Antinociceptive Properties of the New Alkaloid, cis-8,10-Di-N-Propyllobelidiol Hydrochloride Dihydrate Isolated from Siphocampylus verticillatus: Evidence for the Mechanism of Action

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Accepted for publication November 18, 1998

ABSTRACT

The antinociceptive action of the alkaloid cis-8,10-di-N-propyllobelidiol hydrochloride dehydrate (DPHD), isolated from Siphocampylus verticillatus, given i.p., p.o., i.t., or i.c.v., was assessed in chemical and thermal models of nociception in mice, such as acetic acid-induced abdominal constriction, formalin- and capsaicin-induced licking, and hot-plate and tail-flick tests. DPHD given by i.p., p.o., i.t., or i.c.v. elicited significant and dose-related antinociception. At the ID₅₀ level, DPHD was about 2- to 39-fold more potent than aspirin and dipyrone, but it was about 14- to 119-fold less potent than morphine. Its antinociceptive action was reversed by treatment of animals with naloxone, cyprodime, naltrindole, nor-p₃-clorophenylalanine methyl ester hydrochloride; PCPA, dl-p-chlorophenylalanine methyl ester hydrochloride; L-ARG, L-arginine; L-NOARG, N₆-nitro-L-arginine; GABA, γ-aminobutyric acid.
antinociception caused by DPHD, like that caused by morphine, is associated with inhibition of nerve-mediated contractions in the guinea pig ileum and mice vas deferens in vitro, as well against the intestinal transit in mice in vivo.

Materials and Methods

Animals

Male Swiss mice (25–35 g) and albino guinea pigs of either sex (350–550 g), housed at 22 ± 2°C under a 12 h light/12 dark cycle and with access to food and water ad libitum, were used throughout the experiments. Mice were acclimated to the laboratory for at least 1 h before testing and were used once throughout the experiments. The experiments reported were carried out in accordance with the current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain in conscious animals (Zimmermann, 1983).

Pharmacological Analysis

In Vivo Experiments. Acetic acid-induced abdominal constriction. The abdominal constrictons resulting from i.p. injection of acetic acid (0.6%) were similar to that described previously (Corrêa et al., 1996; Vaz et al., 1996). Animals were pretreated with DPHD (29.1–582.2 μmol/kg) or with vehicle (10 ml/kg) 0.5 (i.p.) and 1 (p.o.) h before injection of acetic acid. After the challenge, pairs of mice were placed in separate boxes and the number of abdominal constrictons was cumulatively counted over a period of 20 min after acetic acid injection.

Formalin test. The procedure used was essentially similar to that described previously (Corrêa and Calixto, 1993; Vaz et al., 1996; Santos and Calixto, 1997). Twenty microliters of 2.7% formalin solution (0.92% of formaldehyde), made up in PBS (NaCl 137 mM, KCl 2.7 mM, and phosphate buffer, 10 mM), was injected intraplantarly under the surface of the right hindpaw. Animals were treated with DPHD (8.7–582.2 μmol/kg) or with vehicle (10 ml/kg) by i.p. or p.o. routes, 0.5 and 1 h before formalin injection, respectively. Other groups of animals were treated with DPHD (29.1–291.1 nmol/site) or with vehicle (5 μl/site) by i.c.v. or i.t. routes, as described previously (Hylden and Wilcox, 1980; Vaz et al., 1996, Santos and Calixto, 1997), 10 min before formalin injection. After intraplantar injection of formalin, the animals were immediately placed in a glass cylinder 20 cm in diameter and the time spent licking the injected paw was timed with a chronometer and considered indicative of pain. To investigate whether the antinociceptive activity of DPHD in formalin-induced pain was associated with antiedematogenic activity, at the end of all experiments the animals were sacrificed by cervical dislocation 30 min after formalin injection, and the paw was cut at the knee joint and weighed on an analytical balance (Santos and Calixto, 1997).

Capsaicin-induced pain. In an attempt to provide more direct evidence concerning the possible antinociceptive effect of DPHD on neurogenic pain, we also investigated whether DPHD antagonized capsaicin-induced pain in the mouse paw. The procedure used was similar to that described previously (Corrêa et al., 1996, Santos and Calixto, 1997). Twenty microliters of capsaicin (1.6 μg/paw made in PBS) was injected intraplantarly under the surface of the right hindpaw. Animals were observed individually for 5 min after capsaicin injection. The amount of time spent licking the injected paw was timed with a chronometer and was considered indicative of pain. Animals were treated either with i.p. or p.o. injection of vehicle (10 ml/kg) or DPHD (29.1–582.2 μmol/kg), or with indomethacin (2.8–27.9 μmol/kg i.p.) or morphine (1.5–15.5 μmol/kg s.c.), 0.5 and 1 h before capsaicin injection. Other groups of animals were treated with DPHD (29.1–291.1 nmol/site) or with vehicle (5 μl/site) by i.c.v. or i.t. routes 10 min before capsaicin injection.

Hot-plate test. The hot-plate test was used to measure the response latencies according to the method described previously (Vaz et al., 1996; Beirith et al., 1998). The reaction time was recorded for mice pretreated with vehicle (10 ml/kg i.p., 5 μl/site i.t. or i.c.v.), DPHD (291.1 μmol/kg i.t. or i.c.v.), or morphine (31.0 μmol/kg s.c., 15.5 nmol/site i.t. or i.c.v.) 0.5 h and 10 min before the tests. All animals were selected 1 h before the test on the basis of their reactivity in the model by eliminating those mice that remained on the apparatus (maintained at 50°C) for up to 15 s. Each animal was used as its own control. A latency period (cut-off) of 20 s was defined as complete analgesia.

Tail-flick test. A radiant heat tail-flick analgesiometer was used to measure response latencies according to the method described previously (Vaz et al., 1996; Beirith et al., 1998). The reaction time was recorded for control (saline injection) mice or in animals pretreated with DPHD (291.1 μmol/kg i.p.) or with morphine (31.0 μmol/kg s.c.) 0.5 h before the tests. All animals were selected 24 h before the test on the basis of their reactivity in the model by eliminating those mice that remained on the apparatus for up to 8 s. A latency period (cut-off) of 20 s was defined as complete analgesia.

Rota-rod test. To evaluate the possible nonspecific muscle-relaxant or sedative effects of DPHD, the mice were tested on the rota-rod, as described previously (Vaz et al., 1996; Beirith et al., 1998). The animals were selected 24 h before to the test by eliminating those mice that did not remain on the bar for two consecutive periods of 60 s. Animals were treated with DPHD (291.1 μmol/kg i.p.) or with saline injection (10 ml/kg i.p.) 30 min before being tested. Results are expressed as the times for which animals remained on the rota-rod. The cut-off time used was 60 s.

Gastrointestinal transit. To test the possible effects of DPHD, in the gastrointestinal motility, the mice were fasted for 24 h after the gastrointestinal transit was analyzed, as described previously (Shannon et al., 1997). The animals were treated with DPHD (87.3 μmol/kg i.p.) or with morphine (15.5 μmol/kg s.c.) 0.5 h after being given a standard charcoal meal (0.3 ml) by gavage. The mice were sacrificed 20 min after administration of the charcoal meal and the distance the charcoal meal had traveled was measured. Data were expressed as the percentage of the gut the charcoal meal traveled. Control animals received the same volume of saline injection (10 ml/kg i.p.) 30 min before being tested.

Analysis of the possible mechanism of action of DPHD. To investigate the participation of the opioid system in the antinociceptive effect of DPHD, animals were pretreated with naloxone, a nonselective opioid receptor antagonist (3.0 μmol/kg i.p.); cyprodime, a selective μ opioid receptor antagonist (2.3 μmol/kg i.p.); naltrindole, a selective δ opioid receptor antagonist (2.2 μmol/kg i.p.); or nor-binaltorphimine, a selective κ opioid receptor antagonist (1.4 μmol/kg i.p.) 15 min before administration of DPHD (87.3 μmol/kg i.p.), morphine (15.5 μmol/kg s.c.), or saline (10 ml/kg i.p.) injection, as reported previously (Craft et al., 1995; Frey and Schicht, 1996; Ossipov et al., 1996). The other groups of animals received DPHD, morphine, naloxone, naltrindole, nor-binaltorphimine, or saline 0.5 h before the formalin injection.

To assess the possible participation of G protein (sensitive to pertussis toxin) in the antinociceptive action of DPHD, animals were pretreated with pertussis toxin (1 μg/site i.c.v.) 7 days before the administration of DPHD (87.3 μmol/kg i.p.) or morphine (15.5 μmol/kg s.c., used as positive control; Sánchez-Blázquez and Garzón, 1991; Beirith et al., 1998). Other groups of animals were treated with
saline (5 µl/site i.c.v) and 7 days later received DPHD, morphine, or the saline injection 0.5 h before the formalin injection.

To assess the possible participation of the γ-aminobutyric acid (GABA) system, animals were treated with DPHD (87.3 µmol/kg i.p.), muscimol, a selective GABA<sub>A</sub> receptor agonist (8.7 µmol/kg i.p.), or with boclofen, a selective GABA<sub>B</sub> receptor agonist (4.6 µmol/kg i.p.) 0.5 h before injection of formalin. The animals received bicuculline, a GABA<sub>B</sub> receptor antagonist (1.9 µmol/kg i.p.), or phaclofen, a GABA<sub>A</sub> receptor antagonist (40.0 µmol/kg i.p.), 15 min before administration of DPHD, baclofen, muscimol, or saline injection, as reported previously (Shafizadeh et al. 1997; Beirith et al., 1998).

In separate series of experiments, we also investigated the possible participation of the nitric oxide- l-arginine pathway in the antinociception caused by DPHD. To this end, animals were pretreated with l-arginine (l-ARG), a precursor of nitric oxide (3444 µmol/kg i.p.), and after 15 min they received DPHD (87.3 µmol/kg i.p.); N<sup>N</sup>-nitro- l-arginine (N<sup>N</sup>-NOARG), an inhibitor of nitric oxide synthesis (342.0 µmol/kg i.p.), morphine (15.5 µmol/kg s.c.), or saline (10 ml/kg i.p.) injection, as reported previously (Santos et al. 1995; Vaz et al., 1996). The algesic responses caused by the first and the second phase of the formalin test were recorded 0.5 h after administration of DPHD, l-NOARG, morphine, or saline injection. Other groups of animals received only DPHD, l-NOARG, morphine, l-ARG, or saline injection 0.5 h before formalin injection.

To assess the possible contribution of serotonin to the antinociceptive effect of DPHD, animals were pretreated with DL-<wbr/>p-chlorophenylalanine methyl ester hydrochloride (PCPA), an inhibitor of serotonin synthesis (399.8 µmol/kg i.p.), once a day for 4 consecutive days, before administration of DPHD (87.3 µmol/kg i.p.), morphine (15.5 µmol/kg s.c.), or saline (10 ml/kg i.p.) injection, as reported previously (Santos et al. 1995; Trentin et al., 1997; Beirith et al., 1998). The pain response caused by intraplantar formalin injection was analyzed 0.5 h after drug administration. Other groups of mice received only DPHD, morphine, or saline injection, 0.5 h before formalin injection.

We also investigated the possible role played by K<sub>ATP</sub> channel in the antinociceptive effect caused by DPHD. For this purpose, animals were pretreated with glibenclamide, a K<sub>ATP</sub> channel blocker (81 nmoi/i.c.v.), and 15 min later, they received DPHD, morphine, or saline injection. The algesic responses caused by formalin were recorded 0.5 h after administration of DPHD (87.3 µmol/kg i.p.), morphine (15.5 µmol/kg s.c.), or saline (10 ml/kg i.p.) injection, as reported previously (Raffa and Martinez, 1995; Beirith et al., 1998). Other groups of animals received saline injection (5 µl/site i.c.v.) 15 min before the administration of DPHD, morphine, or saline. Animals received an injection of formalin 0.5 h later.

The possible existence of cross-tolerance between morphine and DPHD was evaluated by treating animals that were pretreated with morphine (15.5 µmol/kg s.c.), DPHD (87.3 µmol/kg i.p.), or saline (10 ml/kg i.p.) by repetitive administration over a 7-day period before testing formalin-induced pain (Ménard et al., 1995). The animals received one injection per day at 9:00 AM for the first 6 days and a single injection at 8:00 AM on day 7 of either morphine or DPHD. Control groups receiving appropriate vehicle were also tested. DPHD was evaluated by treating animals that were pretreated with vehicle received DPHD or morphine.

To examine the possible contribution of endogenous glucocorticoids in the antinociceptive effect caused by DPHD, animals were anesthetized with 2.2.2-tribromoethanol (0.25 g/kg i.p.) and both adrenal glands were removed through dorsal incision, as described previously by Vaz et al. (1996). After surgery, animals were returned to their cages and allowed free access to food and drink, but water was replaced by saline (0.9% NaCl solution) to maintain physiological sodium plasma concentration. Another group of animals was sham-operated and allowed free access to water and food. After 1 week, animals received DPHD (87.3 µmol/kg i.p.) or saline (10 ml/kg i.p.) injection 0.5 h before formalin injection. The sham-operated animals were used as controls.

**In Vitro Experiments.** Guinea pig ileum field stimulation. Albinio guinea pigs were stunned by a blow on the head and were bled. A portion of ileum 10 to 30 cm from the ileo-cecal junction was excised rapidly and was flushed gently with warm Krebs’ solution (composition: NaCl, 118 mM; KCl, 4.8 mM; CaCl<sub>2</sub>, 2.5 mM; MgSO<sub>4</sub>, 1.2 mM; KH<sub>2</sub>PO<sub>4</sub>, 0.9 mM; NaHCO<sub>3</sub>, 25 mM; and glucose, 11 mM; pH 7.4) to remove contents and adhering adipose tissue. Whole segments (1–3 cm long, 6–8 segments per animal) were set up for recording of isometric contractions along their longitudinal axis in a jacketed organ bath containing 5 ml of gassed (5% CO<sub>2</sub> in O<sub>2</sub>) Krebs’ solution maintained at 37°C, under a basal tension of 1 g. Isometric responses were measured by means of TRI-201 force displacement transducers and were recorded on a polygraph (Letica Scientific Instruments, Barcelona, Spain).

After at least 30 min of equilibration, with renewals of the solution every 10 min, preparations were submitted to field stimulation with rectangular 1-ms pulses of supramaximal voltage (ca. 50–70 V), delivered at 0.1 Hz via platinum electrodes (Guimarães and Rae, 1992). Once the twitch contractions evoked by field stimulation attained a steady level, a single cumulative concentration-response curve was obtained for DPHD or morphine (1 nM–10 µM).

**Mouse vas deferens field stimulation.** Male Swiss mice were lightly anesthetized with ether and sacrificed by a blow on the head and cervical dislocation. Both vasa deferentia were removed and freed of adhering connective and adipose tissue, and were then placed in a Petri dish containing warm physiological salt solution (composition: NaCl, 118 mM; KCl, 4.8 mM; CaCl<sub>2</sub>, 2.5 mM; KH<sub>2</sub>PO<sub>4</sub>, 0.9 mM; NaHCO<sub>3</sub>, 25 mM; and glucose, 11 mM; pH 7.2–7.4). Each vas deferens was then transferred to an organ bath containing 5 ml of gassed (5% CO<sub>2</sub> in O<sub>2</sub>) Krebs’ solution (see concentration above) at 37°C, under a basal tension of 0.5 g. Isometric responses were measured by means of TRI-201 force displacement transducers and were recorded on a polygraph (Letica Scientific Instruments).

At least 45 min of equilibration was allowed before any drug additions, during which the bath solution was renewed every 15 min. Field stimulation was induced with trains of four rectangular 0.5-ms pulses of supramaximal voltage (ca. 20–40 V), delivered at 10 Hz every 20 s via platinum electrodes, as described before (Rae and Calixto, 1990; Maas et al., 1995). Once the twitch contractions evoked by field stimulation attained a steady level, a single concentration-response curve was obtained for DPHD or morphine (1 nM–10 µM).

**Drugs**

The following drugs were used: formalin and morphine hydrochloride (Merck, AG, Darmstadt, Germany); DL-<wbr/>p-chlorophenylalanine methyl ester hydrochloride, pertussis toxin, 2,2,2-tribromoethanol, l-ARG, N<sup>N</sup>-nitro-l-arginine, and capsain (Sigma Chemical Co., St. Louis, MO); glibenclamide, naloxone hydrochloride, cypidine hydrobromide, naltrindole hydrochloride, nor-binaltrophenyl dibydrochloride, baclofen, and phaclofen (Research Biochemicals International, National, Natick, MA); and muscimol and bicuculline (Tocris, Balwin, MO). DPHD was isolated from stems and leaves of S. verticillatus in the Chemistry Department of the Federal University of Santa Catarina, Brazil, as described previously (Miguel et al., 1996). Its degree of purity was higher than 98%. Drugs were dissolved in 0.9% NaCl solution, with the exception of indomethacin and capsain, which were dissolved in TWEEN 80 and absolute ethanol, respectively. All drugs were prepared just before use in 0.9% (w/v) NaCl solution. The final concentration of Tween and ethanol did not exceed 5% and did not cause any effect per se.

**Statistical Analysis**

Results are presented as mean ± S.E.M. except the IC<sub>50</sub> values (i.e., the dose or the concentration of drugs reducing the...
pain or twitch responses by 50% in relation to the control value), which are reported as geometric means accompanied by their respective 95% confidence limits. The statistical significance between groups was calculated by means of analysis of variance followed by Dunnett’s multiple comparison test or by Newman-Keuls’ test when appropriate. \( P \) values less than 0.05 (\( P < .05 \)) were considered as indicative of significance. The ID\(_{50}\) or the IC\(_{50}\) values were determined by linear regression from individual experiments with linear regression GraphPad software (1994; San Diego, CA). Hot-plate and tail-flick latencies were converted to percentage of maximum possible effect with the following equation: MPE\% = 100 \times (postdrug latency – predrug latency)/(cut-off time – predrug latency) (Vaz et al., 1996).

### Results

#### Acetic Acid-Induced Abdominal Constriction

The results of Fig. 2 and the data summarized in Table 1 show that DPHD given by i.p. or by p.o. routes produced significant inhibition of acetic acid-induced abdominal contractions. Given orally, DPHD was 2.6-fold less potent than when it was given by i.p. route.

#### Formalin-Induced Pain

The results shown in Fig. 3 and data summarized in Table 1 show that DPHD given by i.p. or p.o. routes caused significant inhibition of the early (0 to 5 min) and the late phase (15 to 30 min) of the formalin-induced licking. DPHD was

### Table 1

Comparison of the mean ID\(_{50}\) values for the antinociceptive actions of DPHD, aspirin, acetaminophen, morphine, and dipyrone in several models of nociception in mice

<table>
<thead>
<tr>
<th>Writhing Test</th>
<th>Route</th>
<th>ID(_{50})</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPHD</td>
<td>i.p.  ((\mu)mol/kg)</td>
<td>165.0 (148.5–183.4)</td>
</tr>
<tr>
<td></td>
<td>p.o.  ((\mu)mol/kg)</td>
<td>421.1 (370.3–502.2)</td>
</tr>
<tr>
<td>Aspirin(^a)</td>
<td>i.p.  ((\mu)mol/kg)</td>
<td>133.1 (73.0–243.3)</td>
</tr>
<tr>
<td></td>
<td>p.o.  ((\mu)mol/kg)</td>
<td>605.1 (516.2–705.0)</td>
</tr>
<tr>
<td>Acetaminophen(^a)</td>
<td>i.p.  ((\mu)mol/kg)</td>
<td>125.0 (104.0–150.0)</td>
</tr>
<tr>
<td></td>
<td>p.o.  ((\mu)mol/kg)</td>
<td>1144.5 (708.0–1846.0)</td>
</tr>
</tbody>
</table>

Formalin test

<table>
<thead>
<tr>
<th>Route</th>
<th>First Phase (ID(_{50}))</th>
<th>Second Phase (ID(_{50}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPHD i.p. ((\mu)mol/kg)</td>
<td>ND</td>
<td>48.3 (39.6–59.1)</td>
</tr>
<tr>
<td>p.o. ((\mu)mol/kg)</td>
<td>ND</td>
<td>249.2 (191.3–324.3)</td>
</tr>
<tr>
<td>i.c.v. (nmol/site)</td>
<td>146.1 (114.5–193.6)</td>
<td>112.6 (44.2–286.5)</td>
</tr>
<tr>
<td>i.t. (nmol/site)</td>
<td>33.5 (13.1–85.6)</td>
<td>56.2 (25.3–125.7)</td>
</tr>
<tr>
<td>Morphine(^a) s.c. ((\mu)mol/kg)</td>
<td>3.7 (2.4–5.4)</td>
<td>4.7 (3.3–7.0)</td>
</tr>
<tr>
<td>i.c.v. (nmol/site)</td>
<td>4.2 (3.5–5.0)</td>
<td>4.1 (3.1–5.0)</td>
</tr>
<tr>
<td>i.t. (nmol/site)</td>
<td>2.4 (1.8–5.0)</td>
<td>1.0 (0.4–2.6)</td>
</tr>
<tr>
<td>Aspirin(^a) i.p. ((\mu)mol/kg)</td>
<td>ND</td>
<td>123.0 (77.0–209.0)</td>
</tr>
<tr>
<td>p.o. ((\mu)mol/kg)</td>
<td>ND</td>
<td>1565.3 (1348.8–1820.6)</td>
</tr>
<tr>
<td>Acetaminophen(^a) i.p. ((\mu)mol/kg)</td>
<td>ND</td>
<td>120.4 (90.0–161.0)</td>
</tr>
<tr>
<td>p.o. ((\mu)mol/kg)</td>
<td>ND</td>
<td>1706.8 (1422.3–2044.2)</td>
</tr>
<tr>
<td>Dipyrone(^b) i.p. ((\mu)mol/kg)</td>
<td>154.5 (99.9–238.8)</td>
<td>263.7 (234.3–296.9)</td>
</tr>
<tr>
<td>i.c.v. (nmol/site)</td>
<td>0.4 (0.3–0.7)</td>
<td>0.4 (0.3–0.5)</td>
</tr>
<tr>
<td>i.t. (nmol/site)</td>
<td>1.3 (0.9–1.8)</td>
<td>0.9 (0.6–1.4)</td>
</tr>
</tbody>
</table>

Capsaicin test

<table>
<thead>
<tr>
<th>Route</th>
<th>ID(_{50})</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPHD i.p. ((\mu)mol/kg)</td>
<td>105.6 (75.7–147.3)</td>
</tr>
<tr>
<td>p.o. ((\mu)mol/kg)</td>
<td>285.3 (211.3–386.1)</td>
</tr>
<tr>
<td>i.c.v. (nmol/site)</td>
<td>40.4 (27.6–59.1)</td>
</tr>
<tr>
<td>i.t. (nmol/site)</td>
<td>154.8 (96.4–248.6)</td>
</tr>
<tr>
<td>Morphine(^a) s.c. ((\mu)mol/kg)</td>
<td>2.6 (2.0–3.4)</td>
</tr>
<tr>
<td>i.c.v. (nmol/site)</td>
<td>1.3 (0.9–3.2)</td>
</tr>
<tr>
<td>Dipyrone(^b) i.p. ((\mu)mol/kg)</td>
<td>207.6 (179.5–240.0)</td>
</tr>
<tr>
<td>i.c.v. (nmol/site)</td>
<td>0.14 (0.11–0.19)</td>
</tr>
<tr>
<td>i.t. (nmol/site)</td>
<td>0.4 (0.3–0.6)</td>
</tr>
</tbody>
</table>

ND Not determined
\(^a\) Data from Vaz et al. (1996).
\(^b\) Data from Beirith et al. (1998).
more potent and efficacious in inhibiting the inflammatory
than the neurogenic component of the formalin pain
response. Given orally, DPHD was 5-fold less potent than when it was given i.p. in relation to the late phase of the formalin
test (Table 1). Independent of the route of administration used, DPHD failed to affect the edematogenic response associated with the second phase of the formalin test (results not shown). The antinociceptive effect of DPHD was long-lasting and significant when given by i.p. (6 h) or p.o. (8 h) routes (results not shown).

The i.c.v. or i.t. injection of DPHD inhibited both phases of formalin-induced licking (Fig. 4). However, at the ID$_{50}$ level, DPHD was about 14- to 112-fold less potent than morphine when assessed against the first and the second phase of the formalin test, respectively (Table 1).

Capsaicin-Induced Pain

The results shown in Fig. 5 and data summarized in Table 1 show that DPHD (given by i.p., p.o., i.c.v., or i.t. routes) or morphine (given by s.c. route) caused significant inhibition of capsaicin-induced licking. However, at the ID$_{50}$ level, DPHD was about 31- to 119-fold less potent than morphine, but it was 2- to 3.5-fold more potent than dipyrone depending on the route of administration used when assessed against capsaicin-induced licking. Interestingly, indomethacin given i.p. had no significant analgesic effect in this model (Fig. 5A).

**Hot-Plate and Tail-Flick Tests**

The results summarized in Table 2 show that DPHD (given by i.p., i.c.v., or i.t. routes) did not cause any significant change in the latency response in either hot-plate test or tail-flick assays. In contrast, morphine (given s.c., i.c.v., or i.t.) caused a significant and marked increase in the pain latency in both algesiometer assays (Table 2).

**Rota-Rod Test**

DPHD given by i.p. route did not significantly affect the motor response of animals. Control response in the rota-rod test was 59.1 ± 0.7 s versus 58.5 ± 1.0 s in the presence of tested compound (n = 7 in each group).

**Effect of Several Classes of Drugs**

**Formalin-Induced Pain.** The results in Fig. 6 show that the pretreatment of animals with naloxone before injection of morphine or DPHD largely reverted the antinociception caused by either morphine or DPHD against both phases of

**TABLE 2**

Effect of morphine and DPHD in the hot-plate and tail-flick tests in mice

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Route</th>
<th>Dose (mmol/kg)</th>
<th>MPE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Tail-flick</td>
<td>Hot-plate</td>
</tr>
<tr>
<td>Control</td>
<td>i.p. (ml/kg)</td>
<td>10</td>
<td>4.1 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>i.c.v. (μl/site)</td>
<td>5</td>
<td>2.4 ± 6.0</td>
</tr>
<tr>
<td></td>
<td>i.t. (μl/site)</td>
<td>5</td>
<td>-2.1 ± 5.0</td>
</tr>
<tr>
<td>DPHD</td>
<td>i.p. (mmol/kg)</td>
<td>291.1</td>
<td>5.1 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>i.c.v. (mmol/site)</td>
<td>291.1</td>
<td>9.8 ± 7.6</td>
</tr>
<tr>
<td></td>
<td>i.t. (mmol/site)</td>
<td>291.1</td>
<td>-8.2 ± 7.0</td>
</tr>
<tr>
<td>Morphine</td>
<td>s.c. (mmol/kg)</td>
<td>31.0</td>
<td>96.0 ± 4.0**</td>
</tr>
<tr>
<td></td>
<td>i.c.v. (mmol/site)</td>
<td>15.5</td>
<td>94.0 ± 6.0**</td>
</tr>
<tr>
<td></td>
<td>i.t. (mmol/site)</td>
<td>15.5</td>
<td>74.0 ± 8.0**</td>
</tr>
</tbody>
</table>

Each group represents mean ± S.E.M. of 6 to 10 animals. Differs significantly from control values. (ANOVA) **p < .01.
the formalin test. However, the pretreatment of animals with cyprodime significantly reverted the antinociceptive effect caused by either morphine or DPHD when assessed against the late (but not the first) phase of the formalin test. In addition, pretreatment of animals with naltrindole or with nor-binaltorphimine before injection of morphine or DPHD significantly reversed the antinociception caused by DPHD, but did not significantly change the antinociceptive action caused by morphine when assessed against both phases of the formalin test (Fig. 6). The pretreatment of animals with t-ARG also significantly reversed the antinociception caused by either t-NOARG or DPHD assessed against both phases of the formalin test. The same treatment with t-ARG also significantly reversed the antinociceptive action of the morphine when assessed against the second (but not the first) phase of the formalin test (Fig. 7).

The results in Fig. 8 show that the pretreatment of animals with PCPA (once a day for 4 days) completely reversed DPHD effects but only partially reversed the antinociception caused by morphine when analyzed against both phases of formalin-induced pain.

The previous treatment of the animals with phaclofen or bicuculine significantly reversed the antinociception caused by baclofen or muscimol but did not significantly change the antinociception caused by DPHD against both phases of formalin test (results not shown).

The K_{ATP} channel blocker glibenclamide did not significantly modify the antinociception caused by DPHD against either phase of formalin-induced nociception (results not shown). However, under the same conditions, glibenclamide significantly antagonized the antinociception caused by morphine in the formalin test (results not shown). The i.c.v. administration of pertussis toxin, an inactivator of Gi protein, caused a significant inhibition of morphine-induced antinociception when assessed against both phases of formalin-induced pain (Fig. 9). Under the same conditions, pertussis

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**Fig. 6.** Effect of pretreatment of animals with naloxone (3.0 μmol/kg i.p.), cyprodime (2.3 μmol/kg i.p.), naltrindole (2.2 μmol/kg i.p.), or nor-binaltorphimine (1.4 μmol/kg i.p.) on the antinociceptive action caused by morphine (15.5 μmol/kg s.c.) and DPHD (87.3 μmol/kg i.p.) on formalin-induced nociception in mice. The total time (mean ± S.E.M.) spent licking the hindpaw was measured in the first phase (0–5 min; A) and against the second phase (15–30 min; B) after intraplantar injection of formalin in the hindpaw. Each column represents the mean of six to eight animals and the vertical lines indicate the S.E.M. The filled columns represent the control values (animals injected with the vehicle) and the asterisks denote the significance levels as compared with control groups (ANOVA); **p < .01.

**Fig. 7.** Effect of pretreatment of animals with L-ARG (3444 μmol/kg i.p.) on the antinociceptive action caused by morphine (15.5 μmol/kg s.c.), L-NOARG (342 μmol/kg i.p.), and DPHD (87.3 μmol/kg i.p.) on formalin-induced nociception in mice. The total time (mean ± S.E.M.) spent licking the hindpaw was measured in the first phase (0–5 min; A) and against the second phase (15–30 min; B) after intraplantar injection of formalin in the hindpaw. Each column represents the mean of six to eight animals and the vertical lines indicate the S.E.M. The open columns represent the control values (animals injected with the vehicle) and the asterisks denote the significance levels as compared with control groups (ANOVA); **p < .01.
toxin also significantly antagonized the antinociceptive action of DPHD against the second (but not the first) phase of the formalin test (Fig. 9).

The results in Fig. 10 show that the pretreatment of animals with morphine (15.5 μmol/kg s.c. once a day for 7 days) produced significant and complete tolerance to the antinociceptive effects caused by morphine or DPHD when compared with the animals pretreated with saline. However, the treatment of animals with DPHD (87.3 μmol/kg i.p. once a day for 7 days) did not significantly change its antinociceptive action, nor did such treatment interfere with morphine action when assessed against both phases of the formalin test (Fig. 10).

The bilateral adrenalectomy of the animals, performed 1 week before experiments, partially but significantly attenuated the antinociception produced by DPHD when assessed against both phases of the formalin test (results not shown).

Responses to Field Stimulation. Electrical field stimulation of the guinea pig ileal segments or mouse vas deferens induced twitch contractions, which were abolished by tetrodotoxin (1 μM; n = 4 for each group, results not shown). Cumulative additions of morphine (1 nM-10 μM) induced concentration-dependent depression of contraction evoked by field stimulation in ilea segments (n = 9 per group), with a mean IC50 value of 25.6 (4.8–137.4) nM and maximal inhibition of 87.5 ± 2.9%. Single additions of morphine (1 nM-10 μM) also caused a concentration-dependent depression of contractions evoked by field stimulation in mouse vas deferens (n = 8 per group), with a mean IC50 value of 2.5 (0.8–8.4) μM and maximal inhibition of 65.0 ± 4.5% (results not shown). In contrast, cumulative or single additions of DPHD (1 nM-10 μM; n = 5–8 per group) caused a discrete (10 to 20%) inhibition of the contractions evoked by field stimulation in either guinea pig ilea segments or in mouse vas deferens (results not shown).

Discussion

We have recently isolated and characterized, by means of X-rays and also by use of several chemical procedures, a novel alkaloid from the aerial parts of S. verticillatus (Campanulaceae), denoted as being the cis-8,10-di-N-propyllobellidol hydrochloride dehydrate (C16H34NO2·Cl ·2H2O) (Miguel et al., 1996). In an earlier study (Trentin et al., 1997), we reported that the extract of S. verticillatus given either
Here we report for the first time the antinociception caused by the major constituent isolated from this plant, the new alkaloid denoted as cis-8,10-di-N-propylbisdiod hydrochloride dehydrate (Miguel et al., 1996). As shown for the extract of *S. verticillatus* (Trentin et al., 1997), this alkaloid, given by p.o., i.p., i.t., or i.c.v. routes produces dose-related and significant antinociception when assessed in chemical assays of nociception, including the neurogenic pain caused by formalin (first phase) and capsaicin in mice. However, given in similar doses, this alkaloid is largely ineffective in producing antinociception when assessed systemically, spinally, and supraspinally in the thermal models of pain, namely, the tail-flick and hot-plate tests.

The fact that this new alkaloid, given by different routes, exhibits significant antinociception when assessed against the neurogenic (first phase of formalin test) and capsaicin-induced algesic response seems to be relevant. It has been well documented that the majority of the nonsteroidal anti-inflammatory drugs so far analyzed are usually ineffective in preventing the neurogenic pain caused by either formalin or capsaicin (Hunskaar and Hole, 1987; Shibata et al., 1989; Malmberg and Yaksh, 1992; Corrêa and Calixto, 1993; Corrêa et al., 1996; Vaz et al., 1996). However, at the ID$_{50}$ level, DPHD was about 14- to 119-fold less potent than morphine, but it was about 2- to 39-fold more potent than aspirin, acetaminophen, or dipyridam, depending on the route of administration used (Vaz et al., 1996, Beirith et al., 1998).

Attempts have also been made in the present study to investigate by use of several in vivo and in vitro pharmacological procedures some of the mechanisms underlying the antinociception caused by this alkaloid. Results of the current study confirm and extend our previous evidence (Trentin et al., 1997) by demonstrating that the activation of the opioid naloxone-sensitive pathway is most likely involved in the antinociception caused by this alkaloid, indicated by the finding that naloxone almost fully reversed the antinociception action of DPHD. By using a more selective opioid antagonist, it was possible to demonstrate that DPHD antinociceptive action involves the $\mu$, $\delta$, and $\kappa$ opioid receptors. This evidence derives from the fact that cyprodeine, naltrindole, or nor-binaltrorphimine significantly inhibited DPHD antinociception according to the formalin test (Craft et al., 1995; Frey and Schicht, 1996; Ossipov et al., 1996). Another piece of evidence suggesting the involvement of opioid-like substances in the antinociception caused by DPHD was its marked cross-tolerance with morphine in animals that had received an s.c. injection of this opioid once a day for 7 consecutive days (Ménard et al., 1995). Interestingly, treatment of animals with DPHD once a day for 7 days did not cause any tolerance to the alkaloid itself, or even cross tolerance with morphine. However, despite similarities with the action of opioid-like drugs, DPHD, surprisingly, was completely devoid of analgesic action when it was assessed in two thermal models of nociception and also in intestinal transit, as well as inhibiting the field-stimulated neurogenic contractions in the isolated guinea pig ileum and mouse vas deferens. Under very similar conditions, morphine caused marked antinociception and reduced intestinal transit, producing concentration-dependent inhibition of the twitch contractions in both guinea pig ileum and mouse vas deferens. The reason for such discrepant findings still remains unclear and was not further investigated in the present study.

In addition, results of the present study provide evidence supporting the involvement of the serotoninergic system in

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**Fig. 10.** Effect of cross-tolerance of animals pretreated with morphine (15.5 $\mu$mol/kg s.c.), DPHD (87.3 $\mu$mol/kg i.p.), or vehicle (10 ml/kg i.p.) on the antinociceptive action caused by morphine (15.5 $\mu$mol/kg s.c.) and DPHD (87.3 $\mu$mol/kg i.p.) on formalin-induced nociception in mice. The total time (mean ± S.E.M.) spent licking the hindpaw was measured in the first phase (0–5 min; A) and against the second phase (15 – 30 min; B) after intraplantar injection of formalin in the hindpaw. Each column represents the mean of six to eight animals and the vertical lines indicate the S.E.M. The filled columns represent the control values (animals injected with the vehicle plus agonist) and the asterisks denote the significance levels as compared with control groups (ANOVA); **p < .01.
the antinociceptive effect of DPHD, as revealed by the finding that pretreatment of animals with PCPA at a dose known to inhibit the cortical content of serotonin and to significantly reverse the morphine antinociception largely antagonized this alkaloid antinociception (Taber and Latranyi, 1981; Vonvoigtlander et al., 1984; Pini et al., 1996; Rattray et al., 1996; Trentin et al., 1997; present study). In addition, our results also support the notion that the l-ARG-nitric oxide pathways might account for the antinociceptive effect of DPHD. This view derives from the fact that treatment of animals with the nitric oxide precursor l-ARG largely reversed the antinociception caused by DPHD (both phases) and by morphine (second phase) as well the antinociceptive effect caused by l-NOARG, a known nitric oxide inhibitor, when assessed in the formalin test. Very similar findings have been reported for morphine- and l-NOARG-induced antinociception (Kawabata et al., 1993; Moore et al., 1996; Vaz et al., 1996; Trentin et al., 1997; present study). However, the antinociception elicited by DPHD seems to be independent of interaction with GABA A or GABA B receptors. These notions are because bicuculine and phaclofen, a selective GABA A and GABA B receptor antagonist under conditions in which it antagonized muscimol- and baclofen-induced antinociception, respectively, did not affect DPHD antinociception (Sawynok, 1984; Malcangi et al., 1991; Vaz et al., 1996; Shafizadeh et al., 1997; present study). The opening of ATP-sensitive potassium channels also does not appear to play a major role in DPHD-induced antinociception because the treatment of animals with glibenclamide, under conditions in which the antinociception caused by morphine was markedly reversed, had no effect on DPHD antinociceptive action when assessed against either phase of the formalin test (Ocaná and Baeyens, 1993; Raffa and Martinez, 1995; Shewade and Ramsawamy, 1995; present study). Also, the antinociception caused by DPHD is not the consequence of possible nonspecific central or peripheral depressant effects, as revealed by the lack of any detectable nonspecific effect in the rotarod test.

The mechanism by which DPHD produces systemic, spinal, or supraspinal antinociception in mice is still not completely understood at this stage of our study. However, the current results show that a large part of its antinociceptive effect was significantly antagonized by i.c.v. treatment of animals with pertussis toxin (1 μg/site, 7 days before the experiments) at a dose which has been demonstrated previously to suppress the antinociceptive effect caused by morphine through ADP ribosylation (Przewlocki et al., 1987; Parolari et al., 1990; Sánchez-Blázquez and Garzón, 1991; Shah et al., 1994, 1997; Hernandez et al., 1995; Tseng and Collins, 1996; present study). Therefore, such results indicate that DPHD antinociception, similar to that of morphine, is coupled to the same signal transduction system, namely G βγ/ρ-pertussis toxin-sensitive mechanisms. Also relevant are the findings showing that DPHD, like the antinociceptive effect caused by the extract of S. verticillatus, is modulated by endogenous glutocorticoids from gland hormones because previous bilateral adrenalectomy of animals carried out 1 week before testing significantly prevented its analgesic action in comparison with sham-operated animals.

In summary, data from the current study extend our previous findings (Trentin et al., 1997) and show that the major naturally occurring constituent isolated from the aerial parts of the Brazilian medicinal plant S. verticillatus, the new alkaloid denoted DPHD, produces systemic, spinal, and supraspinal antinociception when assessed in chemical (acetic acid-, formalin-, and capsaicin-induced pain), but not in thermal, models of nociception in the mouse. Several mechanisms account for its antinociceptive action, such as an interaction with opioid-like substances, i.e., through μ, δ, and κ receptors, involvement of serotoninergic and nitrergic systems, and also a modulatory action exerted by endogenous glucocorticoids. However, an interaction with GABA A or GABA B receptors, or with ATP-sensitive potassium channels, is unlikely to be involved with the antinociception caused by DPHD. Finally, the biochemical mechanism involved in the antinociception produced by DPHD, like that of morphine, seems to involve an interaction with G βγ/ρ-dependent mechanisms sensitive to treatment with pertussis toxin.

Acknowledgments

We are grateful to Rosana Maria Ostroski for in vitro technical assistance. A.R.S. Santos is a Ph.D. student in Pharmacology. A.R.S. Santos and O. G. Miguel thank Conselho Nacional de Desenvolvimento Científico e Tecnológico for fellowship support.

References


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