	CH=CH	1.1 $\pm$ 0.5	65.8 $\pm$ 17.2	22.8 $\pm$ 11.0		60	21
5	OMe	OMe	CH=CH	1910 $\pm$ 930	4410 $\pm$ 1500	5430 $\pm$ 2290		2	3
6	H	OMe	CH ₂ -CH ₂	524 $\pm$ 120	8830 $\pm$ 3410	11600 $\pm$ 6000		17	22
7	OH	H	CH=CH	2.9 $\pm$ 1.1	11.8 $\pm$ 3.1	45.5 $\pm$ 3.1		4	16
8	OMe	H	CH=CH	305 $\pm$ 79	4520 $\pm$ 710	3090 $\pm$ 150		15	10
9	H	H	CH ₂ -CH ₂	22.9 $\pm$ 2.9	241 $\pm$ 27	589 $\pm$ 64		11	26

^a K_i values for standard compounds: DAMGO (μ , 7.6 nM), SNC80 (δ , 0.8 nM), U69593 (κ , 0.3 nM). Mean $\pm$ SEM for displacement of [³H]diprenorphine from three experiments, performed in duplicate.

^b Data from ref 26.

Table 2In Vivo Potency of 6-Desoxymorphine (**7**) in Mice^a

cmpd	ED ₅₀ (sc, mg/kg) ^b		
	TF	PPQ	HP
1 (morphine) ^c	1.92 (0.89–4.14)	0.4 (0.2–0.8)	0.85 (0.39–1.86)
7 (6-desoxymorphine)	0.2 (0.1–0.3)	0.03 (0.018–0.055)	0.33 (0.15–0.72)

^aTF = tail-flick assay; PPQ = *p*-phenylquinone writhing assay; HP = hot plate assay.^bEffective dose 50% (95% C.L.); subcutaneous (*sc*) administration.^cData from ref 29.