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Title Page

DPI-221 [4-((alpha-S)-alpha-((2S,5R)- 2,5-dimethyl-4-(3-fluorobenzyl)-1-piperazinyl)benzyl)-N,N-diethylbenzamide]: a novel non-peptide delta receptor agonist producing increased micturition interval in normal rats.

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Running Title Page

Running Title: Pharmacology of DPI-221

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Abbreviations: MVD, Mouse Vas Deferens; DPDPE, (cyclic [D-Pen^2,D-Pen^5] enkephalin); NTI, naltrindole; DPI-221, 4-((alpha-S)-alpha-((2S,5R)- 2,5-dimethyl-4-(3-fluorobenzyl)-1-piperazinyl)benzyl)-N,N-diethylbenzamide; U69593, (+)-(5α,7α,8β)-N-Methyl-N-(7-(1-pyrrolidinyl)-1 oxaspiro[4,5] dec-8-yl)benzeneacetamide; DAMGO, [D-Ala,*N*-Me-Phe,Gly-ol]-enkephalin; OXY, oxybutynin HCl; CARB, Carbamylcholine chloride / carbachol; OAB, overactive bladder; DPLPE , [2-D-penicillamine, 5-L-penicillamine]-enkephalin; DPLCE, [2-D-penicillamine, 5-L-cysteine]-enkephalin; (±) BW373U86, (±)-4-((alpha-R*)-alpha-((2S*,5R*)-4-allyl-2,5-dimethyl-1-piperazinyl-3-hydroxybenzyl)-N,N-diethylbenzamide

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ABSTRACT

There is a wealth of information from animal models and clinical opioid-analgesic use that indicates a significant role for opioid receptors in the modulation of bladder activity. The novel benzhydrylpiperazine compound DPI-221 was characterized as having delta receptor selectivity using radioligand binding (K_i= 2.0 ± 0.7 nM delta receptor; 1800 ± 360 nM mu receptor; and 2300 ± 680 nM kappa receptor) and agonist activity was demonstrated in the mouse isolated *vas deferens* where DPI-221 inhibited electrically-induced contractions with an IC_{50} value of 88 ± 7.5nM. In the guinea pig isolated ileum, DPI-221 had no effect on electrically-induced contractions at concentrations as high as 1μM. Sterile saline was infused (7 ml/hr) into the bladder of Sprague-Dawley rats, via a transmural catheter, DPI-221 (1.0 to 20 mg/kg p.o.) significantly increased the interval between micturition events while peak void pressure was not significantly decreased by any dose of DPI-221. The micturition effects of 10 mg/kg p.o. DPI-221 were blocked by naltrindole, indicating a delta receptor mechanism of action. In isolated rat bladder strips, DPI-221 was ineffective at relaxing detrusor muscle pre-contracted with carbachol. The most crucial safety aspect of delta agonist administration is the incidence of seizure-like convulsions in rodents. DPI-221 produced no convulsions at doses up to 100 mg/kg p.o. in mice, although rapid bolus i.v. injection of 5 mg/kg produced convulsions in 3% of mice tested. These findings indicate a good safety profile for DPI-221 administered orally, with potent efficacy in modifying bladder activity.

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INTRODUCTION

Overactive bladder (OAB) and the associated urinary incontinence are widely prevalent and yet the exact etiology of overactive bladder has not been fully elucidated. The basic principle of incontinence is an imbalance in the control of detrusor muscle tension in the bladder wall and the maintenance of a tightly closed urethral sphincter (Hudman *et al.*, 2000). The regulation of these systems occurs through afferent Aδ-fibers and (in the case of spinal disruption) C-fibers, while efferent fibers facilitate reflex and voluntary control at spinal, supraspinal and central levels involving the pontine micturition center and the periaquaductal gray matter (Fowler, 2002). However, since the immediate innervation of the bladder is through acetylcholinergic neuromuscular junctions, most pharmacotherapies are based, at least partly, around the muscarinic cholinergic system. Unfortunately, current pharmacological treatments for OAB and urinary incontinence have incomplete efficacy while causing diverse side effects through muscarinic receptor effects or non-specific cardiovascular effects (Ouslander, 2004).

The ability of morphine and other opioids to inhibit reflex activity of the urinary bladder was demonstrated by several groups in the early 1980's (Brent *et al.*, 1983; Dray and Metsch 1984 a, b, c; Hisamitsu and de Groat, 1984; Jubelin *et al.*, 1984). Unlike peripherally-limited opioid antagonists, centrally administered naloxone blocked morphine-induced inhibition of reflex bladder activity (Dray and Metsch 1984a, b, c) indicating a CNS-based mechanism of morphine's action. In the cat urinary bladder, activation of pre-ganglionic neurons, positive for leucine-enkephalin-like immunoreactivity, inhibited post-ganglionic, cholinergic innervation of the urinary bladder via a delta receptor mechanism (de Groat and Kawatani, 1989). These data

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indicate a delta-receptor-mediated inhibitory role of the pre-ganglionic neurons in control of cholinergic regulation of bladder activity. More recently, investigations into the naloxone-sensitive inhibition of micturition events with the widely used analgesic tramadol - an opioid receptor agonist and inhibitor of noradrenaline and serotonin reuptake - continue to highlight the role of the opioid systems in modulation of bladder activity (Pandita *et al.*, 2003; Pehrson and Andersson, 2003).

The opioid receptor system constitutes three receptor subtypes, delta, mu and kappa (Chang *et al.*, 1979), and all three receptors play a role in bladder control and micturition events (Dray and Nunan, 1987; Sheldon *et al.*, 1987; Sheldon *et al.*, 1989; Dray and Nunan, 1985). There is a growing volume of information supporting a role for the delta receptor in these processes. Dray *et al.* (1985) demonstrated that the delta receptor agonists [2-D-penicillamine, 5-L-penicillamine]-enkephalin (DPLPE), [2-D-penicillamine, 5-L-cysteine]-enkephalin (DPLCE) and [2-D-penicillamine, 5-D-penicillamine]-enkephalin (DPDPE) produced dose-related inhibition of reflex bladder contractions when administered either intracerebroventricularily (i.c.v.) or intrathecally (i.t.).

Clinical experience with mu-receptor-based analgesic compounds such as morphine indicates a side-effect profile that includes urinary retention, respiratory depression, muscle rigidity, nausea and vomiting and a high addiction potential - a profile not suitable for the treatment of OAB. In stark contrast, activation of the delta receptor system presents with no addiction or reinforcing potential in rhesus monkeys (Negus *et al.*, 1998; Negus *et al.*, 1995; Negus *et al.*, 1994), and no respiratory depression, muscle rigidity or nausea and vomiting (O'Neill *et al.*, 1997).

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Chang *et al.* (1993) described one of the first non-peptide delta receptor agonists ((±)-4-((alpha-R*)-alpha-((2S*,5R*)-4-allyl-2,5-dimethyl-1-piperazinyl-3-hydroxybenzyl)-N,N-diethylbenzamide – (±) BW373U86) with chemistry suitable for production as a clinical drug. However, (±)BW373U86 produces the most common rodent side-effect of high levels of central delta receptor activation – convulsions (Comer *et al.*, 1993; Broom *et al.*, 2002a &b). These tonic-clonic convulsions are observed with other non-peptide agonists, that are now readily available as research tools, and are a direct function of the rate of drug delivery (Comer *et al.*, 1993; Broom *et al.*, 2002a & b). The right-shift in convulsant threshold of delta receptor agonists produced by increasing the i.v. bolus infusion time strongly suggests that the rate of brain penetration is a critical factor in the determination of this convulsant threshold. The inherently slower absorption rate following oral administration might be expected to improve the convulsant threshold of delta agonists. However, the currently available non-peptide delta agonists are not orally bioavailable and as such are poor drug candidates for many urologic indications. Building on the original chemistry of (±)BW373U86 and the work of Chang *et al.* (1993) we have developed a highly selective, non-peptide delta agonist that is orally available and produces no convulsions in rodents except following rapid bolus intravenous doses. Herein, we report the radioligand binding affinity (Ki), intrinsic activity and convulsant profile of DPI-221 (4-((alpha-S)-alpha-((2S,5R)- 2,5-dimethyl-4-(3-fluorobenzyl)-1-piperazinyl)benzyl)-N,N-diethylbenzamide; Figure 1). The effects of DPI-221 on rat micturition events are investigated, along with the associated delta receptor mechanism of action.

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METHODS

These studies were conducted under approved Institutional Animal Care and Use Committee protocols at Enhance Biotech, Inc. (formerly Ardent Pharmaceuticals, Inc.) and in accordance with USDA regulations.

Receptor Binding Affinity

Membrane preparation for radioligand binding: The brains from male albino Sprague-Dawley rats were obtained from Pel Freez Biologicals (Rogers, AR) and cerebellum from male albino guinea pigs from Accurate Chemical and Scientific Corporation (Westbury, NY). The tissue was rinsed with ice-cold 50 mM Tris-HCl buffer (pH 7.4, 25 ^{o}C) containing the following protease inhibitors: 50 µg/ml soybean trypsin inhibitor, 0.1 mM phenylmethylsulfonyl floride (PMSF) and 1 mM ethylenediaminetetraacetic acid (EDTA), 10 µg/ml Leupeptin, 200 µg/ml Bacitracin, and 0.5 µg/ml Aprotinin. Brains were minced and homogenized in 5 to 10 volumes per gram wet weight in ice-cold 50mM Tris buffer containing protease inhibitors. The homogenate was prepared using a glass-Teflon homogenizer (nominal clearance, 0.13-0.18 mm). The homogenate was centrifuged at 6,000 x g for 15 min at 4^{o}C, and the resulting supernatant centrifuged at 41,000 x g for 30 min at 4°C. The membrane pellet was re-suspended in 10 volumes per gram wet weight of 10 mM Tris-sucrose (0.32 M) buffer and re-homogenized with a Polytron tissue grinder (10 sec, low speed). The homogenate was centrifuged at 41,000 x g for 30 min at 4^{o}C. The resulting membrane pellet was re-suspended in 50 mM Tris buffer with protease inhibitors at a final protein concentration that ranged from 40 µg/ml to 50 µg/ml. The membrane fraction was frozen under liquid N_2 and stored at –80^{o}C

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prior to use in radioligand binding studies. Protein concentration was determined using the method of Bradford (1976).

Radioligand binding*:* Membrane fractions were incubated with DPI-221 ($3x10^{-11}$ M to $6x10^{-5}$ M) or naloxone ($1x10^{-6}$ M) plus 0.1 nM of the delta-opioid receptor agonist [^{3}H] DPDPE (specific activity 50.6 Ci/mmol; n=7), 0.1 nM of the mu-opioid receptor agonist [^{3}H]DAMGO (specific activity 50.0 Ci/mmol; n=7) or 0.1 nM of the kappa-opioid receptor agonist [^{3}H]U69593 (specific activity 41.4 Ci/mmol; n=5) in 2 ml of 10 mM Tris-HCl buffer containing 5 mM $MgCl_2$ and protease inhibitors. Incubation was carried out for 90 min at 25 ^oC in order to permit the complete equilibration of the radioligand with its receptor. The reaction was terminated by rapid filtration through Whatman GF/C glass fiber filters using a cell harvester (model M-48R, Brandel Instruments, Gaithersberg, MD) followed by two 5 ml rinses with ice-cold 50 mM Tris buffer. Non-specific binding was defined as that radioligand bound in the presence of $1x10^{-6}$ M naloxone. Filters were counted by liquid scintillation spectrometry (LS 6500, Beckman Coulter Inc., Fullerton CA) at an efficiency, determined by external standards, of 40 to 45%.

Intrinsic Activity

Vas deferens studies: Tension development in isolated mouse *vas deferens* was measured as described previously (Chang *et al.*, 1993). Following cervical dislocation, *vasa deferentia* were isolated from male CD-1 mice (Charles River, Raleigh, NC) weighing 20-25 g. Muscles were suspended in individual organ baths containing Mg-free Krebs-Henseleit solution (37 ^oC, aerated with O_2-CO_2, 95:5) of the following

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composition (mM): NaCl, 117.5; KCl, 4.75; $CaCl_2$, 2.6; KH_2PO_4, 1.2; $NaHCO_3$, 24.5; and glucose, 11.

The *vas deferens* segments were positioned between platinum electrodes (Radnoti Glassware Technology Inc., Monrovia, CA) and connected to a Grass (Grass-Telefactor, West Warwick, RI) FTO3 isometric force transducer under a resting tension of 0.5 g. Muscles were stimulated to contract by administering 400 msec pulse trains (1 msec duration, supramaximal voltage, 10 Hz) with a Grass S88 stimulator. An IC_{50} value for DPI-221 inhibition of electrically-induced contractions was produced through the addition of cumulative concentrations ($1x10^{-10}$ to $1x10^{-7}$M; n=8) of DPI-221 to the bathing solution (Chang *et al*., 1993).

Guinea Pig Ileum studies: Tension developed in response to electrical stimulation of guinea pig isolated ileum was recorded as described previously by Gengo *et al.* (2003a). Male albino guinea pigs (Charles River, Raleigh, NC) weighing 300-500g were euthanized by decapitation and an 8cm section of ileum removed and divided into 2-3cm segments. Indivudual segments were suspended in standard organ baths (Radnoti) and continuously bathed in Krebs-Henseleit solution (37°C, aerated with O_2/CO_2 95:5) of the following composition: 117.5 mM NaCl, 4.75 mM KCl, 2.4 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 24.5 mM $NaHCO_3$, and 11 mM glucose. Tissues were suspended from Grass FTO3 isometric force transducers under a resting tension of 1 g and contractions were elicited by a field stimulation of 0.1Hz pulses of 0.5 ms duration at supramaximal voltage using platinum electrodes and a Grass S88 stimulator. DPI-221 effects on electrically-induced contractions of the ileum were examined through the addition of cumulative concentrations ($1x10^{-7}$ to $1x10^{-6}$M; n=7) of DPI-221 to the bathing

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solution. The maximum DPI-221 concentration tested was limited by the solubility of the compound in these assay conditions.

Direct Effects On Detrusor Tension

Bladder Isolated Rings: Male Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis) were anaesthetized with isoflurane in an N_2O/O_2 vehicle (65% N_2O; 35% O_2) and then killed by exsanguination. The bladder was removed and placed into warm (37°C) Kreb's solution which was made up fresh daily and consisted of the following (mM in distilled water - pH 7.2): NaCl, 119.0; KCl, 4.4; $NaHCO_3$, 20.0; NaH_2PO_3, 1.2; $MgCl_2$, 1.2; $CaCl_2$, 2.5; glucose, 11.0.

Bladders were dissected free of any adhering fat or serosa and then cut into longitudinal strips of muscle ~4 x 1 x 0.5 mm. These were suspended in a Perspex organ bath of 10.0 ml volume using a fine silk suture. The bladder strips were constantly perfused at a rate of 1 ml/min with Kreb's solution aerated with 95% oxygen and 5% carbon dioxide and the temperature was maintained at 37°C throughout the experiments. The bladder muscle strips were attached to an isometric force transducer (Kent Scientific Corp., Torrington, CT) connected to a personal computer and held at a resting tension of 2 g for a 3-4 hour equilibration period. Data was acquired using Workbench software (Strawberry Tree, Sunnyvale, CA)

DPI-221 on carbachol pre-contracted muscles: After equilibration to the resting tension, an EC_{50} concentration of carbachol (360 nM) was used to pre-contract the muscle strips. The carbachol-induced increase in tone was allowed to plateau to a stable

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pre-contracted state before DPI-221 solutions were added directly to the tissue bath to produce bath concentrations of 1, 3 and 10 μM. Each concentration was allowed to establish a stable tension and then a sufficient additional volume of DPI-221 was added to produce the next highest bath concentration of interest. Tissue viability was confirmed with 10 μM oxybutynin as a positive control added to the tissue bath after completion of DPI-221 concentrations.

Micturition Events in Conscious Restrained Rats

Cystometry: The cystometry methods used were similar to those of Pandita *et al.*, (2003) Male Sprague Dawley (Harlan, Indianapolis, Indiana) rats weighing 200 to 250 grams were anesthetized with pentobarbital, 65 mg/kg i.p. and placed in a supine position. A 10 mm long midline incision was made in the abdominal wall and the urinary bladder was cannulated, via an incision at the dome, with a polyethylene cannula (PE50) that was permanently sutured in place. The cannula was subcutaneously positioned and exteriorized in the retro-scapular region. Compounds were administered intravenously (i.v.) or orally (p.o.) via catheters inserted into the jugular vein or stomach (respectively) and exteriorized in the retro-scapular area. Rats were treated with Penicillin G intramuscularly 1 mg/kg (1658 Units) to prevent infection and allowed to rest 3 days after implantation before testing commenced.

Three days after cannula implantation animals were placed, conscious, into Plexiglass tube-shaped rat restrainers (Plas-Labs Inc., Lansing, Michigan) where they remained for the duration of the study. Maximal restraint times were 4 hours for dose response studies and 6 hours for the duration of action study. Once animals were restrained, the bladder cannula was attached to a syringe pump and sterile saline was pumped into the bladder at

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a rate of 7 ml/hr. A pressure probe (Grass PT200) was attached to the bladder infusion line with a three way hub to record micturition pressure changes. Data was acquired using PowerLab hardware (ADInstruments) and software, Chart 4, on a personal computer.

Dose-response curve: A stable baseline of all micturition values (interval, baseline pressure, and peak developed pressure) was maintained for 1 hour prior to administration of any test compound. Data was produced only from animals with micturition intervals greater than 2 minutes and less than 15 minutes. A single dose of DPI-221 (0.5 [n=11], 1 [n=10], 2 [n=8], 10 [n=15] or 20 mg/kg [n=5]) or pH–matched 5% dextrose vehicle [n=13] was administered via the stomach cannula (p.o.) – cannula patency was confirmed at the end of the study for all animals with a positive control dose of the muscarinic antagonist oxybutynin. DPI-221 was slowly administered over the course of 1 minute and data was acquired for 3 hours (or until any effects had obviously reached a plateau). Data was collected over a 1 hour pre-drug baseline and over a 1 hour period starting 1 hour after drug administration. The mean peak developed voiding pressure and the mean interval between pressure peaks over the 1 hour data collection window were measured and expressed as the percent of the pre-drug baseline values for each animal.

Duration of action of DPI-221: In order to assess the duration of action of DPI-221, rats received a single p.o. (stomach cannula) dose of 10 mg/kg of DPI-221 and bladder pressures were recorded for up to 4 hours after dosing. Data was analyzed in 40 minute bins.

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Naltrindole blockade of DPI-221 micturition effects: In order to investigate whether the effects of DPI-221 on micturition events was sensitive to blockade of the delta receptor, the delta receptor-selective antagonist naltrindole was used in an intravenous dosing regimen designed and reliably used in our laboratories to provide stable levels of receptor blockade over an extended (> 2 hour) experimental period. Naltrindole (NTI) was administered intravenously (0.75 mg/kg) 35 minutes prior to oral dosing of DPI-221 at the lower of the maximally effective doses previously tested (10 mg/kg p.o.), 5 minutes after DPI-221 dosing [n=8] or pH-matched 5% dextrose vehicle [n=8], and a third time 40 minutes later. Control DPI-221 or dextrose vehicle groups [n=15 and 13 respectively] received 3 injections of saline as the vehicle for NTI doses. The sequential dosing was used to maintain an effective inhibitory concentration of NTI in the blood at all times during the course of the experiment. Data was analyzed from a 1 hour window sampled 1 hour after DPI-221 dosing.

Convulsant Effects in Mice

The convulsant effects of DPI-221 were investigated using male CD-1 mice (Harlan, Indianapolis, Indiana). Using individual dosing groups of 10 animals, DPI-221 was administered as a rapid bolus intravenously (tail vein) at doses of 3, 5 and 10 mg/kg (n = 30, 29 and 70) or via oral gavage / feeding tube over ~1 minute at doses of 60 and 100 mg/kg p.o. (n = 10 and 10) and at 30 and 100 mg/kg s.c. (n = 10 and 10). Animals were observed for 1 hour following i.v. dosing and 2 hours following p.o. dosing. A convulsion was recorded if a mouse had uncontrollable clonic (or tonic-clonic) movements that encompassed its entire body, usually followed by a brief cataleptic

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period. Convulsion incidence was expressed as a percentage of the number of animals receiving the dose.

Arterial Blood Gas and Antinociception in Rats

Male albino Wistar Hannover rats (Harlan, Madison, WI) weighing 200-300 g were anesthetized with 2% isoflurane in a 30% O_2 and 70% N_2O vehicle. Catheters were inserted in the femoral artery and external jugular vein. 60 minutes after surgery, DPI-221 was administered at 10 mg/kg i.v. or 60 mg/kg p.o. (by gavage) and antinociception was measured using the tail pinch method and arterial blood gas analyzed at 0, 4, 8 16 and 32 minutes following DPI-221 dosing as per the methods of Gengo *et al.* (2003b).

Sources of drugs

[^{3}H]DPDPE, [^{3}H]DAMGO, and [^{3}H]U69593 were purchased from Dupont-New England Nuclear, a division of Perkin-Elmer (Boston, MA). Purities were greater than 98%. NTI and all other chemicals were reagent grade and purchased from Sigma Chemical Co. (St. Louis, MO). Novel compounds described in this study were synthesized at Ardent Research Laboratories (Durham, NC) and Burroughs Wellcome Co. (Research Triangle Park, NC) using standard protocols.

DPI-221 was manufactured at Ardent Pharmaceuticals, Inc. as a free-base and all solutions were made based on dry weight of free-base (MW 487.66) that was then dissolved in 1 ml of ethanol and either converted to an HCl salt and dried under nitrogen for aqueous dosing solution preparation, or diluted directly from the ethanol stock for *in vitro* studies.

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Calculations and Statistics

Receptor binding and intrinsic activity data were analyzed by non-linear regression of the concentration-response curves to determine the K_i or IC_{50} values using the computer program Prism (GraphPad Software Inc., San Diego, CA).

Micturition event recordings provided significant differences in group sizes and variances owing to cannula twisting or chewing by the rats. Once the pressure trace was compromised no further analysis was performed on that trace. Micturition parameters for DPI-221 doses were expressed as the percent change from baseline and compared with vehicle control using a Kruskal-Wallis analysis of variance followed by Dunn's *post hoc* test. Data from the duration of action study was compared with baseline and between measurement time points using the same tests.

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RESULTS

Receptor binding affinity

In competition radioligand binding assays, DPI-221 demonstrated delta receptor selectivity (Figure 2) with binding affinities of (K_i ± s.e.m.): 2.0 ± 0.7 nM at the delta receptor (n = 7); 1800 ± 360 nM at the mu receptor (n = 7); and 2300 ± 680nM at the kappa receptor (n = 5). The data were best fit using a one site sigmoidal model with a pseudo-Hill slope of close to 1.0.

Intrinsic Activity

DPI-221 produced a concentration-dependent inhibition of electrically stimulated *vas deferens* contractions with a corresponding IC_{50} (± s.e.m.) value of 88 ± 7.5 nM (n = 8; Figure 3). DPI-221 produced no discernable effect on the electrically-induced contraction of the guinea pig isolated ileum at any concentration tested (Figure 3).

Direct Effects On Detrusor Tension

At concentrations up to and including the maximum achievable concentration in physiological buffering solutions (10 μM) DPI-221 produced no significant relaxation of carbachol-induced contraction of bladder muscle strips. The muscarinic antagonist oxybutynin (10 μM) completely relaxed the tissues, indicating the viability of each preparation. A representative trace for a single bladder strip is presented in Figure 4.

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Micturition Events in Conscious Restrained Rats

Dose-Response Curves

During the baseline period, a minimum of 10 micturition intervals were recorded (or one hour for micturition intervals < 6 minutes) to provide stable baseline interval and peak void pressure values. The average baseline interval was 5 ± 0.5 minutes. Orally administered DPI-221 increased micturition interval relative to baseline in a dose-dependent manner (Figure 5) and this increase was significantly different from controls at doses of 1.0, 2.0, 10.0 and 20.0 mg/kg ($p<0.05$ except 10 mg/kg where $p<0.01$). While not statistically significant, 0.5 mg/kg produced an increase in interval over baseline (15 ± 6.7%) that was 50% of the maximal effect seen at both 10 and 20 mg/kg (32 ± 10.2% and 32 ± 10.4% respectively (Figure 5). The data presented in Figure 5 for the peak void pressure reveals a dose-related decrease in peak pressure up to doses of 10 and 20 mg/kg. However, statistical significance from control was not observed at any dose tested. Basal vesicular pressure across all groups ranged from 5 – 25 mmHg at the start of the study, and through the duration of a 4 hour study, basal pressure drifted by a maximum of ± 6 mmHg. When the percent change from baseline was compared across dose groups with an analysis of variance, there was no significant effect of DPI-221 on the slow drift in basal pressure with time.

Duration of action of DPI-221

Oral administration of DPI-221 (10 mg/kg) followed by data sampling in 40 minute windows demonstrated a slow increase in micturition interval that reached a peak at around 2 hours after dosing (Figure 6). Micturition interval remained stable at ~25% above baseline for the rest of the recording period (up to 120 minutes). The variability of

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the later time points was larger than that seen at initial time points owing to diminishing pressure trace integrity over the 6 hour study period. Bladder pressure traces were rendered unreadable by animals that twisted or chewed cannulae. The effect of DPI-221 on peak void pressure described above was supported in this study. There was also a statistical trend in the effect of DPI-221 ($p<0.05$ v. baseline) that paralleled the time course of the significant effects on micturition interval (Figure 6).

NTI blockade of DPI-221 micturition effects

DPI-221 (10 mg/kg p.o.) produced a significant increase in micturition interval and a small decrease in micturition pressure (Figure 7). The delta selective antagonist naltrindole had no effect on either micturition interval or pressure when administered alone. However, when naltrindole was dosed prior to DPI-221, and during the first hour of DPI-221 exposure, naltrindole completely blocked the effects of 10 mg/kg p.o. DPI-221 on micturition interval and pressure (Figure 7).

Convulsion incidence in mice

Intravenous administration of DPI-221 as a rapid bolus dose of 5 and 10 mg/kg produced convulsions in 3% and 23% of animals respectively. DPI-221 (3 mg/kg i.v.) produced no convulsions. When administered orally or subcutaneously, however, at doses as high as 100 mg/kg no convulsions were observed at any dose by either route of administration.

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Arterial Blood Gas and Antinociception in Rats

There were no antinociceptive effects of DPI-221 administered either intravenously or orally. Similarly there was no deviation in arterial blood gases from normal, control levels with DPI-221 dosed either i.v. or p.o.

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DISCUSSION

DPI-221 is a highly delta-receptor-selective compound with good agonist potency in the standard mouse *vas deferens* assay for delta receptor activity and no mu (or kappa) receptor activity *in vitro* (guinea pig ileum assay) or *in vivo* - as evidenced by the lack of antinociceptive (tail pinch test) or respiratory depressant effects in rats. When administered orally to conscious, restrained rats DPI-221 significantly prolonged micturition interval without altering basal or peak void pressure at doses as low as 1.0 mg/kg. This effect is thought to be more indicative of changes in micturition urge and neuronal control than of inhibition of muscle activity. The maximal effect produced by DPI-221 was an average extension of micturition interval of 32% (10 and 20 mg/kg p.o.). The size of this effect is thought to be somewhat constrained by the physiological limitations of rat bladder size (up to ~1.5 ml for 200 –230g rats; Jeong and Lee, 2000) and the 7mL/hr rate of bladder filling. At the higher doses tested (10 and 20 mg/kg p.o.) a small decrease in void pressure was observed with DPI-221. In isolated bladder strips, DPI-221 had no effect on carbachol-induced tension, indicating that the decreased void pressure was probably not the result of a direct muscarinic effect on detrusor tone or contractility. Throughout the course of the study, basal bladder pressure remained stable and was not affected by DPI-221 treatment, suggesting that the decreased void pressure does not indicate retention of urine due to ineffective voiding contractions. DPI-221 effects on bladder compliance, voiding resistance, absolute void volume or its indirect effects on bladder contractility can not be determined from the data presented here. The physiological relevance of the decreased peak void pressure at higher doses of DPI-221 is not clear from this data.

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The effects of DPI-221 as a delta-selective agonist support the growing volume of evidence that the delta receptor system plays an important role in the regulation of reflex and voluntary bladder activity. Activation of the opioid system with morphine and antagonists such as naloxone centrally (Dray and Metsch, 1984a, b, and c) as well as at spinal and supraspinal levels (Dray and Nunan; 1985; 1987) alters reflex activity of the urinary bladder in intact animals. Building on this earlier evidence that had used somewhat generalized (if mu favoring) opioid receptor tools, Sheldon *et al.*, (1987; 1989) and Dray *et al.* (1985) utilized peptide-derived compounds with greater selectivity for mu, delta or kappa receptors to further elucidate the differential roles of these receptor subtypes. These studies, in combination with leucine-enkephalin immunolocalization in pre-ganglionic neurons of the bladder-associated ganglia in cats (de Groat and Kawatani, 1989), and the i.c.v. and i.t. efficacy of DPDPE (and DPLPE, DPLCE) in inhibiting reflex bladder contractions (Dray *et al.*, 1985) and potentiating the effects of morphine (Sheldon *et al.*, 1989), clearly indicate a role for the delta receptor in control of the urinary bladder. As mentioned previously, however, there have been no non-peptide compounds available for clinical use or testing prior to this time owing to their poor oral availability and rapid CNS penetration (i.e. low convulsion threshold). The compound introduced herein, DPI-221, is a novel delta receptor agonist with characteristics far more suited to development as a clinical therapeutic agent.

It is well established in clinical settings, particularly in the analgesic use of morphine, that mu agonist agents induce urinary retention. This was illustrated in our rat model as a large increase in basal pressure to above that of peak voiding pressure and a complete ablation of all voiding pressure changes (Holt *et al.*, 2005). The effects of DPI-221 on micturition interval without altering pressure also contrasts starkly with the effects of

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anti-muscarinic agents, such as oxybutynin, in the same conscious rat model. Oxybutynin produced variable changes in micturition interval, tending towards decreased interval, and dose-dependently decreased peak void pressure in this rat model (Holt *et al.*, 2005). These findings would suggest that the delta receptor mechanism may actually offer a cleaner therapeutic tool for limiting micturition urge and frequency without significantly altering bladder mechanics or producing urinary retention.

One of the critical advantages of DPI-221 over other currently available delta receptor agonists is its oral efficacy in models of micturition events. This is a crucial factor in the development of this compound as a drug in clinical use and is a major improvement over the original compounds from this chemical class, such as BW373U86. In addition to the obvious clinical suitability of an oral medication for overactive bladder, the infusion-rate dependency of delta receptor-mediated convulsions (Comer *et al.*, 1993) would theoretically be avoided by the relatively slow rate of gastric absorption as compared to intravenous bolus administration. This theoretical premise appears to hold true in the case of DPI-221. Intravenous doses of DPI-221 produce convulsions when administered in a rapid bolus of 5 or 10 mg/kg, whereas an oral dose of 60 or 100 mg/kg or s.c. dose of 100 mg/kg produced no convulsions. The obvious interpretation of these findings is that oral administration does not carry the convulsion-based safety concerns of current delta receptor agonists. However, these findings are also pertinent to further considerations of the mechanism of action of DPI-221.

DPI-221 increased micturition interval in intact animals but had no effect on carbachol-developed tension in isolated rat bladder detrusor muscle strips. This would seem to discount any direct action of DPI-221 on bladder muscle or at the cholinergic neuromuscular junction as the mechanism of interval prolongation. However, although

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oral dosing of DPI-221 does not produce convulsions, the presence of convulsions following intravenous dosing indicates that DPI-221 can cross the blood-brain barrier. As such, no inference can be made as to the localization of effect to more peripheral sites, such as at the pre-ganglionic level described by de Groat and Kawatani (1989), or central sites such as the pontine micturition center or periaquaductal grey area (Fowler, 2002). What remains clear from the studies presented here, however, is that DPI-221 modulates micturition interval through a naltrindole-sensitive, delta receptor-mediated mechanism, and that the effects on micturition interval last beyond 4 hours after oral dosing. Taken together with the suitability of DPI-221 for formulation and development, the lack of convulsant effects after oral dosing, and the high potency in extending micturition interval, these findings indicate that DPI-221 would be an appropriate candidate for development as a clinically useful therapy for overactive bladder and urinary incontinence.

In light of the delta selectivity of DPI-221 and the naltrindole sensitivity of its micturition effects, demonstration of the therapeutic efficacy and clinical utility of DPI-221 would provide a unique opportunity to validate the animal models of a delta receptor mechanism as chemically meaningful and therapeutically useful. Alongside the clinical development of DPI-221, follow-on animal studies will further define the mechanism and localization of action of the delta-mediated effects of DPI-221 on urinary bladder activity.

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FOOTNOTES

Some of this data was presented at Experimental Biology, 2004 : Watson MJ, Gengo PJ, Pendergast WJ, Chang JP, Wei V, Sanford IJ, Millard DJ and Chang K-J, 2004, The Effects of a Novel δ-Opioid Receptor Agonist on Micturition in Rats. *Faseb J.*, **18 (5)**, Program #635.5; Abstract #3191.

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LEGENDS FOR FIGURES

Figure 1. The chemical structure of DPI-221 [4-((alpha-S)-alpha-((2S,5R)- 2,5-dimethyl-4-(3-fluorobenzyl)-1-piperazinyl)benzyl)-N,N-diethylbenzamide].

Figure 2. Receptor binding competition curves. Membrane fractions were incubated with 0.1 nM of the delta receptor agonist [^{3}H] DPDPE (■; n = 7), 0.1 nM of the mu receptor agonist [^{3}H]DAMGO (▲; n = 7), or 0.1 nM of the kappa receptor agonist [^{3}H]U69593 (▼; n = 5). Displacement of bound ligand by DPI-221 is expressed in terms of the remaining bound ligand as a percentage of total specific binding in control conditions (% of control).

Figure 3. The concentration-response effects of DPI-221 on electrically stimulated contractions of the mouse *vas deferens* (*delta* receptor dominated; ■; n = 8) or guinea pig ileum (*mu* and *kappa* receptor dominated; ▲; n = 7). DPI-221 was added to the tissue bath in increasing concentrations to produce a cumulative concentration-response curve for opioid receptor-induced relaxation.

Figure 4. A sample trace of the effect of DPI-221 on the carbachol-induced tension of a bladder strip isolated from a male Sprague-Dawley rat. DPI-221 (1 and 10 μM) had no significant effect on carbachol-induced tension (500 nM). Oxybutynin (10 μM) completely antagonized carbachol-induced tension.

Figure 5: DPI-221 P.O. Micturition Interval and Pressure Dose Response Curve

In catheterized rats undergoing constant infusion (7 ml/hr) of sterile saline into the bladder DPI-221 (p.o.) dose dependently increased the time interval between micturition events. DPI-221 (p.o.) produced a non-significant trend towards decreasing micturition developed bladder pressure. * denotes $p < 0.05$ v. vehicle control; ** denotes $p < 0.01$ v. vehicle control.

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Figure 6: The duration of action of DPI-221

In male catheterized rats undergoing constant infusion (7 ml/hr) of sterile saline into the bladder, DPI-221 (10mg/kg p.o.) increased micturition interval (■) and decreased micturition pressure (▼). Maximum effect was reached approximately 120 minutes after gastric infusion. * denotes $p<0.05$ v. baseline.

Figure 7: Naltrindole blockade of DPI-221 effects on micturition parameters

In male catheterized rats undergoing constant infusion (7 ml/hr) of sterile saline into the bladder the increase in micturition interval in response to DPI-221 (10 mg/kg p.o.) was blocked by the delta opioid antagonist naltrindole (NTI; 0.75 mg/kg x 3). The small decrease in pressure induced by DPI-221 was also antagonized by NTI**.** * denotes $p < 0.05$ v. vehicle control; ** denotes $p < 0.01$ v. vehicle control.

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Figure 1

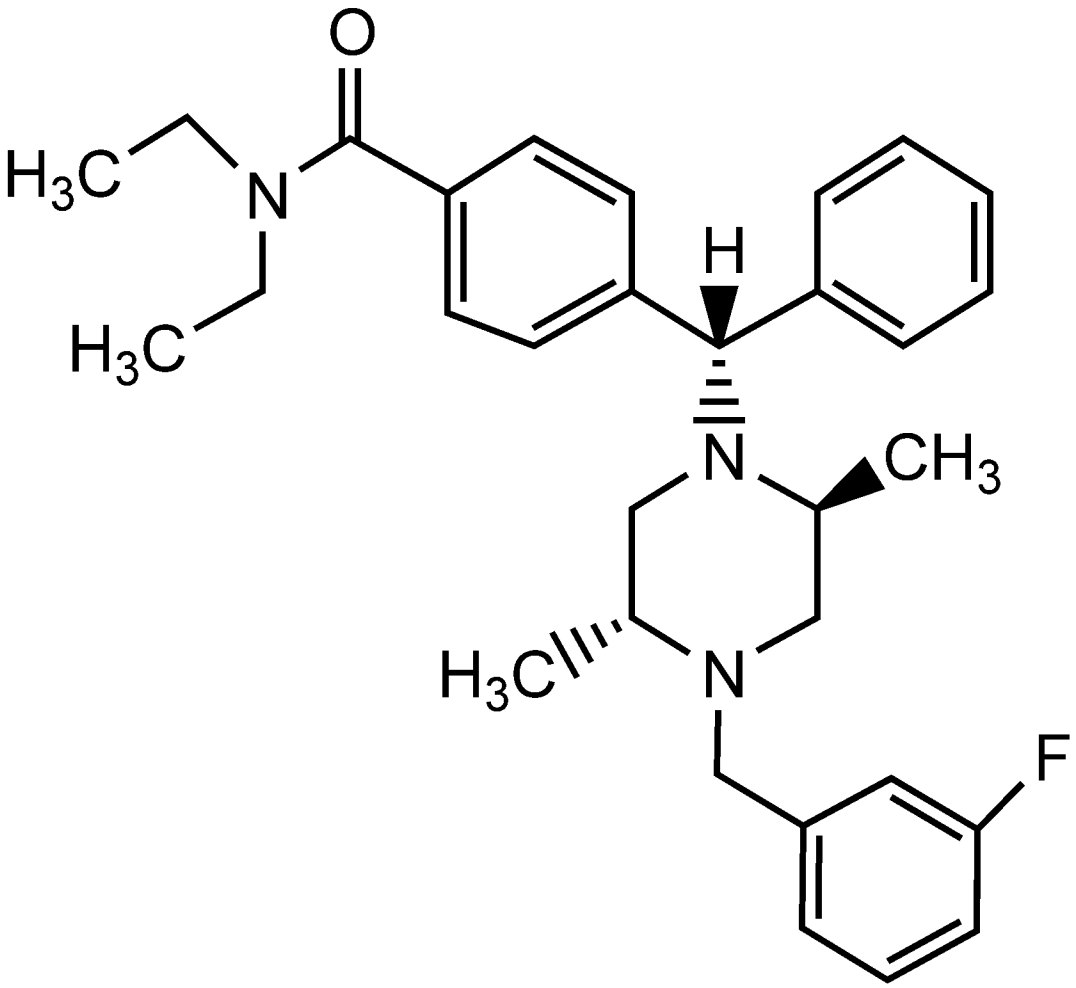

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Figure 2

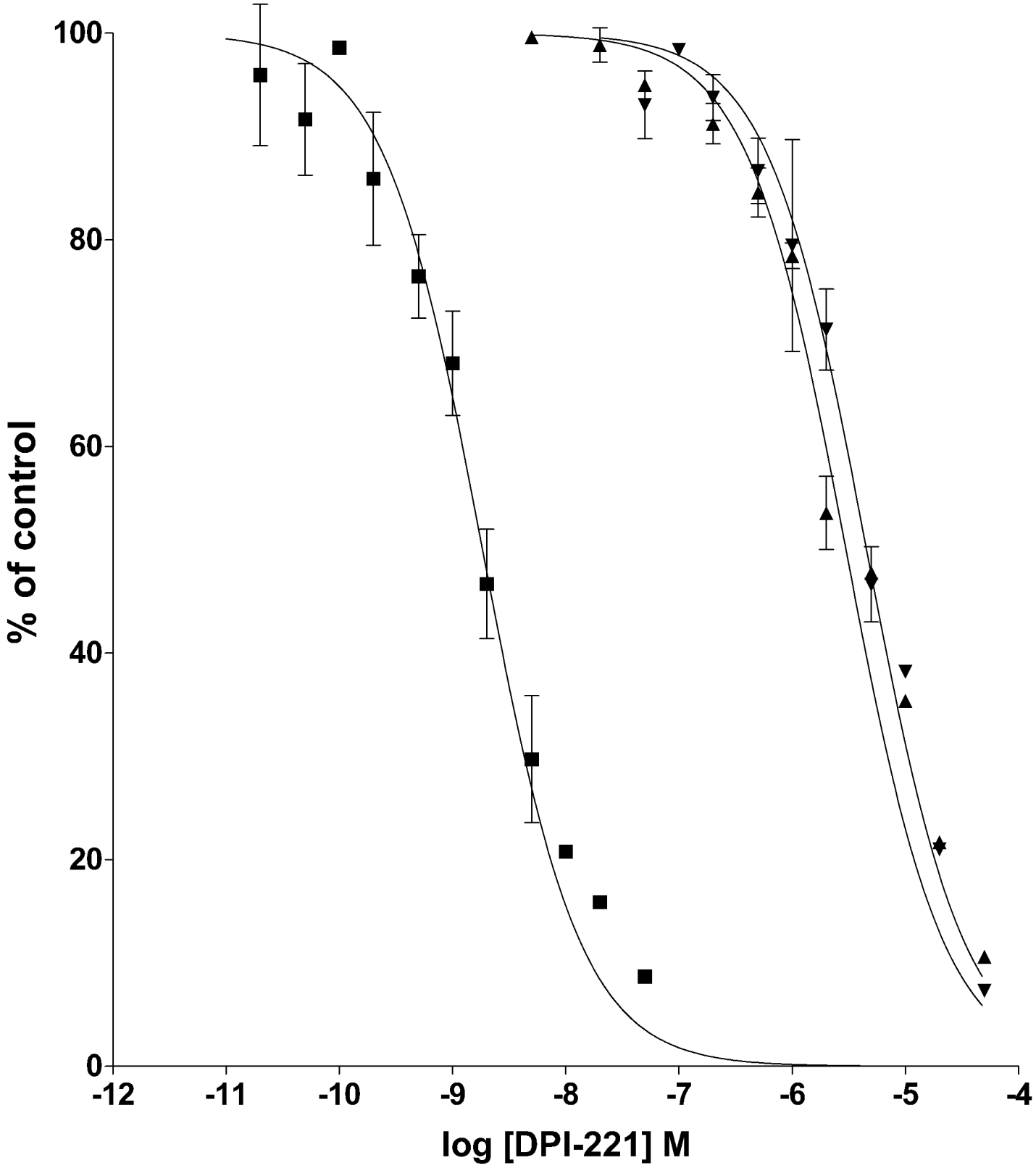

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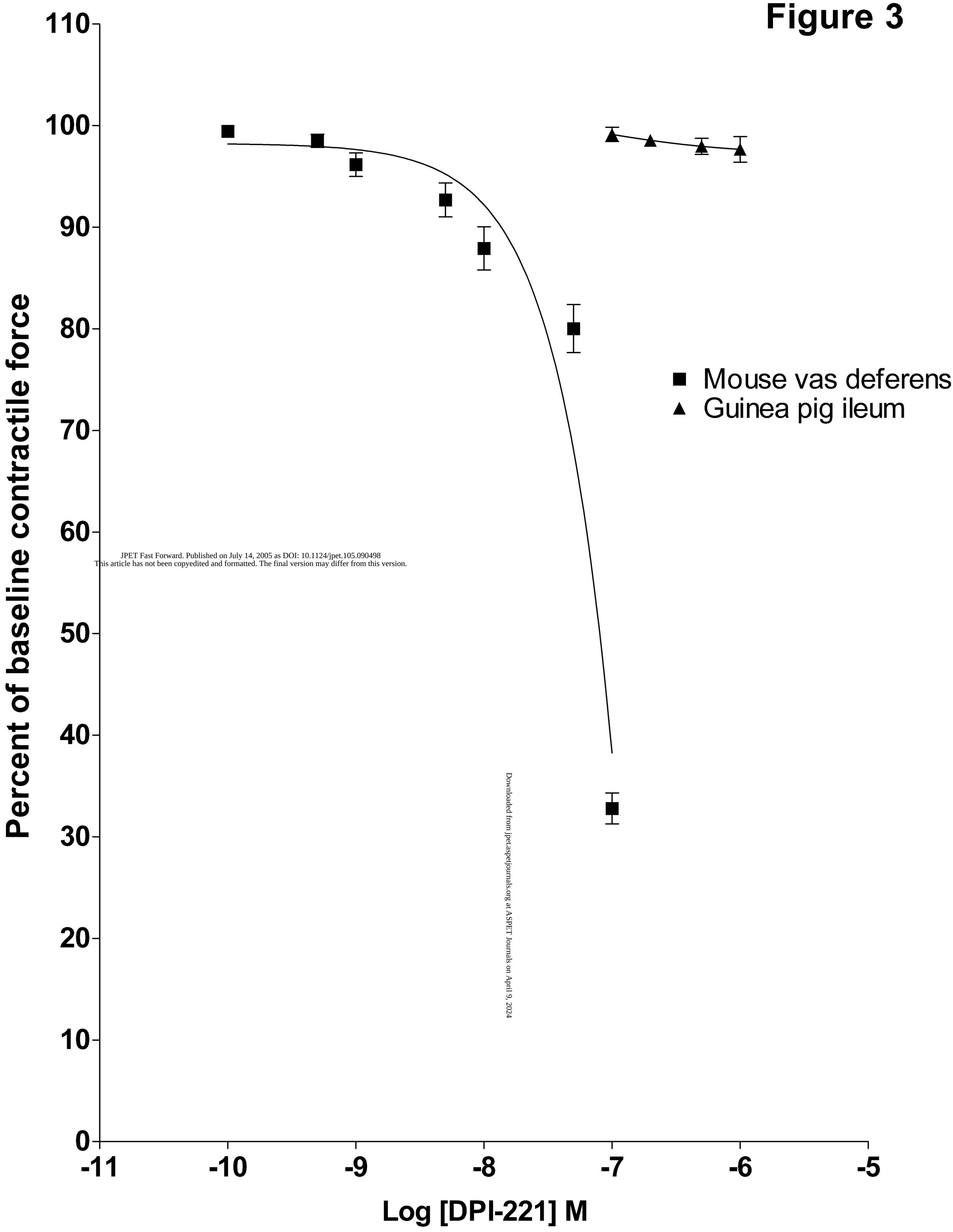
Figure 3



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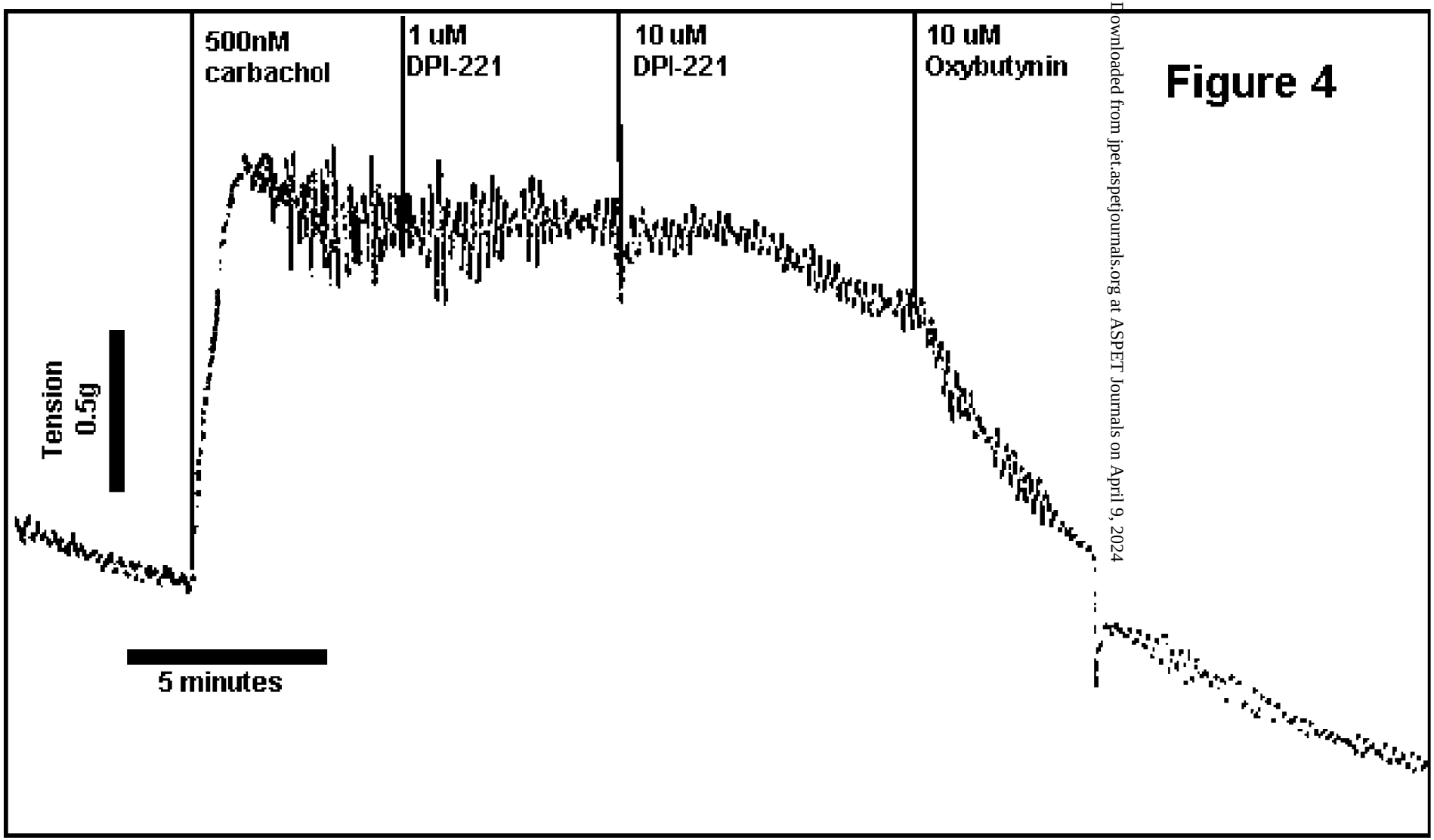

Figure 4

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Figure 5

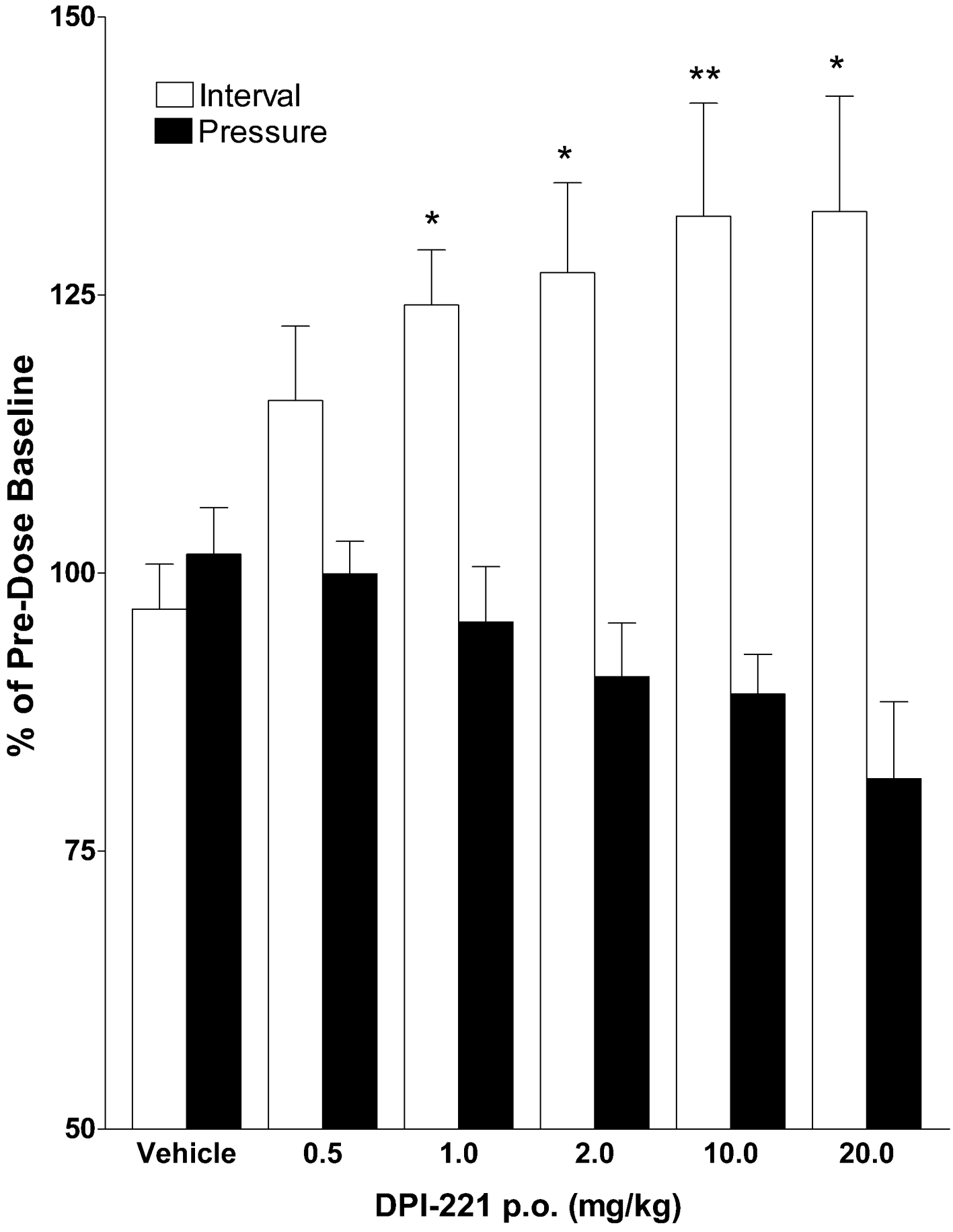

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Figure 6

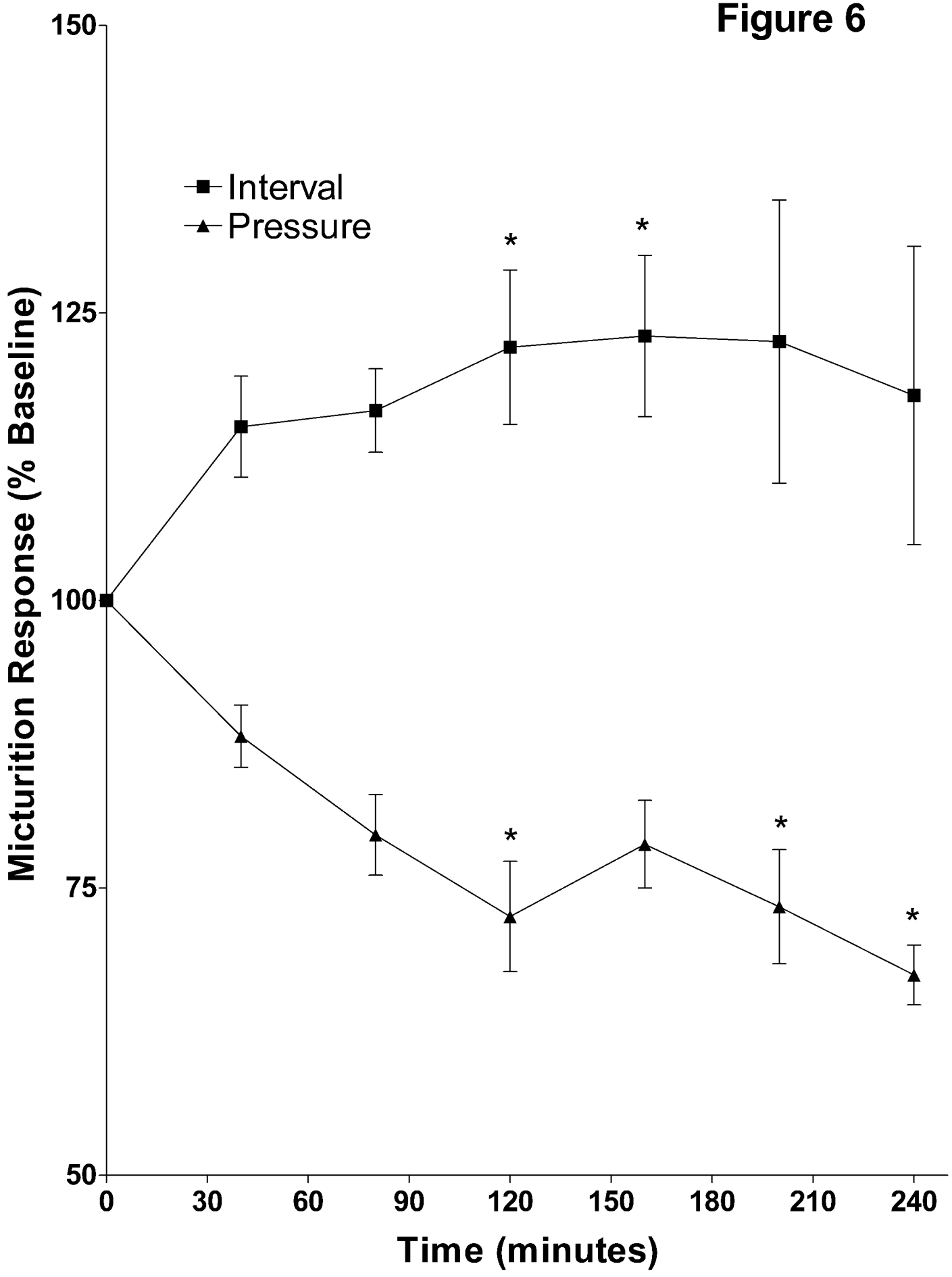

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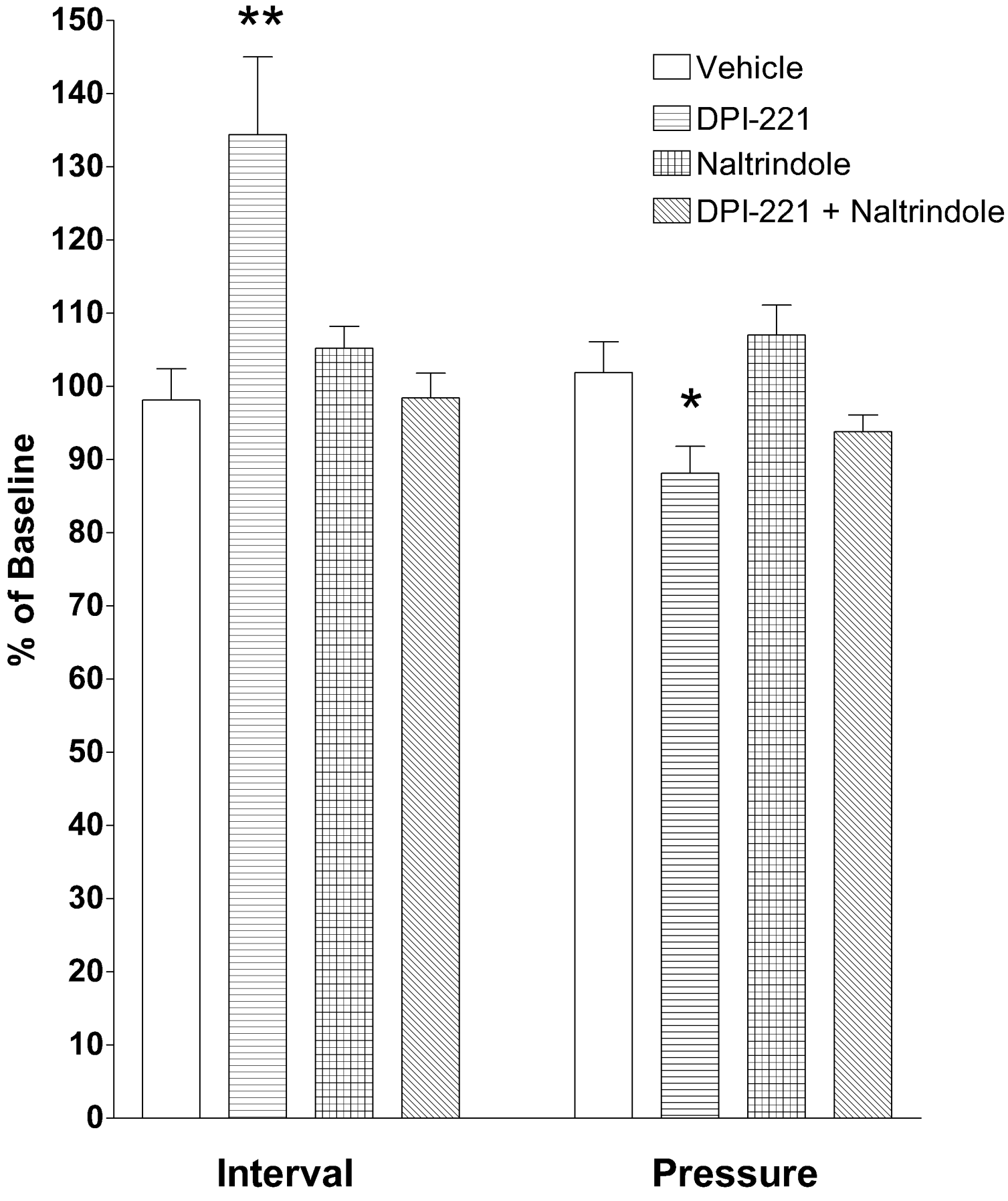
Figure 7



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