In vivo Activity of Norhydrocodone: An Active Metabolite of Hydrocodone

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Running Title: Norhydrocodone: An Active Metabolite of Hydrocodone.

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Number of text pages: 35

Number of tables: 3

Number of figures: 5

Number of references: 34

Number of words in the abstract: 248

Number of words in the introduction: 531

Number of words in the discussion: 863

Abbreviations: HYC, Hydrocodone; HYM, Hydromorphone; NHYC, Norhydrocodone; CYP, Cytochrome P450; DMAGO, [D-Ala²,N-MePhe⁴,Gly-ol⁵]Enkephalin; DPDPE, [D-Pen²,D-Pen⁵]Enkephalin; CL, Confidence limit.

Recommended section: Neuropharmacology
Abstract

Hydrocodone is primarily metabolized to hydromorphone and norhydrocodone. Although hydromorphone is a known active metabolite of hydrocodone, the in vivo activity of norhydrocodone is not well-documented. In the current study, the pharmacodynamics of norhydrocodone were evaluated and compared to hydrocodone and hydromorphone. Binding studies established that norhydrocodone, similar to hydrocodone and hydromorphone is a $\mu$-selective opioid ligand. In vivo analgesia (tailflick) studies demonstrated that, following subcutaneous (SC), intrathecal (IT) and intracerebroventricular (ICV) administration, norhydrocodone produced analgesia. Following SC administration, norhydrocodone was $\approx 70$-fold less potent and hydromorphone was $\approx 5.4$-fold more potent than hydrocodone in producing analgesia. Following IT administration, norhydrocodone produced a shallow analgesia dose-response curve and maximal effect of 15-45%, while hydrocodone and hydromorphone produced dose-dependent analgesia. IT Hydromorphone was $\approx 174$-fold more potent than IT hydrocodone. Following ICV administration, norhydrocodone had similar potency to hydrocodone in producing analgesia, while hydromorphone was $\approx 96$-fold more potent than hydrocodone. Analgesia induced by the three drugs following SC, IT and ICV administration was antagonized by SC naltrexone, confirming that it is opioid receptor-mediated. SC norhydrocodone-induced analgesia was completely blocked by ICV naltrexone, indicating that norhydrocodone-induced analgesia is likely a supraspinal effect. Seizure activity was observed following IT administration of all three drugs. Norhydrocodone and hydromorphone were $\approx 3.7-4.6$-fold more potent than hydrocodone in inducing seizure activity. Naltrexone did not antagonize opioid-induced seizure activity, suggesting seizures were non-opioid receptor-mediated. Taken together,
norhydrocodone is an active metabolite of hydrocodone and may contribute to therapeutic and toxic effects following hydrocodone administration.
Introduction

Hydrocodone, a semi-synthetic opioid, is metabolized primarily by CYP-mediated pathways (Otton et al., 1993; Hutchinson et al., 2004). In humans, rodents and dogs, hydromorphone and norhydrocodone are the primary metabolites of hydrocodone (Cone et al., 1978; Li et al., 2013). In humans, more than 50% of total hydrocodone clearance is via CYP2D6- and CYP3A4-mediated pathways leading to formation of hydromorphone and norhydrocodone, respectively (Hutchinson et al., 2004). Other metabolites of hydrocodone found in lower concentrations in humans include 6α- and 6β-hydrocodol, which are formed via non-CYP-mediated pathway (Cone et al., 1978; Li et al., 2013). The metabolites of hydrocodone, except norhydrocodone, have been examined and demonstrated to possess some analgesic activity (Small et al., 1938). It has been suggested that hydrocodone is a prodrug and requires metabolism to hydromorphone, which is an active opioid agonist (Lurcott, 1998). However, hydromorphone may not play a critical role in the pharmacologic effect of hydrocodone, since inhibition of conversion of hydrocodone to hydromorphone had no significant influence on the analgesic potency of hydrocodone in rats (Tomkins et al., 1997) or abuse liability of hydrocodone in humans (Kaplan et al., 1997). At present, the in vivo contribution of the second major metabolite, norhydrocodone, to the pharmacodynamics of hydrocodone is not well-documented.

The reported elimination half-life for hydrocodone following oral administration in humans is 3-9h (Otton et al., 1993; Hao et al., 2011) and for hydromorphone following intravenous and oral administration in humans the t1/2 is 2-4h (Vallner et al., 1981; Parab et al., 1988). The reported half-life for norhydrocodone following oral hydrocodone administration is 8h (Hao et al., 2011). Studies analyzing human plasma and urine following oral hydrocodone
indicated that norhydrocodone concentration exceeds that of hydromorphone, and in some cases, that of the parent compound (Hao et al., 2011; Valtier and Bebarta, 2012). In addition, the examination of urine specimens revealed that norhydrocodone is detected for a significantly longer period compared to both hydrocodone and hydromorphone (Valtier and Bebarta, 2012). Overall, this suggests that chronic administration of hydrocodone might lead to accumulation of the nor-metabolite.

While norhydrocodone has been suggested to be an inactive metabolite (Smith, 2009), one in vitro study examining G-protein activation by N-demethylated codeine congeners compared to the parent molecules, demonstrated that norhydrocodone had lower potency but similar efficacy as hydrocodone for μ- and δ-opioid receptor-mediated G-protein activation (Thompson et al., 2004). Since, these data indicate that norhydrocodone activates opioid receptor signaling and, as noted above, is a major metabolite of hydrocodone, we hypothesized that norhydrocodone may possess in vivo activity and might contribute towards the pharmacodynamics of hydrocodone. In addition, based on the reports of other opioid nor-metabolites (normeperidine, noroxycodone) having toxic properties (Umans and Inturrisi, 1982; Leow and Smith, 1994), we could not rule out that norhydrocodone may also have untoward characteristics.

In the current study, hydrocodone, hydromorphone and norhydrocodone were evaluated for analgesic and neurotoxic activity in a mouse model. Subcutaneous (SC), intrathecal (IT) and intracerebroventricular (ICV) routes of administration were employed to examine each opioid agonist. Overall, the results indicate that, like hydrocodone and hydromorphone, norhydrocodone is a μ-selective opioid ligand and that the in vivo effects of norhydrocodone include both opioid receptor-mediated analgesia and non-opioid receptor-mediated neuroexcitatory effects.
Materials and Methods

Subjects

Male Swiss Webster mice, weighing 28 ±2g (mean ±SD), obtained from Taconic Farms (Germantown, NY) were used throughout. Animals were housed 10 per cage with food and water available ad-libitum. Mice were allowed to acclimatize to the home cage for at least 24h prior to experimentation. All protocols were approved by the St. John's University Institutional Animal Care and Use Committee.

Receptor Binding Studies

Binding studies were performed as previously described (Yoburn et al., 1995; Sirohi et al., 2007). Mice (10-12/group) were sacrificed and spinal cords were removed and homogenized at 20,000rpm (Brinkmann Polytron Homogenizer, Westbury, NY) in 15mL of ice-cold 50mM Tris buffer, pH 7.4. Homogenates were centrifuged at 15,000 rpm for 15min at 4°C, and the pellet was resuspended and centrifuged again. Pellets were resuspended in 35mL of the Tris buffer and incubated for 30min at 25°C. Homogenates were centrifuged again and resuspended in 50mM potassium phosphate buffer, pH 7.2. Protein was determined (Bradford, 1976) using reagent from Bio-Rad (Richmond, CA). For saturation studies, spinal cord homogenate was assayed in triplicate in tubes containing 0.08-10nM [³H]DAMGO, 0.08-10nM [³H]DPDPE, or 0.16-10nM [³H]U69,593 (PerkinElmer Life Sciences, Boston, MA). For competition studies, 1nM [³H]DAMGO, 1nM [³H]DPDPE or 3nM [³H]U69,593 binding was assayed in triplicate in the absence and presence of hydrocodone (0.1nM-1mM), hydromorphone (0.001nM-1mM), or norhydrocodone (0.01nM-1mM). For saturation and competition studies, non-specific binding was determined in triplicate in the presence of 1µM cold levorphanol ([³H]DAMGO, [³H]DPDPE) or 10µM naloxone ([³H]U69,593). All tubes were incubated for 90min at 25°C;
then incubation was terminated by the addition of ice-cold 50mM potassium phosphate buffer and samples were filtered over GF/B filters (Brandel, Gaithersburg, MD). Tubes were washed three times with phosphate buffer and filters were counted. For \(^{3}H\)U69,593 binding, filters were soaked in 0.5%w/v polyethylenimine (Sigma-Aldrich, Saint Louis, MO) for 90min before use. Binding data were analyzed by Prism ver. 5.0 (Graphpad Software, La Jolla, CA) using non-linear regression. \(K_D\) and \(B_{\text{max}}\) values were calculated for saturation studies. \(K_i\) values were determined for competition studies (Cheng and Prusoff, 1973). All binding data were best fit by one-site model.

**In vivo Studies**

**General Procedure**

Three routes of administration were used: SC, IT and ICV. Mice were injected with hydrocodone, hydromorphone or norhydrocodone based on doses determined in preliminary experiments. The following procedures were used for all three routes.

Mice were tested for analgesia using the tailflick assay at 5–240min post injection (see Analgesia assay; below) to determine the time of peak analgesic effect. Dose-response studies were conducted for each drug at the time of peak analgesic effect. Finally, the effect of naltrexone on opioid-induced analgesia was determined. Quantal data were used for dose-response functions and to determine ED\(_{50}\)s for analgesia and graded data was collected for time-action and naltrexone antagonism studies. The naltrexone antagonism studies were designed to test analgesia at the time of peak effect for both the opioid agonists (hydrocodone, hydromorphone, norhydrocodone) and the opioid antagonist (naltrexone). The time of peak effect for SC naltrexone has been shown to be 45min (Sirohi et al., 2007).
Mice injected IT with hydrocodone, hydromorphone and norhydrocodone were observed for seizure activity for up to 30min (see Seizure activity assay below). The effect of SC and IT naltrexone on IT hydrocodone, hydromorphone and norhydrocodone-induced seizures was studied. In these studies agonists were injected IT at the time of peak effect for naltrexone. Quantal data were collected for seizure dose-response functions to determine ED50s and to examine the effect of naltrexone on opioid-induced seizures.

**SC Analgesia Studies**

Mice (N=5–6/group) were injected with hydrocodone (1.25mg/kg), hydromorphone (0.3mg/kg) or norhydrocodone (4-200mg/kg) and tested for analgesia at 5–240min post injection to determine the time of peak analgesic effect. Next, mice (N=5-6/dose) were injected with hydrocodone (0.625-3mg/kg), hydromorphone (0.1-0.4mg/kg) or norhydrocodone (4-200mg/kg) and tested for analgesia at the time of peak effect and ED50s estimated. Lastly, mice (N=6-11/group) were injected SC with either saline or naltrexone (1mg/kg) and then injected immediately with hydromorphone (0.3mg/kg) or norhydrocodone (100mg/kg); or 30min later injected with hydrocodone (2mg/kg). Forty-five min after saline or naltrexone injections at the time of peak effect of each opioid and naltrexone, mice were tested for analgesia.

**IT Analgesia Studies**

Mice (N=5–9/group) were injected with hydrocodone (1-5µg/mouse), hydromorphone (0.005-0.5µg/mouse) or norhydrocodone (0.01-1µg/mouse) and tested for analgesia at 5–240min post injection to determine the time of peak analgesic effect. Next, mice (N=6-11/dose) were injected with hydrocodone (0.1-5µg/mouse), hydromorphone (0.001-0.05µg/mouse) or norhydrocodone (0.001-1µg/mouse) and tested for analgesia at the time of peak effect and ED50s estimated. Lastly, mice (N=6-14/group) were injected SC with either saline or naltrexone
(1mg/kg or 10mg/kg) and then 35min later injected with hydrocodone (5µg/mouse) or hydromorphone (0.05µg/mouse); or 40min later injected with norhydrocodone (0.1µg/mouse).

Forty-five min after saline or naltrexone injections at the time of peak effect of each opioid and naltrexone, mice were tested for analgesia. In the IT analgesia studies it was noted that high doses of norhydrocodone (>1µg/mouse) induced seizure activity in mice.

**ICV Analgesia Studies**

Mice (N=5–6/group) were injected with hydrocodone (1-20µg/mouse), hydromorphone (0.01-0.1µg/mouse) or norhydrocodone (1-10µg/mouse) and tested for analgesia at 10–180min post injection to determine the time of peak analgesic effect. Next, mice (N=6-12/dose) were injected with hydrocodone (0.5-20µg/mouse), hydromorphone (0.01-0.1µg/mouse) or norhydrocodone (0.25-4µg/mouse) and tested for analgesia at the time of peak effect and ED50s estimated. In addition, mice (N=5-11/group) were injected SC with either saline or naltrexone (1mg/kg) and then 15min later injected with hydromorphone (0.1µg/mouse); or 30min later injected with hydrocodone (10µg/mouse) or norhydrocodone (4µg/mouse). Forty-five min after saline or naltrexone injections at the time of peak effect of each opioid and naltrexone, mice were tested for analgesia.

It was hypothesized that SC norhydrocodone-induced analgesia was predominantly a supraspinal effect and therefore we examined the effect of ICV naltrexone on SC norhydrocodone. Mice (N=5/group) were injected SC with norhydrocodone (200mg/kg) and then 30min later injected ICV with either saline or naltrexone (10µg/mouse). Forty-five min after SC norhydrocodone injections mice were tested for analgesia.
IT Seizure Studies

As noted above (IT analgesia studies), mice injected IT with doses of norhydrocodone >1µg/mouse demonstrated seizure activity. To explore this informal observation, dose-response studies for IT opioid agonist-induced seizures were conducted (see Seizure activity assay below). Mice (N=5-10/dose) were injected IT with hydrocodone (5-50µg/mouse), hydromorphone (0.5-7.5µg/mouse) or norhydrocodone (1-10µg/mouse) and observed for seizure activity and ED$_{50}$s estimated. Next, mice (N=6-15/group) were injected SC with either saline or naltrexone (10 or 30mg/kg) and then 45min later injected IT with hydrocodone (50µg/mouse), hydromorphone (5µg/mouse) or norhydrocodone (10µg/mouse) and observed for seizure activity. In addition, mice (N=5-6/group) were injected with IT naltrexone (10µg/mouse) alone or IT naltrexone (10µg/mouse) and IT hydrocodone (50µg/mouse), hydromorphone (5µg/mouse) or norhydrocodone (10µg/mouse) and observed for seizure activity.

Intrathecal (IT) Injection

Mice were injected IT using a modification of the method of Hylden and Wilcox (1980). Mice were briefly (≈2min) anesthetized with isoflurane:oxygen (4:96) and injected (2-4µl) in the lumbar intrathecal space using a 30-gauge needle attached to a 10µl glass syringe (Hamilton, Reno, NV, USA). The needle was held in place for 10s following each injection.

Intracerebroventricular (ICV) Injection

Mice were injected ICV using previously described methods (Pedigo et al., 1975; Yoburn et al., 1988). Mice were briefly (≈ 2min) anesthetized with isoflurane:oxygen (4:96) and a small scalp incision was made in order to expose the skull. The incision was closed following injection with a surgical staple. Drug solutions (4µl) were injected 2mm lateral and 2mm posterior to
bregma using a 2.5mm long 27-gauge needle attached to a 25µl glass syringe (Hamilton, Reno, NV, USA). The needle was held in place for 30s following each injection.

**Analgesia Assay**

Analgesia (antinociception) was determined using the tailflick assay (Tailflick Model 37360, UGO Basile, Comerio, VA, Italy). The ventral surface of the tail was exposed to heat ≈ 2cm from the tip of the tail. The heat stimulus was adjusted so that the baseline tailflick latency would typically be 2-4s (actual mean ±SD baseline latency = 2.4 ±1.1s). Baseline testing was performed for each animal before drug administration. During baseline testing, if the animal did not remove its tail from the heat source by 10s, the test was terminated and the animal was excluded from further study. Following drug treatment, if a mouse did not remove its tail from the heat source by 10s, the test was terminated, a latency of 10s was recorded and the mouse was defined as analgesic. All testing was conducted by a tester who was unaware of the treatment of an individual mouse.

**Seizure activity Assay**

Mice were observed for seizure activity for up to 30min following IT injection of opioid agonists. The effects included: agitation, hind limb extension and rotatory movements, followed by development of tonic-clonic seizures and in some cases loss of righting reflex (Lutfy et al., 1994). The onset of seizure activity was always within 15min of IT injection. The duration of seizures was not determined. A tester unaware of individual mouse treatment observed the animals and identified a mouse as positive for seizure activity. Seizure activity was collected as quantal data.
Drugs

Hydrocodone bitartrate, hydromorphone hydrochloride and naltrexone hydrochloride were obtained from Spectrum Chemicals Inc. (Gardena, CA). Norhydrocodone hydrochloride was obtained from Lipomed Inc. (Cambridge, MA). Drugs for all the injections (SC, IT, ICV) were dissolved in 0.9% saline and doses are expressed as the free base.

Data Analysis

Receptor binding studies were analyzed using one-way analysis of variance (ANOVA) and post-hoc Student-Newman-Keuls tests (Prism ver. 5.0, Graphpad Software, La Jolla, CA). Quantal dose-response data were analyzed using the BLISS-21 computer program (Department of Statistics, University of Edinburgh, Edinburgh, UK). This program uses Probit analysis (Finney, 1973) to calculate ED$_{50}$s, standard errors, 95% confidence intervals and relative potency estimates (ratio of respective ED$_{50}$s). For analyzing the effect of naltrexone on SC, IT or ICV opioid-induced analgesia data, significant differences were calculated using the Student’s t-test or one-way ANOVA and post-hoc Student-Newman-Keuls tests (Prism ver. 5.0, Graphpad Software, La Jolla, CA). For analyzing the effect of naltrexone on IT opioid-induced seizures data, chi-square analysis was employed (Prism ver. 5.0, Graphpad Software, La Jolla, CA). In all cases, the level of significance was p<0.05.
Results

Receptor Binding Studies

Opioid receptor (μ, δ, κ) saturation binding studies were performed in mouse spinal cord tissue homogenate. The mean (±SD) B$_{max}$ and K$_D$ from three independent experiments were: [3H]DAMGO (μ): 164(±39)fm/mg protein, 1.4(±0.4)nM; [3H]DPDPE (δ): 31(±8)fm/mg protein, 1.2(±0.2)nM; [3H]U69,593 (κ): 28(±1)fm/mg protein, 3.3(±0.4)nM. Competition binding studies for hydrocodone, hydromorphone and norhydrocodone indicated that each opioid was relatively μ-selective, with lowest affinity for κ-receptors (Table 1). At μ- and κ-opioid receptors, there was a significant (p<0.05) difference in potency among all three opioids with potency for hydromorphone > hydrocodone > norhydrocodone (μ: F$_{2,9}$=31.11; κ: F$_{2,6}$=16.31). At δ-opioid receptor, the order of potency was the same, although the difference was just outside the significance level (F$_{2,6}$=4.58, p=0.06).

SC Analgesia Studies

The time of peak effect for analgesia following SC administered hydrocodone, hydromorphone and norhydrocodone was estimated as 15, 45 and 45min, respectively (Fig. 1A). Dose-response studies for SC opioid-induced analgesia were conducted at the time of peak effect for each opioid and ED$_{50}$s were determined (Fig. 1B, Table 2). The order of potency for SC opioid-induced analgesia was hydromorphone > hydrocodone > norhydrocodone. Hydromorphone was significantly more potent (≈ 5.4-fold), and norhydrocodone was significantly less potent (≈ 70-fold), than the parent compound in producing analgesia following SC administration (p<0.05, Probit analysis). Mice were injected with naltrexone (1mg/kg, SC) and hydrocodone (2mg/kg, SC), hydromorphone (0.3mg/kg, SC) or norhydrocodone (100mg/kg,
SC) and tested for analgesia at the time of peak effect for both antagonist and agonist (see Materials and Methods). Naltrexone significantly antagonized SC hydrocodone, hydromorphone and norhydrocodone-induced analgesia, confirming that systemic opioid-induced analgesia is opioid receptor-mediated (Fig. 1C).

**IT Analgesia Studies**

The time of peak effect for analgesia following IT administered hydrocodone, hydromorphone and norhydrocodone was estimated as 10, 10 and 5min, respectively (Fig. 2A). To examine the possible role of the IT injection protocol itself on tailflick latency, mice (N=5) were injected with saline (IT) and tested for analgesia at 5, 10, 15 and 30min. Following injection, there was no significant change from baseline tailflick latency (F4,20=1.21, p>0.05), suggesting the IT injection protocol did not alter nociception. Dose-response studies for IT opioid-induced analgesia were conducted at the time of peak effect for each opioid and ED50s were estimated (Fig. 2B, Table 2). Hydromorphone was significantly more potent (≈ 174-fold) than hydrocodone in producing analgesia following IT administration (p<0.05, Probit analysis). IT norhydrocodone produced a shallow dose-response function for spinal analgesia and the ED50 could not be estimated, although 15%-45% of mice were analgesic at IT doses ≥ 0.001μg/mouse. Norhydrocodone (>1µg/mouse, IT) induced seizure activity in mice that precluded the examination of analgesic effects at higher doses (see IT Seizure Studies below). Next, mice were injected with naltrexone (1 or 10mg/kg, SC) and hydrocodone (5µg/mouse, IT), hydromorphone (0.05µg/mouse, IT) or norhydrocodone (0.1µg/mouse, IT) and tested for analgesia at the time of peak effect for both antagonist and agonist. Naltrexone (1mg/kg, SC) significantly (p<0.05) antagonized IT hydrocodone and IT hydromorphone-induced analgesia. A higher dose of SC naltrexone (10mg/kg) was required to significantly antagonize IT norhydrocodone-induced
analgesia ($F_{2,29}=3.63, p<0.05$). Antagonism by naltrexone confirms that IT opioid-induced analgesia is opioid receptor-mediated (Fig. 2C).

**ICV Analgesia Studies**

The time of peak effect for analgesia following ICV administered hydrocodone, hydromorphone and norhydrocodone was estimated as 15, 30 and 15min, respectively (Fig. 3A). To examine the possible role of the ICV injection protocol on tailflick latency, mice (N=13) were injected with saline (ICV) and tested for analgesia at 10, 15 and 30min. Following injection, there was no significant change from the baseline tailflick latency ($F_{3,48}=1.71, p>0.05$). Dose-response studies for ICV opioid-induced analgesia were conducted at the time of peak effect for each opioid agonist and ED$_{50}$S were determined (Fig. 3B, Table 2). The order of potency for ICV opioid-induced analgesia was hydromorphone $>$ norhydrocodone $\approx$ hydrocodone. Hydromorphone was significantly more ($\approx$ 96-fold) potent than hydrocodone in producing analgesia following ICV administration ($p<0.05$, Probit analysis). There was no significant difference in the potency of ICV norhydrocodone and ICV hydrocodone for induction of analgesia ($p>0.05$, Probit analysis). Mice were injected with naltrexone (1mg/kg, SC) and hydrocodone (10µg/mouse, ICV), hydromorphone (0.1µg/mouse, ICV) or norhydrocodone (4µg/mouse, ICV) and tested for analgesia at the time of peak effect for both antagonist and agonist. Naltrexone significantly antagonized ICV hydrocodone, hydromorphone and norhydrocodone-induced analgesia, confirming that supraspinal opioid-induced analgesia was opioid receptor-mediated (Fig. 3C).

It was hypothesized that systemic norhydrocodone-induced analgesia was predominantly a supraspinal effect. In order to test this hypothesis, mice were injected SC with norhydrocodone...
(200mg/kg, SC) and naltrexone (10µg/mouse, ICV). ICV administration of naltrexone completely antagonized the analgesic effect of systemic norhydrocodone (Fig. 4), suggesting that SC norhydrocodone-induced analgesia is primarily mediated via supraspinal opioid receptors.

**IT Seizure Studies**

As noted above (see *IT Analgesia Studies*), norhydrocodone (>1µg/mouse) was observed to produce seizure activity in mice after spinal injection. Therefore, dose-response studies of IT opioid-induced seizures were conducted and ED50s were determined (Fig. 5A, Table 3). The order of potency for opioid-induced seizures following IT administration was norhydrocodone ≈ hydromorphone > hydrocodone. Norhydrocodone and hydromorphone were significantly more potent (≈ 4.6- and 3.7-fold, respectively) than the parent compound in induction of seizures (p<0.05, Probit analysis). Next, the effect of SC and IT naltrexone on opioid-induced seizures was examined. Naltrexone (10mg/kg, SC and 10µg/mouse, IT) did not antagonize seizures induced by IT opioid agonists (Fig. 5B). In addition, a higher dose of SC naltrexone (30mg/kg) was also unable to significantly antagonize seizures produced by norhydrocodone (10µg/mouse, IT). Naltrexone (10µg/mouse, IT) alone did not induce any seizure activity (data not shown). At the doses used, no seizure activity was observed following SC or ICV administration of hydrocodone, hydromorphone and norhydrocodone.
Discussion

In the current study, we examined the pharmacodynamics of hydrocodone and its major metabolites hydromorphone and norhydrocodone in the mouse. Hydrocodone and hydromorphone-induced pharmacological effects have been well-studied in humans and rodents (Small et al., 1938; Murray and Hagen, 2005; Peckham and Traynor, 2006; Susce et al., 2006; Kumar et al., 2008; Madia et al., 2009). In addition, some reports suggest that hydromorphone, an active metabolite of hydrocodone, is primarily responsible for hydrocodone-induced in vivo effects (Otton et al., 1993; Lurcott, 1998). To date, the in vivo effects of norhydrocodone are not well-documented. If norhydrocodone is active in vivo, it may contribute to the pharmacodynamic profile of hydrocodone since plasma concentrations of norhydrocodone in humans have been reported to be greater than that of hydromorphone (Hao et al., 2011). Further, norhydrocodone is detected in urine for significantly longer than hydromorphone and hydrocodone (Valtier and Bebarta, 2012).

The present binding studies demonstrated that norhydrocodone, hydrocodone and hydromorphone are μ-selective opioid ligands, although norhydrocodone has lower affinity at μ and κ-opioid receptors compared to hydrocodone (Table 1). In vitro norhydrocodone has been shown to be moderately lower in potency, with similar efficacy, to hydrocodone at μ and δ-opioid receptors (Thompson et al., 2004). While binding studies and in vitro pharmacodynamic data raise the possibility that norhydrocodone might possess in vivo activity and contribute to the opioid receptor-mediated effects of hydrocodone, to our knowledge, the in vivo activity of norhydrocodone has not been thoroughly examined.
Norhydrocodone was active in vivo following SC, IT and ICV administration. The potency and maximal analgesic effect of norhydrocodone compared to the parent varied depending on the route of administration, with SC injection resulting in low potency (≈ 70-fold less) but similar maximal analgesic effect (Fig. 1B, Table 2). Spinally, norhydrocodone produced reduced analgesic effect (15-45%) compared to hydrocodone (67%) and hydromorphone (100%) and its ED₅₀ could not be accurately determined (Fig. 2B, Table 2). Supraspinally, norhydrocodone has similar potency compared to hydrocodone and produced equal maximal analgesic effect (Fig. 3B, Table 2). Hydromorphone was the most potent opioid analgesic of the three drugs for all routes of administration (Table 2). SC naltrexone significantly antagonized SC, IT and ICV hydrocodone, hydromorphone and norhydrocodone-induced analgesia confirming that analgesia was opioid receptor-mediated (Fig. 1C, 2C, 3C).

The analgesic properties of SC norhydrocodone appear to be mostly mediated by supraspinal opioid receptors. First, as noted above, IT norhydrocodone was associated with a reduced analgesic effect, whereas ICV norhydrocodone produced full analgesia (Fig. 2B, 3B). Second, SC norhydrocodone-induced analgesia was blocked by ICV naltrexone injection (Fig. 4). Taken together, these results suggest that systemic norhydrocodone induces opioid receptor-mediated analgesia, primarily via supraspinal opioid receptors. The low potency of SC norhydrocodone may be related to reduced access of the drug to the CNS, primarily supraspinal sites.

Spinal opioid-induced neuroexcitation has been reported in humans and rodents (Frenk et al., 1984; Parkinson et al., 1990). Studies have demonstrated that opioid nor-metabolites (e.g. normeperidine, noroxycodone) are more potent than parent compounds in inducing seizure activity in rodents (Umans and Inturrisi, 1982; Leow and Smith, 1994). In the present study,
hydrocodone, hydromorphone and norhydrocodone induced seizures following spinal administration. IT norhydrocodone and hydromorphone were roughly equipotent in inducing seizures. IT hydrocodone was less potent than IT norhydrocodone and IT hydromorphone in producing seizure activity (Fig. 5A, Table 3). No seizure activity was observed following SC or ICV administration of these compounds, within the range of doses tested. Apparently, IT seizure activity is not opioid receptor-mediated since it was not antagonized by SC or IT naltrexone (Fig. 5B). These results agree with previous reports examining neuroexcitation following a range of opioid agonists (Umans and Inturrisi, 1982; Frenk et al., 1984; Yoburn et al., 1990; Leow and Smith, 1994).

The present results raise the possibility that neuroexcitatory effects may develop following hydrocodone administration. Although there is at least one report of neuroexcitation following hydrocodone treatment (Lauterbach, 1999), it is not known if this is related to the parent or the metabolites. In a study in which formation of hydromorphone is inhibited, and norhydrocodone potentially increased, there was an increase in unpleasant effects of oral hydrocodone (e.g. dysphoria, physical unpleasantness; Otton et al., 1993). It is possible that the unpleasant effects might be a precursor to frank neuroexcitatory actions, although this is not known. Nevertheless, following acute oral hydrocodone, the plasma and urine levels of norhydrocodone invariably exceed that of the hydromorphone and sometimes that of hydrocodone (Hao et al., 2011; Valtier and Bebarta, 2012). Chronic hydrocodone administration might result in substantial levels of the nor-metabolite that may mediate unpleasant subjective effects and perhaps incipient neuroexcitatory action. Further studies are required to examine this possibility.
In summary, norhydrocodone is a $\mu$-selective opioid ligand and produces analgesia which appears to be primarily supraspinal. Following spinal injection, it induces non-opioid receptor-mediated neuroexcitation and is less effective in producing analgesia compared to hydrocodone and hydromorphone. Overall, the observation that norhydrocodone possesses \textit{in vivo} activity raises the possibility that it might play a role in therapeutic as well as toxic effects following acute or chronic hydrocodone treatment. Further studies are needed to evaluate the concentration of norhydrocodone in CNS following hydrocodone administration to address these issues.
Acknowledgements

The authors are grateful to Dr. M.T. Turnock for continuous encouragement and discussions during this study. Shruti Jaswal and Mili R. Gajjar provided technical and procedural assistance. The authors acknowledge Aarti Manyal for helpful discussions and support.
Authorship Contributions

Participated in research design: Yoburn and Navani

Conducted experiments: Navani

Performed data analysis: Yoburn and Navani

Wrote or contributed to the writing of the manuscript: Yoburn and Navani
References


Cheng Y and Prusoff WH. (1973) Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition (IC_{50}) of an enzymatic reaction. *Biochem Pharmacol* 22:3099-3108.


Footnotes

This project was supported by the Department of Pharmaceutical Sciences, College of Pharmacy and Health Sciences, St. John’s University.
Figure Legends

Figure 1: Hydrocodone (HYC), hydromorphone (HYM) and Norhydrocodone (NHYC) induced analgesia following SC administration

(A) Mice were injected SC with HYC, HYM or NHYC and tested for analgesia at 5–240min post injection. HYC and HYM time-action studies are results of a single experiment. NHYC time-action was examined with a range of doses (see Materials and Methods) and a representative dose is presented. B=Baseline tailflick latency. (B) For dose-response studies, mice were injected with HYC, HYM or NHYC and tested for analgesia at the time of peak effect. HYC, HYM and NHYC dose-response curves are results of single experiment (see Table 2 for ED50s). (C) Mice were injected SC with either saline or NTX and then injected SC with HYC, HYM or NHYC. Mice were tested for analgesia at the time of peak effect of each opioid and NTX. Data presented for antagonism of analgesia are the results of 1 (HYC, NHYC) or 2 combined (HYM) independent experiments. *Significantly different (p<0.05) compared to saline injected animals.

Figure 2: Hydrocodone (HYC), hydromorphone (HYM) and Norhydrocodone (NHYC) induced analgesia following IT administration

(A) Mice were injected IT with HYC, HYM and NHYC and tested for analgesia at 5-240min post injection. Time-action studies were conducted with a range of doses for each opioid (see Materials and Methods) with similar results. Representative data from a single dose are presented. B=Baseline tailflick latency. (B) For dose-response studies, mice were injected with HYC, HYM or NHYC and tested for analgesia at the time of peak effect. HYC and HYM dose-response curves are results of a single experiment and the NHYC dose-response curve is the
mean result of 3 independent experiments (see Table 2 for ED50s). (C) Mice were injected SC with either saline or NTX and then injected IT with HYC, HYM or NHYC. Mice were tested for analgesia at the time of peak effect of each opioid and NTX. Data presented for antagonism of analgesia are the results of 1 (HYC, HYM) or 2 combined (NHYC) independent experiments. *Significantly different (p<0.05) compared to saline injected animals.

Figure 3: Hydrocodone (HYC), hydromorphone (HYM) and Norhydrocodone (NHYC) induced analgesia following ICV administration

(A) Mice were injected ICV with HYC, HYM or NHYC and tested for analgesia at 10–180min post injection. Time-action studies were conducted with a range of doses for each opioid (see Materials and Methods) with similar results. Representative data from a single dose are presented. B=Baseline tailflick latency. (B) For dose-response studies, mice were injected with HYC, HYM or NHYC and tested for analgesia at the time of peak effect. HYC and HYM dose-response curves are from a single experiment. The NHYC dose-response curve is the mean of 2 independent experiments (see Table 2 for ED50s). (C) Mice were injected SC with either saline or NTX and then injected with HYC, HYM or NHYC. Mice were tested for analgesia at the time of peak effect of each opioid and NTX. Data presented for antagonism of analgesia are the results of 1 (HYC, HYM) or 2 combined (NHYC) independent experiments. *Significantly different (p<0.05) compared to saline injected animals.

Figure 4: The effect of ICV naltrexone (NTX) on SC norhydrocodone (NHYC) induced analgesia
Mice were injected SC with NHYC and ICV with either saline or NTX and tested for analgesia at the time of peak effect of NHYC. Data presented are the results of a single experiment. *Significantly different (p<0.05) compared to saline injected animals.

**Figure 5: Hydrocodone (HYC), hydromorphone (HYM) and Norhydrocodone (NHYC) induced seizure activity following IT administration**

(A) For dose-response studies, mice were injected IT with HYC, HYM or NHYC and observed for seizure activity for up to 30min. HYC, HYM and NHYC dose-response curves are the mean results of 2-3 independent experiments for each drug (see Table 3 for ED50s). (B) Mice were injected SC with either saline or NTX and then injected IT with HYC, HYM or NHYC at the time of peak effect of NTX and observed for up to 30min for seizure activity. In addition, mice were injected with IT NTX and IT HYC, HYM or NHYC simultaneously and observed for up to 30min for seizure activity. Data presented for antagonism of seizures are results of 1 (HYC, HYM) or 3 combined (NHYC) independent experiments.
Tables

Table 1: Opioid receptor $K_i$ and selectivity ratios for Hydrocodone (HYC), Hydromorphone (HYM) and Norhydrocodone (NHYC) in mouse spinal cord tissue homogenate

<table>
<thead>
<tr>
<th>Drug</th>
<th>Mean $K_i$ (nM) (95%CL)</th>
<th>Selectivity ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\mu$</td>
<td>$\delta$</td>
</tr>
<tr>
<td>HYC</td>
<td>56 (40-80)</td>
<td>1429 (974-2097)</td>
</tr>
<tr>
<td>HYM</td>
<td>1.3 (0.9-2.0)</td>
<td>35 (23-55)</td>
</tr>
<tr>
<td>NHYC</td>
<td>142 (96-210)</td>
<td>2166 (1472-3188)</td>
</tr>
</tbody>
</table>

Data are means of three independent experiments. Selectivity ratios were determined by dividing the respective $K_i$ values. See Material and Methods for details.
Table 2: ED$_{50}$ estimates for Hydrocodone (HYC), Hydromorphone (HYM) and Norhydrocodone (NHYC) induced analgesia

<table>
<thead>
<tr>
<th>Route of administration</th>
<th>HYC</th>
<th>HYM</th>
<th>NHYC</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC (mg/kg)</td>
<td>1.37 (0.93-1.96)</td>
<td>0.26 (0.18-0.36)</td>
<td>95.77 (63.70-146.21)</td>
</tr>
<tr>
<td>IT (μg/mouse)</td>
<td>1.18 (0.42-4.28)</td>
<td>0.0068 (0.0022-0.021)</td>
<td>ND</td>
</tr>
<tr>
<td>ICV (μg/mouse)</td>
<td>3.54 (1.67-7.26)</td>
<td>0.037 (0.019-0.075)</td>
<td>1.98 (1.30-3.39)</td>
</tr>
</tbody>
</table>

Mice were injected SC, IT or ICV with an opioid agonist and tested for analgesia as described in the Material and Methods. (ND=Not Determinable)
Table 3: ED$_{50}$ estimates for Hydrocodone (HYC), Hydromorphone (HYM) and Norhydrocodone (NHYC) induced seizure activity

<table>
<thead>
<tr>
<th>Route of administration</th>
<th>HYC</th>
<th>HYM</th>
<th>NHYC</th>
</tr>
</thead>
<tbody>
<tr>
<td>IT ($\mu$g/mouse)</td>
<td>19.43 (12.63-31.63)</td>
<td>5.25 (3.54-8.27)</td>
<td>4.27 (2.89-6.76)</td>
</tr>
</tbody>
</table>

Mice were injected IT with an opioid agonist and observed for seizure activity as described in the Material and Methods.
Figure 1
Figure 2
Figure 3

A) Mean Tail Flick Latency (s)

B) Percent Analgesic

C) Mean (+SEM) Tail Flick Latency (s)

- HYC (5 μg/mouse)
- HMY (0.05 μg/mouse)
- NHYC (2.5 μg/mouse)

Dose (μg/mouse)

- Saline
- NTX (1mg/kg, SC)

* Significant difference
Figure 4
Figure 5