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Differences in the Antinociceptive Effects and Binding Properties of Propranolol and Bupranolol Enantiomers

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Abstract: **Recent efforts have suggested that the β-adrenergic receptor (β-AR) system may be a novel and viable therapeutic target for pain reduction; however, most of the work to date has focused on the β_2-adrenergic receptor (AR). Here, we compared the antinociceptive effects of enantiomeric configurations of propranolol and bupranolol, two structurally similar nonselective β-blocking drugs, against mouse models of inflammatory and chronic pain. In addition, we calculated in silico docking and measured the binding properties of propranolol and bupranolol for all 3 β-ARs. Of the agents examined, S-bupranolol is superior in terms of its antinociceptive effect and exhibited fewer side effects than propranolol or its associated enantiomers. In contrast to propranolol, S-bupranolol exhibited negligible β-AR intrinsic agonist activity and displayed a full competitive antagonist profile at $\beta_1/\beta_2/\beta_3$-ARs, producing a unique blockade of β_3-ARs. We have shown that S-bupranolol is an effective antinociceptive agent in mice without negative side effects. The distinctive profile of S-bupranolol is most likely mediated by its negligible β-AR intrinsic agonist activity and unique blockade of β_3-AR. These findings suggest that S-bupranolol instead of propranolol may represent a new and effective treatment for a variety of painful conditions.**

***Perspective:** The S enantiomer of bupranolol, a β-receptor antagonist, shows greater antinociceptive efficacy and a superior preclinical safety profile and it should be considered as a unique β-adrenergic receptor compound to advance future clinical pain studies.*

Key words: *Pain, propranolol, bupranolol, β-adrenergic receptors, antinociception.*

Current treatment options for chronic pain display limited efficacy and are associated with problematic side effects. Thus, novel treatment strategies and therapeutic targets are needed to address a profound unmet medical need that affects the lives of hundreds of millions worldwide.[43] Within recent years, the β-adrenergic receptor (β-AR) system has emerged as a promising therapeutic target for drug development and pain management. The endogenous ligands for β-ARs are catecholamines such as epinephrine ($\beta_2 > \beta_1 \ggg \beta_3$) and norepinephrine ($\beta_3 \approx \beta_1 > \beta_2$),[24,60] with the functional roles of β-ARs best characterized as affecting cardiovascular, airway, uterine, and metabolic functions. However, β-ARs are also densely distributed in the nervous system, making them prime targets for altering memory, mood, and pain (see Molinoff[44] for a review). Among the β-AR subtypes, β_2-ARs are believed

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to be the most relevant for pain. β_2-ARs are 1) located on peripheral nociceptors,[1] 2) located in discrete spinal cord regions that directly participate in nociceptive transmission,[46] and 3) essential for the antiallodynic action of antidepressant drugs.[9] Furthermore, stimulation of β_2-ARs on peripheral afferents sensitizes nociceptors[1] and enhances pain signaling through the release of proinflammatory cytokines from the central nervous system[27,28] as well as from peripherally located adipocytes and macrophages.[58,60]

Evidence from human studies indicates that sequence variation in the gene encoding for the β_2-AR (*ADRB2*) is associated with individual differences in the susceptibility to several chronic pain conditions. For instance, we have previously shown[15] that haplotypic variants within the *ADRB2* gene locus are associated with the development of temporomandibular joint disorder (TMD), a chronic musculoskeletal pain condition. Genetic variations in *ADRB2* have also shown associations with chronic neck pain,[55] irritable bowel syndrome,[36] and fibromyalgia.[61]

Clinically, propranolol, a lipophilic nonselective β-AR antagonist (ie, β-blocker), has shown promise with respect to pain management, especially in the treatment of migraine headaches,[52] fibromyalgia,[38] and temporomandibular disorders.[59] Despite the promising clinical usefulness of β-blockers, including propranolol, there is still a paucity of experimental research demonstrating the effectiveness of β-blockers in pain reduction. Even although propranolol is the prototypic β-blocker used for clinical pain management of migraine,[40] many adverse effects are associated with this drug, including drowsiness, fatigue, depression, and cognitive changes (see Freitag[21] for a review). Propranolol is typically administered as a racemic mixture to treat hypertension and normalize tachycardia responses.[19] However, there is emerging evidence that for propranolol and bupranolol, a structurally similar β-blocker, the S-enantiomers of both compounds show greater cardiosympatholytic activity.[3,39,56]

Here, we investigated whether enantiomers of 2 nonselective β-blocking drugs, propranolol and bupranolol, were effective in multiple algesiometric assays in mice. In addition, we explored at the cellular and structural level whether racemic mixtures and optically pure enantiomers of propranolol and bupranolol produced β_1-, β_2-, or β_3-AR blockade in a manner that matches the effects on both sensory and motoric behaviors in mice.

Methods

Mice

All behavioral experiments were performed on naive, adult (6–12 weeks of age), CD-1 (ICR:Crl) mice of both sexes, bred in house from breeders obtained from Charles River (Boucherville, Quebec, Canada). All mice were housed with their same-sex littermates (2–4 animals per cage) in standard shoebox cages, maintained in a temperature-controlled (20°C ± 1°C) environment (14/10 hour light/dark cycle), where they had access to food (Harlan Teklad 8604) and water ad libitum. All animal experiments were approved by McGill University and were in accordance with the Canadian Council on Animal Care and with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines for reporting experiments involving animals.[33,41]

Drugs

The HCl salts of racemic bupranolol, S-bupranolol, and R-bupranolol were provided by Algynomics Inc (Chapel Hill, NC), a company specializing in personalized pain medicine, of which 3 authors [J.S.M., L.D., and W.M.] are either equity stock holders or cofounders (as stated in the disclosure). Fig 1 shows the chemical structures of propranolol and bupranolol and indicates the position of their stereocenters. Racemic propranolol and the R-enantiomers and S-enantiomers of propranolol were purchased from Sigma Aldrich (St. Louis, MO) and dissolved in saline.

Behavioral Assays

All mice were habituated to the testing environment for at least 20 minutes before testing commenced. In all experiments, mice were assigned randomly to drug and dose, and experimenters were blinded to drug and dose. Sample sizes in all pain assays were n = 6–12 mice/dose/drug.

Rota-Rod Test

Drug effects on motor coordination were tested using an accelerating Rota-Rod treadmill (Acceler Rota-Rod 7650; UgoBasile, Gemonio, Varese, Italy) for mice.[29] Mice were placed on the Rota-Rod, which accelerated from 4 to 40 revolutions/min over a period of 5 minutes, and the time spent on the rotating drum was recorded for each mouse. On the test day, 1 drug-free baseline trial was performed and then the mice were treated with drugs and retested 3 times at 20-minute intervals. Performance was quantified by calculating the percentage of maximal ataxia 60 minutes after drug administration compared with the baseline scores: ((Baseline – Post-drug/Baseline) × 100).

Formalin Test

Mice were injected with drugs (see later discussion) and then allowed to habituate for 20 minutes within Plexiglas cylinders (30 cm high, 15 cm diameter) placed

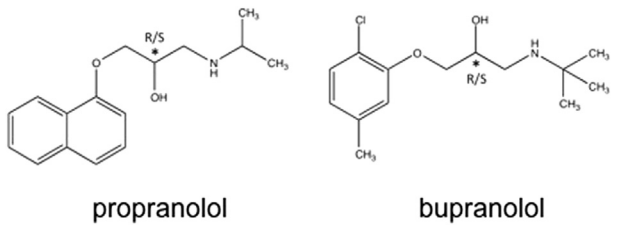

Figure 1. Chemical structures of R/S propanolol (left) and bupranolol (right). The position of the stereocenter is indicated by the star.

on a glass tabletop. Then, 20 μL of 5% formalin was injected subcutaneously into the plantar surface of the left hind paw using a 100-μL microsyringe with a 30-gauge needle. Mice were then returned to the cylinders and videotaped undisturbed for 60 minutes. Videos were later coded offline by a blinded observer, and the first 10 seconds of every minute was sampled for the presence of licking/biting (positive sample) of the left hind paw. The early phase was defined as the percentage of positive samples during the first 0 to 10 minutes after injection of formalin; the late phase was defined as the percentage of positive samples during the period 10 to 60 minutes after injection.

Radiant Heat Paw Withdrawal Test

Mice were placed on a glass floor within small Plexiglas cubicles as described earlier, and a focused high-intensity projector lamp beam was directed from below onto the midplantar surface of the hind paw.[25] The commercial device (IITC Model 336; Harvard Apparatus, Holliston, MA) was set to 20% active intensity. Latency to withdraw from the stimulus was measured to the nearest .1 seconds. Baseline measurements consisted of testing both hind paws twice on 3 separate occasions separated by at least 30 minutes. After drug administration, both hind paws were tested only once at the indicated time point.

von Frey Test

The up-down method of Dixon was used.[6] Mice were placed on a perforated metal floor (with 5-mm diameter holes placed 7 mm apart) within small Plexiglas cubicles ($9 \times 5 \times 5$ cm high), and a set of 8 calibrated von Frey fibers (Stoelting Touch Test Sensory Evaluator Kit 2 to 9; Stoelting Co, Wood Dale, IL; ranging from ≈.015 *g* to ≈1.3 *g* of force) were applied to the plantar surface of the hind paw until the fibers bowed and then held for 3 seconds. The threshold force required to elicit withdrawal of the paw (median 50% withdrawal) was determined twice on each hind paw (and averaged) for all baseline measurements (except for the spared nerve injury [SNI] experiments), with sequential measurements separated by at least 20 minutes. After drug administration, 1 measurement per hind paw was taken at the indicated time point.

Carrageenan Hypersensitivity

λ-Carrageenan (2%; 20 mg/mL; Sigma, Oakville, ON, Canada) was suspended by sonication in saline and injected subcutaneously in a volume of 20 μL into the left plantar hind paw using a 100-μL microsyringe with a 30-gauge needle. Mice were tested for thermal and mechanical sensitivity of both hind paws using the radiant heat paw withdrawal and von Frey tests, respectively, before and 3 hours after carrageenan injection. All drugs were injected immediately after the postcarrageenan (3 hour) test, and postdrug measurements were taken 20, 40, and 60 minutes later.

SNI

SNI, an experimental nerve injury designed to produce neuropathic pain, was performed under isoflurane/oxygen anesthesia, as described previously.[14] Mice were tested for mechanical sensitivity before and after surgery using the von Frey test, except that the spared sural region was targeted by applying the fibers to the lateral aspect of the hind paw. Drug administration immediately followed baseline measurements for SNI-induced mechanical allodynia 7 days postoperatively, and postdrug measurements were taken at 20, 40, and 60 minutes.

In Silico Docking Calculations

The structure of the engineered β_2-AR at 2.8 Å resolution (PDB: 3NY8)[4,62] and the structural models of β_1-AR and β_3-AR subtypes were used for the docking calculations. The homology models of β_1-AR and β_3-AR were derived from the template structure of β_2-AR at 3.2-Å resolution[49] using I-TASSER.[51,69] The raw models obtained were optimized with Chiron[48] (http://troll.med.unc.edu/chiron/index.php), a software for protein structure refinement developed in the Dokholyan Laboratory. The quality of the 3 final protein structures was comparable with high-resolution crystal structures, as assessed by Gaia[35] (http://troll.med.unc.edu/chiron/index.php). Docking calculations were performed using MedusaDock,[17,18] a tool developed in house that simultaneously models the flexibility of both ligand and protein. For each system analyzed, we retrieved a reliable binding pose of the investigated molecules (Fig 1) by combining the values of the MedusaScore,[67] a physical force field–based scoring function accounting for the protein-ligand interaction energy, with a hierarchical cluster analysis of the top-ranked ligand conformations. Specifically, in each single β-AR subtype, we performed 200 independent docking calculations for every compound and collected their top-scored conformations. We clustered the ensemble of docking solutions according to the root-mean-square deviation (RMSD) computed over the heavy atoms of the ligand. We identified the optimal number of highly populated clusters by applying the average linkage method and the Kelley penalty index[31] to minimize the number of clusters and the spread of internal values in each cluster. The clustering level with the lowest Kelley penalty represents a condition in which the clusters are highly populated and concurrently maintain the smallest internal spread of RMSD values. The centroid of the most populated cluster was chosen as the representative conformation of the ligand bound to the protein. This procedure demonstrates high reliability because it reproduces the binding pose of the original cocrystallized molecule in β_2-AR with an RMSD computed over the heavy atoms of the ligand of 1.4 Å (data not shown).

Cell Culture and Transfection

HEK293T (human embryonic kidney cells expressing large T antigen of SV40) cells were transfected with an expression vector containing β_1-, β_2-, or β_3-ARs, using a calcium phosphate transfection protocol or Lipofectamine 2000 (Invitrogen, Waltham, MA). Cells were cotransfected with GloSensor-22F vector (GloSensor cAMP Assay kit;

Promega, Madison, WI) and pIRES-GFP to ensure transfection. Twenty-four hours after transfection, cells were transferred into a white tissue culture treated poly-L-lysine–coated 384-well plate (20,000 cells/well) with a clear bottom. The next day, green fluorescent protein expression was verified before the cyclic adenosine monophosphate (cAMP) production experiment was performed.

cAMP Production Assay

For the cAMP production experiment, cell culture medium was replaced with GloSensor reagent according to the manufacturer's instructions (GloSensor cAMP Assay kit). The well plates were incubated with the reagent for 2 hours at room temperature. Generally, cells were then treated with β-AR antagonists (propranolol, R-bupranolol or S-bupranolol; 10^{-12}–10^{-5} M) for 30 minutes before undergoing a challenge with the β-AR agonist, isoproterenol (ie, EC_{50} concentration [half-maximal effective concentration] of isoproterenol for each receptor type). Some experiments were conducted in the absence of antagonist to determine the effects of isoproterenol alone, with concentrations ranging between 10^{-14} and 10^{-5} M. To determine cAMP levels, we measured luminescence after 10 minutes of agonist treatment using the Victor3 multilabel reader (Perkin Elmer, Waltham, MA). Each dose-response curve was normalized individually against the maximum response where applicable. Experiments were performed on 2 or 3 plates with samples run in triplicate or quadruplicate (n = 7–11 technical replicates; 2 or 3 biological replicates).

Schild Plot and pA_2 Assays

Cells were pretreated for 30 minutes with antagonists at 3 or 4 different concentrations within the log part of the dose-response curve previously determined. Then, the cells were treated with 10^{-14} to 10^{-7} M isoproterenol to determine the degree to which the EC_{50} value shifted to the right in the presence of the competitive antagonist. Schild plot and pA_2 plots were used as measures of antagonist affinity and selectivity for receptor subtypes, respectively.[45] The pA_2 values for each antagonist were calculated by plotting the $\log((A'/A) - 1)$ against $-\log B$, where A is the EC_{50} without any antagonist, and A' refers to each EC_{50} value in the presence of a certain concentration (B) of an antagonist. The pA_2 value is determined as the point where the curve fitted to the plot intercepts the x-axis ($y = 0$), and it is the –log of the concentration of antagonist that is required for a 2-fold shift in the EC_{50} of the agonist. Assuming that the test agent is a competitive antagonist that acts at a single receptor in the test system and does not show partial agonist properties, then, the pA_2 plot has a slope equivalent to –1. pA_2 values are therefore useful in ranking the order of potencies of competitive antagonist and assessing receptor selectivity.

cAMP Production in Response to the Antagonists

cAMP production was measured as described earlier. After GloSensor reagent loading, the cells were treated at various concentrations of isoproterenol or antagonists alone for 10 minutes. Luminescence was measured after 10 minutes of agonist treatment using the Victor3 multilabel reader (Perkin Elmer).

Statistical Analyses

A criterion level of $\alpha = .05$ was adopted for all experiments. Behavioral data were analyzed by analysis of variance followed by Dunnett and Tukey post hoc analyses where appropriate. Half-maximal antinociceptive doses (AD_{50}s) and ataxia ED_{50}s were calculated using the method of Tallarida and Murray[57] as implemented by FlashCalc 40.1 software (M. Ossipov, University of Arizona).

Results

Differential Sedation Caused by Propranolol and Bupranolol Isomers

To identify the highest nonsedating dose of β-AR antagonists that could be used in our behavioral assessment of pain inhibition; the mouse Rota-Rod assay was used. We assessed the latency to fall from the Rota-Rod after administration of racemic propranolol, racemic bupranolol, and their respective enantiomers. We found that some of the β-AR antagonists caused sedation and ataxia, but only when high doses were administered (Fig 2A). Compared with saline-treated mice, racemic propranolol and both of its enantiomers impaired Rota-Rod performance (ie, time to ataxia) at 60 mg/kg, whereas none of the bupranolol compounds produced ataxia at this dose. Bupranolol and R-bupranolol produced ataxia at very high doses (>90 mg/kg); S-bupranolol produced no ataxia at any dose up to and including 120 mg/kg. S-Propranolol significantly impaired Rota-Rod latencies with doses as low as 30 mg/kg. ED_{50}s and associated confidence intervals for maximal ataxia obtained 60 minutes after drug administration are shown in Table 1. In some cases, doses of R-bupranolol (90 and 120 mg/kg), racemic propranolol (60 mg/kg), and R-propranolol (30 and 60 mg/kg) resulted in mortality before Rota-Rod testing (Supplementary Table 1). Lethality after S-bupranolol administration was not observed, even when high doses (120 mg/kg) of S-bupranolol were used.

Formalin-Induced Pain Is Inhibited by the S-Enantiomers of Bupranolol and Propranolol

We next examined whether different enantiomers of propranolol and bupranolol were antinociceptive in the formalin test of tonic/inflammatory pain. Robust inhibition of formalin-induced licking was observed at varying doses of propranolol, bupranolol, and their enantiomers, R-propranolol, S-pupranolol, R-bupranolol, and S-bupranolol (Figs 2B and 2C). All drugs except R-bupranolol dose-dependently inhibited formalin-induced licking, with S-bupranolol showing the greatest effect in both the early and late phases. AD_{50}s and associated

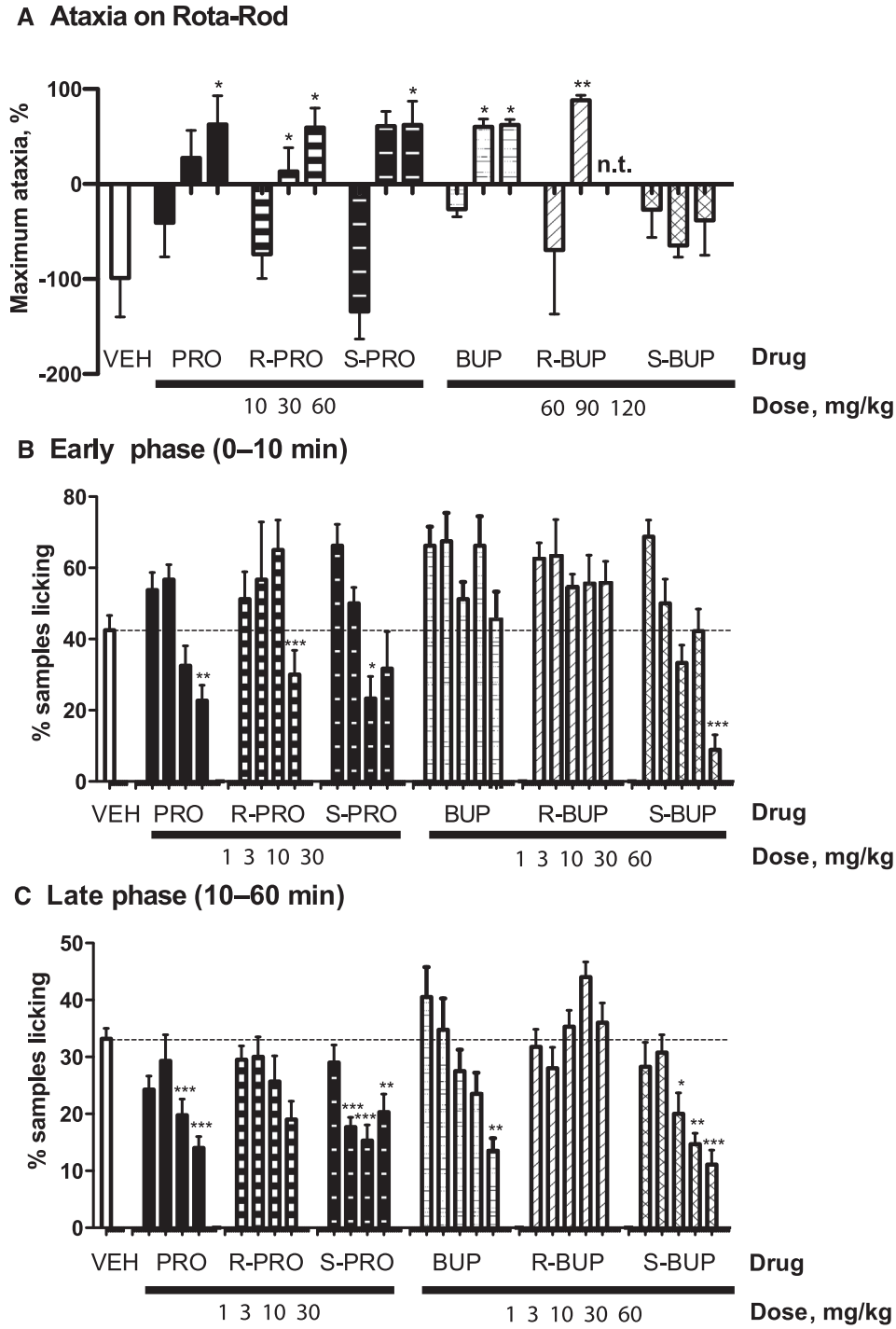

Figure 2. The antinociceptive effects of propranolol and bupranolol enantiomers. **(A)** The sedation or ataxia produced by racemic or enantiomeric versions of the nonselective β-AR antagonists propranolol or bupranolol (propranolol [PRO], $F_{3,14} = 4.0$, $P < .05$; R-propranolol (R-PRO), $F_{3,15} = 7.1$, $P < .01$; S-propranolol [S-PRO], $F_{3,15} = 14.5$, $P < .001$; bupranolol [BUP], $F_{3,14} = 15.5$, $P < .001$; R-bupranolol [R-BUP], $F_{2,7} = 4.8$, $P < .05$). The only antagonist that was found not to be sedating, even at a very high dose, was S-bupranolol (S-bupranolol, $F_{3,13} = .92$, nonsignificant [n.s.]). Bars represent the percentage of maximal ataxia when measured against baseline ± standard error of the mean. n.t. = no trial. **(B)** Antagonists reduce the amount of licking in the formalin test during the early phase (0–10 minutes) but only when high (and in most cases, sedating) doses are used (propranolol, $F_{4,39} = 7.2$, $P < .001$; R-propranolol, $F_{4,33} = 2.2$, n.s, S-propranolol, $F_{4,33} = 6.9$, $P < .001$; bupranolol, $F_{5,46} = 3.5$, $P < .001$; R-bupranolol, $F_{5,43} = 2.0$, n.s.; S-bupranolol, $F_{5,50} = 9.6$, $P < .001$) **(C)** Antagonists reduce the amount of licking during the late phase (10–-60 minutes) of the formalin test (propranolol, $F_{4,39} = 10.2$, $P < .001$; R-propranolol, $F_{4,33} = 2.2$, n.s.; S-propranolol, $F_{4,33} = 10.1$, $P < .001$; bupranolol, $F_{5,46} = 6.1$, $P < .001$; R-bupranolol, $F_{5,43} = 3.0$, n.s.; S-bupranolol, $F_{5,50} = 9.8$, $P < .001$). S-Bupranolol was equal to, if not better than, racemic and S-propranolol in blocking formalin-induced nociception. Sample sizes in all groups for Rota-Rod testing range from n = 5 or 6 and for formalin testing range from $n = 8$–10 mice/dose/drug. *$P < .05$, **$P < .01$, ***$P < .001$ compared with vehicle (VEH); all P-values corrected for multiple comparisons.

Table 1. Half-Maximal Doses to Achieve Ataxia and Analgesia (AD_{50}s; 95% Confidence Intervals in Parentheses) of Propranolol, R-Propranolol, S-Propranolol, Bupranolol, R-Bupranolol, and S-Bupranolol

Drug	Ataxia	Early Phase	Late Phase
Propranolol	4.8 (1.8–12.7)	45.0 (25.8–78.0)	62.0 (15.7–242.0)
R-Propranolol	13.3 (9.8–18.3)	>1000	121.0 (39–380)
S-Propranolol	22.2 (18.3–29.4)	35.0 (18.3–67)	25.5 (7.8–83.0)
Bupranolol	88.0 (74–105)	>1000	99.0 (46–212)
R-Bupranolol	74.0 (62–87)	>1000	>1000
S-Bupranolol	166.0 (93–294)	51.0 (30.2–85.0)	26.6 (14.1–50)

NOTE. Mice were tested in the Rota-Rod test to assess ataxia and the formalin assay to quantify analgesia induced by drug treatment (based on changes from saline-treated means). All doses are in mg/kg.

confidence intervals for maximal analgesia observed in the early and late phases are shown in Table 1.

S-Bupranolol Produces Antiallodynic Effects in Inflammatory and Chronic Neuropathic Pain Assays

Because S-bupranolol was the most effective compound against formalin-induced pain without affecting sedation or causing mortality, we tested its antinociceptive properties in other pain assays. Mice injected with a high dose (60 mg/kg) of S-bupranolol displayed increased thermal thresholds to noxious radiant heat test at 20 minutes after injection, but no effect was observed on mechanical thresholds (Figs 3A and 3B). S-Bupranolol (60 mg/kg) produced a complete and sustained (>60 minute) reversal of both thermal and mechanical hypersensitivity produced by the inflammatory agent λ-carrageenan; lower doses produced a transient reversal of both thermal and mechanical hypersensitivity (Figs 3C and 3D). Mechanical allodynia after SNI was also reversed by a high dose (60 mg/kg) of S-bupranolol, but not after a lower dose (30 mg/kg) (Fig 3E).

In Silico Docking

The β_2-AR binding site has a narrow cleft of nonpolar residues and few polar amino acids (Fig 4A),[34,62] and has similar structural features as those identified for both β_1-ARs and β_3-ARs. The representative binding conformations for propranolol and bupranolol enantiomers for the β_2-ARs exhibit similar binding modes as shown for several β_2-AR ligands.[7,24,34,62,64] The β-hydroxyethylamine moiety establishes a network of hydrogen bonds with $D133^{3.32}$, $N312^{7.39}$, and $Y316^{7.43}$, and the aromatic portion of the molecule is engaged in nonpolar interactions with $F193^{5.32}$, $F289^{6.51}$, $F290^{6.52}$, $V114^{3.33}$, and $V117^{3.36}$ (Figs 4B and 4C) (italic superscripts refer to the Ballesteros-Weinstein numbering convention[2] for G-protein coupled receptors). R-Enantiomers and S-enantiomers of both molecules exhibit a similar interaction pattern at both β_1-AR and β_3-AR binding sites, and the bound conformations are similar for each receptor subtype (see RMSD values in Supplementary Table 2). The main difference between the selected docking solutions is in the orientation of the hydroxyl group that determines its ability to interact via a hydrogen bond with $N^{7.39}$ and $D^{3.32}$ residues in the 3 β-ARs. In β_2-AR, the hydroxyl group of R-propranolol and R-bupranolol is engaged in a hydrogen bond with the amidic oxygen of the $N312^{7.39}$ side chain, and it interacts with the carboxyl oxygen of $D133^{3.32}$ in S-propranolol and S-bupranolol (Figs 2B and 2C). In β_1-AR, the hydroxyl group of both propranolol enantiomers faces the amidic oxygen of the $N363^{7.39}$ side chain (Supplementary Fig 1A); the same interaction is observed for R-bupranolol, whereas in S-bupranolol, the hydroxyl group establishes a hydrogen bond with the carboxyl oxygen of $D138^{3.32}$ (Supplementary Fig 1B). For β_3-AR, the hydroxyl group of both propranolol and bupranolol enantiomers interacts with the carboxyl oxygen of $D117^{3.32}$ (Supplementary Figs 2A and 2B). Moreover, in the binding site of β_3-AR, S-bupranolol presents a flipped orientation of the aromatic ring with respect to the R-enantiomer that does not affect the hydrophobic interactions with $F309^{6.52}$. Thus, there are subtle differences in the docking of R-enantiomers and S-enantiomers within β-AR binding sites.

Inhibition of Isoproterenol-Induced cAMP Production

The inhibition of isoproterenol-evoked cAMP production by racemic propranolol, R-bupranolol, or S-bupranolol was examined for all 3 β-ARs. In these studies, we first verified the agonistic effects of isoproterenol on all 3 β-ARs and determined the concentrations giving half-maximal responses (ie, EC_{50} values) (Fig 5A). These values were generally very low for all 3 receptors, within the picomolar to nanomolar range (see Supplementary Table 3A).

Next, dose-response curves were determined for the antagonists in the presence of isoproterenol, namely racemic propranolol, R-bupranolol, or S-bupranolol. We used a specific concentration close to the EC_{50} value of isoproterenol for the stimulation of each receptor type: .22 nM for β_1-AR, 15 pM for β_2-AR, and 2.2 nM for β_3-AR. All 3 compounds behaved as antagonists for β_1-AR (Fig 5B and Supplementary Table 3B). Comparison of the concentrations that produced a 50% inhibition (ie, IC_{50} values) showed that racemic propranolol and S-bupranolol are the most potent of the test agents. However, closer inspection of the unnormalized data values revealed that the maximum antagonistic effect

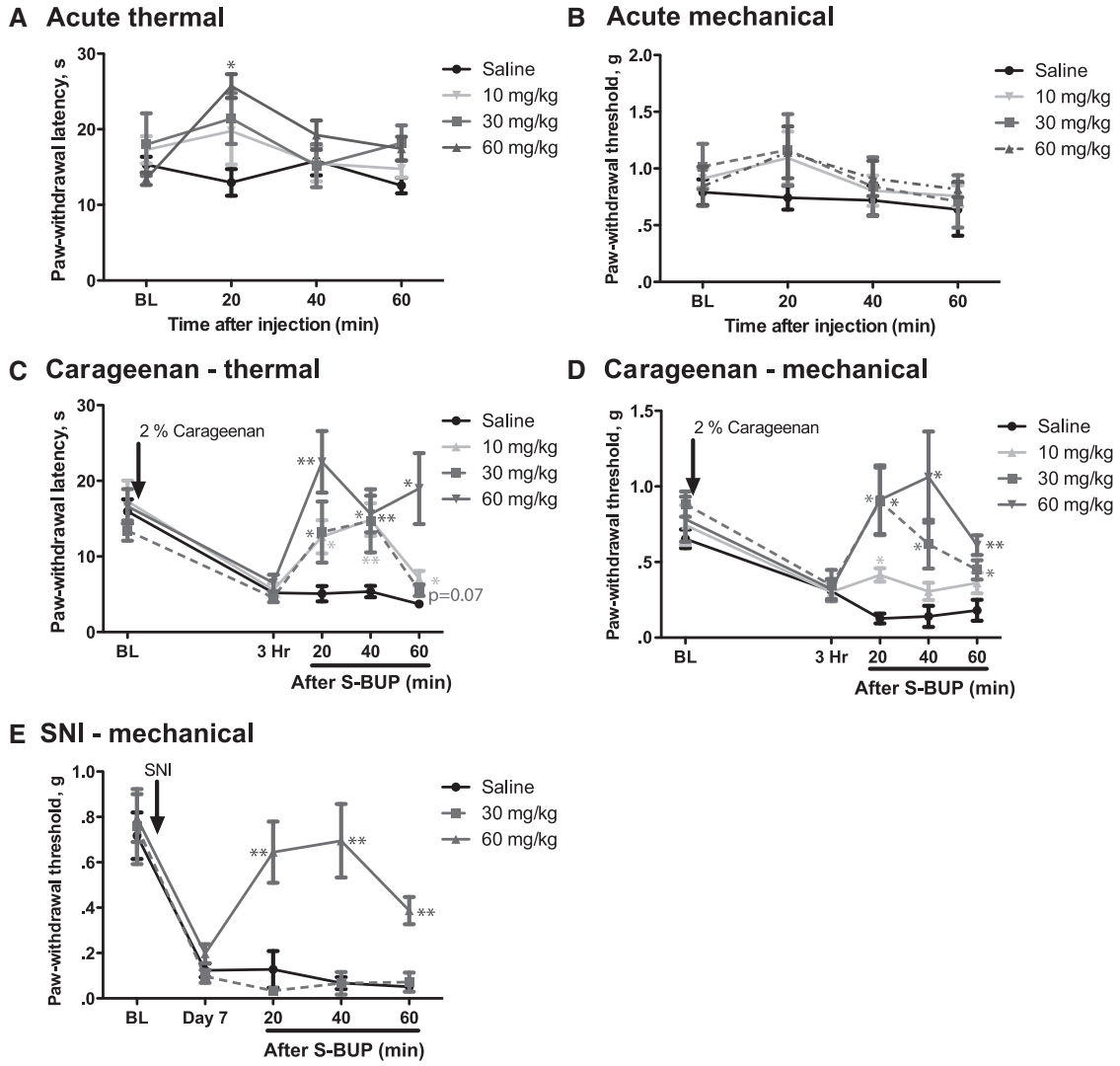

Figure 3. The effect of S-bupranolol on different pain modalities/assays (acute, inflammatory, and chronic). S-Bupranolol has minimal and no efficacy against acute thermal (**A**; Dose × Time Repeated Measure: $F_{9,78} = 2.1$, $P < .05$) and mechanical (**B**; Drug × Time Repeated Measures; $F_{9,72} = .4$, nonsignificant) thresholds, respectively. Symbols represent mean ± standard error of the mean (SEM)latency (seconds; in **A**) or threshold (g; in **B**) to withdraw from stimulus. Different doses of S-bupranolol reverse thermal (**C**; Dose × Time Repeated Measures: $F_{12,80} = 2.6$, $P < .0010$) and mechanical (**D**; Drug × Repeated Measures: $F_{12,80} = 2.2$, $P < .05$) hypersensitivity induced by carrageenan. Symbols represent mean ± SEM latency (seconds; in **C**) or threshold (g; in **D**) to withdraw from stimulus before carrageenan (BL), 3 hours after carrageenan (3 Hr), and 20 to 60 minutes after drug (S-BUP) administration. **(E)** S-Bupranolol attenuates mechanical allodynia induced by SNI when a high dose (60 mg/kg) is used (Dose × Time Repeated Measures: $F_{8,60} = 4.0$, $P < .001$). Symbols represent mean ± SEM threshold (*g*) to withdraw from stimulus before surgery (BL), 7 days after surgery (day 7), and 20 to 60 minutes after drug (S-BUP) administration. Sample sizes in all groups range from n = 7–10 mice/dose/drug. $*P < .05$, $**P < .01$, $***P < .001$ compared with baseline (in **A**), 3 hours after carrageenan (in **C** and **D**), or day 7 after SNI (in **E**).

of racemic propranolol is about 80% of that of S-bupranolol (see Supplementary Fig 3A). For β_2-AR, the IC_{50} values (half-maximal inhibitory concentrations) of all antagonists were similar (Fig 5C and Supplementary Table 3C), ranging from 6.7 nM for S-bupranolol to 29 nM for R-bupranolol. For β_3-AR, all compounds showed antagonistic properties; however, S-bupranolol gave nearly a full sigmoidal inhibition curve for β_3-AR (Fig 5D, Supplementary Fig 3C and Supplementary Table 3D) but did not fully block the formation of cAMP even at the highest concentration tested. Partial (~20%) inhibition was observed for propranolol. Very high concentrations of R-bupranolol were required to produce a steep (ie, within 1 log unit) inhibition of cAMP production, suggesting a possible nonselective inhibition. Therefore, the β_3-AR-associated IC_{50} values obtained for R-bupranolol and racemic propranolol may not be reliable. Also, the data point at 10 μM for racemic propranolol was discarded, because it seemed to increase cAMP production (Supplementary Fig 3C), providing additional evidence for a nonspecific effect of propranolol at β_3-ARs.

pA_2 Values of Antagonists

Next, to determine the relative potencies and receptor selectivity of the β-AR antagonists, Schild plots were generated (Fig 6A, Supplementary Figure 4-6 and

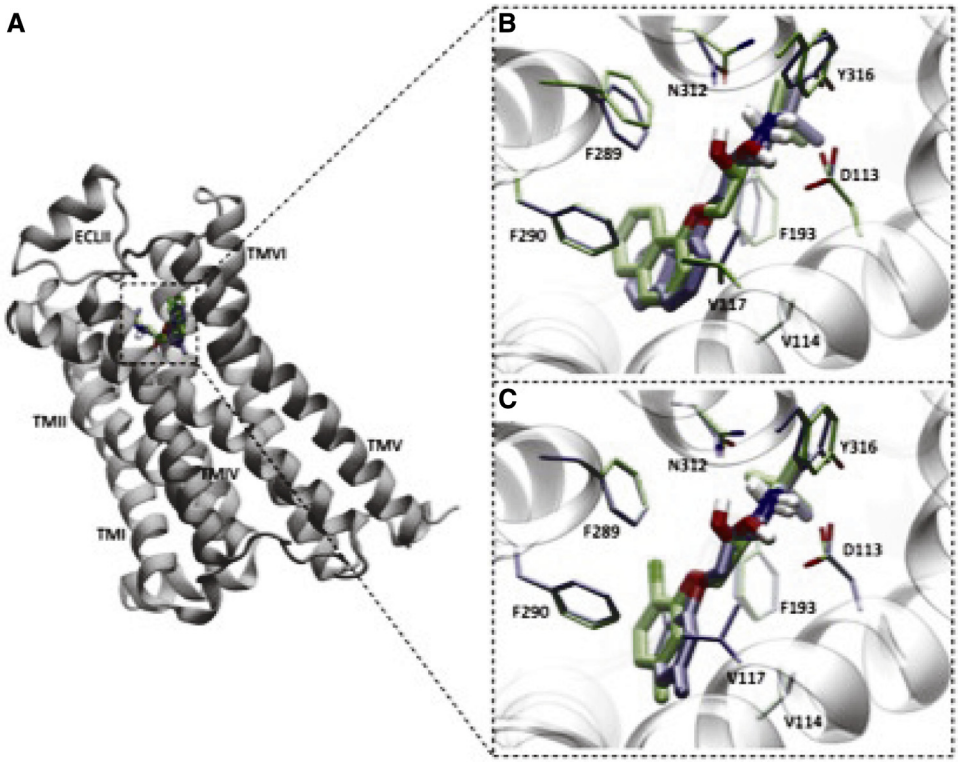

Figure 4. R-/S-Propranolol and bupranolol docking solutions. **(A)** The crystallographic structure of β_2-AR is shown in gray cartoon. The 4T lysozime construct (4TL) has been removed from the figure for the sake of clarity. **(B)** Zoomed view of R-enantiomers and S-propranolol and of **(C)** R-/S-bupranolol. Carbon atoms are represented in green and blue for R-enantiomers and S-enantiomers, respectively. The same color code is adopted to indicate the side chains of β_2-AR amino acids when in complex with the 2 enantiomers. TMI–TMVI = Transmembrane bundle 1–6; ECLIII = Extracellular Loop 3; F = Phenylalanine; N = Asparagine; D = Aspartate; Y = Tyrosine; V = Valine.

Supplementary Table 4) to obtain pA_2 values. According to the pA_2 plots, racemic propranolol and S-bupranolol have similar effects on β_1-ARs and are more potent than R-bupranolol (Fig 6A). All of the compounds have pA_2 slopes equivalent to –1 (ie, 95% confidence intervals overlap with –1), suggesting that the antagonism is competitive, selective, and does not show partial agonist or intrinsic sympathomimetic activity (ISA) for all compounds tested. This can be concluded from the lack of cAMP signal at the beginning of the curve, when the cells are under the effect of the antagonists, and the added isoproterenol (10^{-14} M final concentration) does not trigger significant cAMP production. The lack of ISA at β_1-ARs was also evident by examining individual dose-response curves in the assay in which cells were treated with antagonists alone (Supplementary Figs 7A and 7B).

However, for β_2-ARs, reliable Schild analyses were not obtained. Although the pA_2 values of the antagonists are almost equal, slopes differ significantly from –1 (except for R-bupranolol; Fig 6B). A pA_2 slope that deviates significantly from –1 indicates that the antagonism is not competitive, that the test antagonist is acting at more than 1 receptor, or that some other factor (eg, a partial agonist) is obscuring the effect. It is also evident from the Schild plots and dose-response curves of antagonists alone (Supplementary Figs 5, 7C and 7D) that all compounds show partial agonistic effects on β_2-ARs. The presence of ISA activity and the differences in the pA_2 slopes suggest that racemic propranolol and the R-enantiomers and S-enantiomers of bupranolol are able to stimulate β_2-ARs receptors at high concentrations, with propranolol showing the greatest ISA at β_2-ARs.

Similar to β_1-AR, racemic propranolol and S-bupranolol were the most potent antagonists for β_3-ARs according to their pA_2 values (Fig 6C). The slopes of the pA_2 plots were equivalent to –1 (eg, within the 95% confidence intervals) for racemic propranolol and the enantiomers of bupranolol. However, as with β_2-AR, comparing the pA_2 values may not be the most suitable way to rank the antagonists, because they have different properties: R-bupranolol showed weak antagonism for β_3-ARs, and propranolol displayed mixed agonist-antagonist characteristics (Supplementary Figs 7E and 7F). S-Bupranolol was the only compound that can be reliably classified as a competitive antagonist, with potent antagonistic effects on β_3-AR and with a negligible ISA effect.

Discussion

In the present study, we demonstrated that enantiomers of 2 nonselective β-AR antagonists, bupranolol and propranolol, display different antinociceptive efficacies in mice and have different affinities and properties for β-AR subtypes. In the formalin test, the S-enantiomers were substantially more potent than the R-enantiomers.

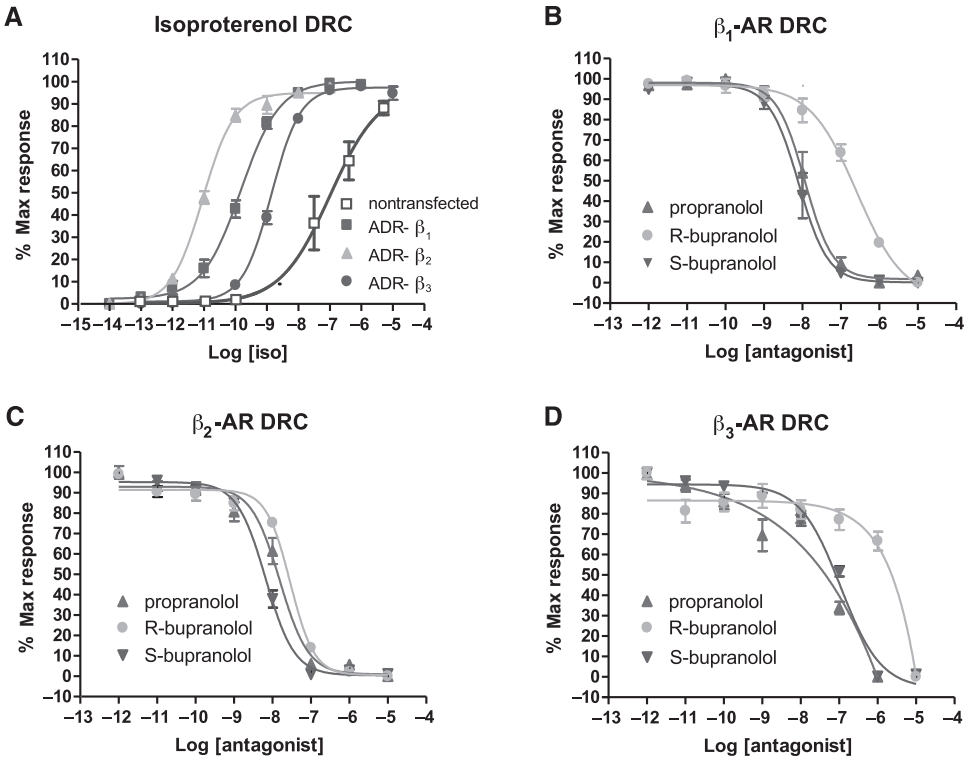

Figure 5. Dose-response curves (DRC) of isoproterenol (iso) and the β-AR antagonists used. **(A)** Dose-response curves of isoproterenol for each β-AR. ADR = adrenergic receptor. **(B–D)** Inhibition dose-response curves of antagonists at EC_{50} of isoproterenol for β_1-AR (**B**; n = 9), β_2-AR (**C**; n = 11), and β_3-AR (**D**; n = 7), respectively.

In contrast to R-bupranolol, S-bupranolol was equally as effective in blocking nociceptive behaviors as propranolol and S-propranolol in the late phase of the formalin test. However, S-propranolol and propranolol were found to be more sedative (or ataxia producing) on the Rota-Rod, and likely confounded the licking behavior scored in the formalin test. Thus, it is difficult to determine whether formalin-evoked behaviors decreased because of antinociceptive effectiveness or sedation.

The antinociceptive effects of S-bupranolol are generalizable to other pain assays, as we also demonstrated efficacy in tests of inflammatory and neuropathic pain. S-Bupranolol was not effective against acute thermal (except transiently at a high dose) or mechanical pain. This is consistent with previously published experimental pain work showing that intravenous propranolol has a minimal effect on decreasing heat pain in healthy men.[53] In the clinic, analgesia is not typically observed in patients treated with propranolol for hypertension at a dosage that typically ranges from 40 to 80 mg per day. However, pain resulting from angina in cardiac patients is significantly reduced with higher propranolol doses,[20,23] and nonselective β-blockers either reduce pain sensitivity to noxious stimuli or pain scores in clinical studies.[8,11,47] To a certain extent, our data are analogous to findings seen in the clinical setting such that β-AR blockade is most effective in alleviating hypersensitivity without raising experimentally assessed pain thresholds. This is important in light of recent research demonstrating that blocking β-ARs reduces

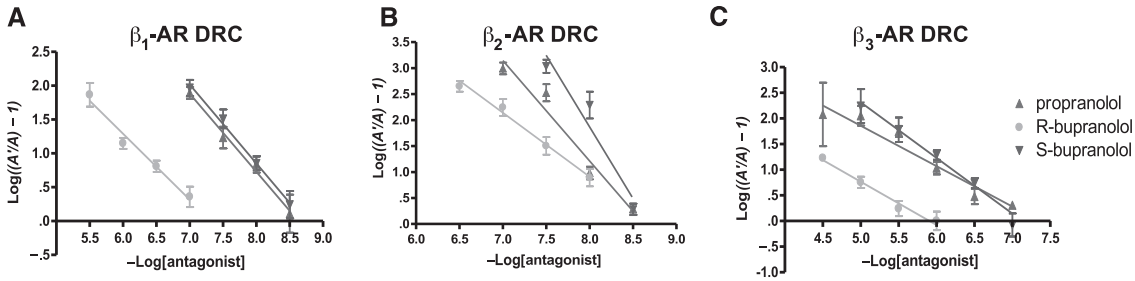

Figure 6. pA_2 plots for the antagonists of β_1-AR (**A**; n = 6), β_2-AR (**B**; n = 9), and β_3-AR (**C**; n = 9). Dose-response curves for isoproterenol were determined in the presence of various concentrations of antagonists, and the EC_{50} values obtained (*A*′) were compared with the EC_{50} values without antagonist **(*A*)**. Log*((A′/A)–1)* was plotted against the negative logarithm of antagonist concentration (–log [antagonist]). The *x*-intercept determines the estimated pA_2 value, assuming also that –1 is within the 95% confidence interval of the slope. Higher pA_2 values indicate higher affinity of the antagonist. Refer to Supplementary Table 3 for a more detailed summary of the findings.

pain associated with a variety of conditions, including chronic TMD,[59] irritable bowel syndrome,[68] and peripheral nerve damage.[42] The hypersensitivity resulting from tissue or nerve injury leads to a hyperadrenergic state[37] and it is possible that the antinociceptive properties of β-AR blockade are revealed only when adrenergic activity is increased. In this regard, β-AR blockade may act to downregulate β-AR[37] or reduce epinephrine-induced sensitization of sensory terminals.[32] Of course, this is not the only explanation because hypersensitivity resulting from the sensitization of P2X2/3 receptors is also reversed by β-AR blockade.[63]

Although propranolol and bupranolol are sympatholytic drugs, we do not believe that these drugs are important only for sympathetically maintained pain syndromes such as complex regional pain syndrome. The R-enantiomers of both compounds were associated with greater side effects and lethality. Relative to propranolol, both the R-enantiomers and S-enantiomers of bupranolol produced less sedation/ataxia, with the R-enantiomer of bupranolol producing greater Rota-Rod deficits than the corresponding S-enantiomer. These findings demonstrate that S-bupranolol is superior to the other enantiomers tested based on both antinociceptive response and side effects, and we believe that β-AR blockade with bupranolol will provide an efficient analgesic strategy for a wide range of pain disorders.

Furthermore, the cellular assays revealed different pharmacodynamic properties of propranolol, bupranolol, and associated enantiomers toward $\beta_1/\beta_2/\beta_3$-ARs. We found that R-bupranolol is a relatively potent β_2-AR antagonist and it has weak or essentially little antagonistic properties at β_1/β_3-ARs, respectively, and weak antinociceptive properties. This is striking considering that β_2-ARs are considered to be important for the analgesia mediated by β-AR blockade.[1,5] In contrast, S-bupranolol and S-propranolol displayed the strongest antinociceptive properties in the formalin assay and showed high protein-ligand interactions for the representative binding poses for $\beta_1/\beta_2/\beta_3$-ARs. At the cellular level, the inhibitory effects of S-bupranolol were similar to propranolol at blocking $\beta_1/\beta_2/\beta_3$-ARs. Our data along with previously published data suggest that β_1-ARs and β_3-ARs may be just as important, if not more important than β_2-ARs in producing antinociception in mice. In rats, esmolol, a selective β_1-AR blocker, can suppress nociception induced by formalin[13] and is effective as an adjunct for perioperative pain management in human patients by reducing postoperative opioid requirement.[12] Increased catecholamine levels resulting from reduced enzymatic activity of catechol-*O*-methyltransferase increase pain sensitivity,[16] which can be blocked by antagonists of β_2-ARs and β_3-ARs. Furthermore, the stimulation of β_3-ARs on dorsal root ganglion neurons evokes the release of the pronociceptive molecule ATP after peripheral nerve injury.[30] The current data do not indicate whether β_3-ARs are exhibiting their effects via peripheral or central mechanisms; however, these receptors are expressed at very low levels in the central nervous system.[50] Stimulation of peripheral β_2/β_3-ARs produces hyperalgesia and the activation of β-AR in the spinal cord produces analgesia (see Segall et al[54] for a review), supporting a bias toward a peripheral site of action for β_3-antagonists. However, we cannot exclude a central site of action, which is consistent with the anxiolytic effects of β-blocking drugs.[26] When coupled with our finding that S-bupranolol is a full β_3-AR antagonist, its superior effects in producing antinociception are consistent with earlier findings that combined treatment with selective β_2/β_3-ARs produces greater antinociception than blocking β_2/β_3-AR separately.[45]

Although bupranolol and propranolol are clearly nonselective β-AR antagonists, they are not believed to exhibit ISA.[66] In contrast to previous findings, we observed that propranolol increased cAMP production, indicative of clear ISA at all β-AR receptor subtypes reaching up to 15%, 37%, and 45% of responses to isoproterenol at $\beta_1/\beta_2/\beta_3$-ARs, respectively. In contrast to propranolol, the R-enantiomers and S-enantiomers of bupranolol produced weak ISA at β_1/β_2-ARs (S-enantiomer>R-enantiomer) and exhibited very weak or no β_3-AR ISA. S-Bupranolol was the only compound examined that can be classified as a relatively potent, nonselective competitive antagonist without notable ISA for β_3-ARs. Thus, compared with propranolol, the receptor mechanisms mediating the antinociceptive effects of S-bupranolol are less confounded because of the weak or no ISA exhibited at $\beta_1/\beta_2/\beta_3$-ARs. ISA is typically regarded as a beneficial feature of β-blockers because it minimizes the bradycardia found in elderly patients,[22] and patients tend to tolerate β-blockers with ISA better than those without.[10] However, our results suggest that the absence of ISA activity (particularly at β_3-ARs) by S-bupranolol may be an important feature for antinociception without significant side effects.

In silico docking calculations were performed to explore the binding properties of propranolol and bupranolol enantiomers with respect to the 3 β-AR subtypes. The binding mode of propranolol and bupranolol enantiomers discloses minimal structural differences, especially in the interaction with $N^{7.39}$ or $D^{3.32}$ residues of $\beta_1/\beta_2/\beta_3$-AR. The estimated binding energies of the top-ranked protein-ligand conformations indicate that all stereoisomers can effectively bind the 3 receptors. Although characterized by a flipped orientation, the aromatic moiety of S-bupranolol is engaged in hydrophobic interactions with the same protein residues (ie, F341, V139) as all the other molecules. Consequently, the estimated energy of binding for S-bupranolol (ie, –38.4 ± .6 kcal/mol) is not significantly different from R-bupranolol (ie, –37.8 ± .1 kcal/mol) or propranolol enantiomers (ie, –38.7 ± .1 and –39.2 ± .6 for S-enantiomers and R-enantiomers, respectively). These data suggest that all the stereoisomers under investigation can equally bind the targeted receptor, confirming the common limitation of current docking scoring functions in quantifying fine differences in the affinity of potent ligands to a given target.[65] These findings also suggest that the differences in the potencies observed for the different enantiomers in the cellular and behavioral assays do not result from differences in ligand binding affinities for a given β-AR

receptor but instead result from different capacities to engage downstream signaling pathways. Binding assays alone are not able to differentiate the relative potencies of these agents at β-AR and emphasize the importance of conducting in vitro cellular and in vivo assessments.

These findings suggest that a nonselective antagonist of $\beta_1/\beta_2/\beta_3$-ARs (which expresses little ISA, produces little ataxia, and expresses a high therapeutic index) would be more effective as a strategy to produce analgesia compared with blocking individual β-ARs. S-Bupranolol fits this unique profile compared with racemic propranolol, R-propranolol, S-propranolol, and R-bupranolol. S-Bupranolol is effective in producing antinociceptive behaviors across several algesiometric assays in mice and in blocking $\beta_1/\beta_2/\beta_3$-ARs without producing significant sedation or lethality. S-Bupranolol has a superior preclinical safety profile and shows greater antinociceptive efficacy compared with the other test agents and should be considered as a unique β-AR compound to advance future clinical pain studies.

Supplementary Data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jpain.2015.09.004.

References

1. Aley KO, Martin A, McMahon T, Mok J, Levine JD, Messing RO: Nociceptor sensitization by extracellular signal-regulated kinases. J Neurosci 21:6933-6939, 2001

2. Ballesteros JA, Weinstein H: Integrated methods for the construction of three-dimensional models and computational probing of structure-function relations in G protein-coupled receptors. In: Stuart CS (ed): Methods in Neurosciences. San Diego, CA, Academic Press, 1995, pp 366-428

3. Barrett AM, Cullum VA: The biological properties of the optical isomers of propranolol and their effects on cardiac arrhythmias. Br J Pharmacol 34:43-55, 1968

4. Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, Shindyalov IN, Bourne PE: The Protein Data Bank. Nucleic Acids Res 28:235-242, 2000

5. Cadirci E, Suleyman H, Hacimuftuoglu A, Halici Z, Akcay F: Indirect role of beta2-adrenergic receptors in the mechanism of analgesic action of nonsteroidal antiinflammatory drugs. Crit Care Med 38:1860-1867, 2010

6. Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL: Quantitative assessment of tactile allodynia in the rat paw. J Neurosci Methods 53:55-63, 1994

7. Cherezov V, Rosenbaum DM, Hanson MA, Rasmussen SG, Thian FS, Kobilka TS, Choi HJ, Kuhn P, Weis WI, Kobilka BK, Stevens RC: High-resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor. Science 318:1258-1265, 2007

8. Chia YY, Chan MH, Ko NH, Liu K: Role of beta-blockade in anaesthesia and postoperative pain management after hysterectomy. Br J Anaesth 93:799-805, 2004

9. Choucair-Jaafar N, Yalcin I, Rodeau JL, Waltisperger E, Freund-Mercier MJ, Barrot M: Beta2-adrenoceptor agonists alleviate neuropathic allodynia in mice after chronic treatment. Br J Pharmacol 158:1683-1694, 2009

10. Cohen TJ, Cayenne S, Black M, Doolittle S, Ibrahim B, Quan W: The effects of intrinsic sympathomimetic activity on beta-blocker efficacy for treatment of neurocardiogenic syncope. J Invasive Cardiol 11:457-460, 1999

11. Collard V, Mistraletti G, Taqi A, Asenjo JF, Feldman LS, Fried GM, Carli F: Intraoperative esmolol infusion in the absence of opioids spares postoperative fentanyl in patients undergoing ambulatory laparoscopic cholecystectomy. Anesth Analg 105:1255-1262, 2007. table of contents

12. Coloma M, Chiu JW, White PF, Armbruster SC: The use of esmolol as an alternative to remifentanil during desflurane anesthesia for fast-track outpatient gynecologic laparoscopic surgery. Anesth Analg 92:352-357, 2001

13. Davidson EM, Doursout MF, Szmuk P, Chelly JE: Antinociceptive and cardiovascular properties of esmolol following formalin injection in rats. Can J Anaesth 48:59-64, 2001

14. Decosterd I, Woolf CJ: Spared nerve injury: an animal model of persistent peripheral neuropathic pain. Pain 87: 149-158, 2000

15. Diatchenko L, Anderson AD, Slade GD, Fillingim RB, Shabalina SA, Higgins TJ, Sama S, Belfer I, Goldman D, Max MB, Weir BS, Maixner W: Three major haplotypes of the β2 adrenergic receptor define psychological profile, blood pressure, and the risk for development of a common musculoskeletal pain disorder. Am J Med Genet B Neuropsychiatr Genet 141B:449-462, 2006

16. Diatchenko L, Slade GD, Nackley AG, Bhalang K, Sigurdsson A, Belfer I, Goldman D, Xu K, Shabalina SA, Shagin D, Max MB, Makarov SS, Maixner W: Genetic basis for individual variations in pain perception and the development of a chronic pain condition. Hum Mol Genet 14: 135-143, 2005

17. Ding F, Dokholyan NV: Incorporating backbone flexibility in MedusaDock improves ligand-binding pose prediction in the CSAR2011 docking benchmark. J Chem Inf Model 53:1871-1879, 2013

18. Ding F, Yin S, Dokholyan NV: Rapid flexible docking using a stochastic rotamer library of ligands. J Chem Inf Model 50:1623-1632, 2010

19. Doggrell SA: The membrane stabilizing and β1-adrenoceptor blocking activity of (+)- and (-)-propranolol on the rat left atria. Gen Pharmacol 21:677-680, 1990

20. Flamenbaum W, Weber MA, McMahon FG, Materson BJ, Carr AA, Poland M: Monotherapy with labetalol compared with propranolol. Differential effects by race. J Clin Hypertens 1:56-69, 1985

21. Freitag FG: The cycle of migraine: patients' quality of life during and between migraine attacks. Clin Ther 29:939-949, 2007

22. Frishman WH: Clinical significance of β1-selectivity and intrinsic sympathomimetic activity in a β-adrenergic blocking drug. Am J Cardiol 59:33F-37F, 1987

23. Frishman WH, Shapiro W, Charlap S: Labetalol compared with propranolol in patients with both angina pectoris and systemic hypertension: a double-blind study. J Clin Pharmacol 29:504-511, 1989

24. Hanson MA, Cherezov V, Griffith MT, Roth CB, Jaakola VP, Chien EY, Velasquez J, Kuhn P, Stevens RC: A specific cholesterol binding site is established by the 2.8 A structure of the human β2-adrenergic receptor. Structure 16: 897-905, 2008

25. Hargreaves K, Dubner R, Brown F, Flores C, Joris J: A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. Pain 32:77-88, 1988

26. Hudson CF: An evaluation of once daily long acting propranolol hydrochloride (Inderal LA and Half-Inderal LA) in the treatment of anxiety. A double-blind placebo-controlled general practice study. Br J Clin Pract 42:419-426, 1988

27. Johnson JD, Cortez V, Kennedy SL, Foley TE, Hanson H 3rd, Fleshner M: Role of central β-adrenergic receptors in regulating proinflammatory cytokine responses to a peripheral bacterial challenge. Brain Behav Immun 22: 1078-1086, 2008

28. Johnson JD, Zimomra ZR, Stewart LT: β-adrenergic receptor activation primes microglia cytokine production. J Neuroimmunol 254:161-164, 2013

29. Jones BJ, Roberts DJ: A rotarod suitable for quantitative measurements of motor incoordination in naive mice. Naunyn Schmiedebergs Arch Exp Pathol Pharmakol 259:211, 1968

30. Kanno T, Yaguchi T, Nishizaki T: Noradrenaline stimulates ATP release from DRG neurons by targeting β3-adrenoceptors as a factor of neuropathic pain. J Cell Physiol 224:345-351, 2010

31. Kelley LA, Gardner SP, Sutcliffe MJ: An automated approach for clustering an ensemble of NMR-derived protein structures into conformationally related subfamilies. Protein Eng 9:1063-1065, 1996

32. Khasar SG, McCarter G, Levine JD: Epinephrine produces a beta-adrenergic receptor-mediated mechanical hyperalgesia and in vitro sensitization of rat nociceptors. J Neurophysiol 81:1104-1112, 1999

33. Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG, Group NCRRGW: Animal research: reporting in vivo experiments: the ARRIVE guidelines. Br J Pharmacol 160:1577-1579, 2010

34. Kolb P, Rosenbaum DM, Irwin JJ, Fung JJ, Kobilka BK, Shoichet BK: Structure-based discovery of β2-adrenergic receptor ligands. Proc Natl Acad Sci U S A 106:6843-6848, 2009

35. Kota P, Ding F, Ramachandran S, Dokholyan NV: Gaia: automated quality assessment of protein structure models. Bioinformatics 27:2209-2215, 2011

36. Kushnir VM, Cassell B, Gyawali CP, Newberry RD, Kibe P, Nix BD, Sabzpoushan A, Kanuri ND, Sayuk GS: Genetic variation in the β-2 adrenergic receptor (ADRB2) predicts functional gastrointestinal diagnoses and poorer health-related quality of life. Aliment Pharmacol Ther 38:313-323, 2013

37. Light AR, Vierck CJ, Light KC: Myalgia and fatigue: translation from mouse sensory neurons to fibromyalgia and chronic fatigue syndromes. In: Kruger L, Light AR (eds): Translational Pain Research: From Mouse to Man. Frontiers in Neuroscience. Boca Raton, FL, CRC Press, 2010

38. Light KC, Bragdon EE, Grewen KM, Brownley KA, Girdler SS, Maixner W: Adrenergic dysregulation and pain with and without acute β-blockade in women with fibromyalgia and temporomandibular disorder. J Pain 10:542-552, 2009

39. Malinowska B, Kiec-Kononowicz K, Flau K, Godlewski G, Kozlowska H, Kathmann M, Schlicker E: Atypical cardiostimulant β-adrenoceptor in the rat heart: stereoselective antagonism by bupranolol but lack of effect by some bupranolol analogues. Br J Pharmacol 139:1548-1554, 2003

40. Martin VT, Wernke S, Mandell K, Ramadan N, Kao L, Bean J, Liu J, Zoma W, Rebar R: Symptoms of premenstrual syndrome and their association with migraine headache. Headache 46:125-137, 2006

41. McGrath JC, Drummond GB, McLachlan EM, Kilkenny C, Wainwright CL: Guidelines for reporting experiments involving animals: the ARRIVE guidelines. Br J Pharmacol 160:1573-1576, 2010

42. McLachlan EM, Hu P: Inflammation in dorsal root ganglia after peripheral nerve injury: effects of the sympathetic innervation. Auton Neurosci 182:108-117, 2014

43. Institute of, Medicine: Relieving Pain in America: A Blueprint for Transforming Prevention, Care, Education, and Research. Washington, D.C., Institute of Medicine, 2011

44. Molinoff PB: α- and β-adrenergic receptor subtypes properties, distribution and regulation. Drugs 28(Suppl 2): 1-15, 1984

45. Nackley AG, Tan KS, Fecho K, Flood P, Diatchenko L, Maixner W: Catechol-O-methyltransferase inhibition increases pain sensitivity through activation of both β2- and β3-adrenergic receptors. Pain 128:199-208, 2007

46. Nicholson R, Dixon AK, Spanswick D, Lee K: Noradrenergic receptor mRNA expression in adult rat superficial dorsal horn and dorsal root ganglion neurons. Neurosci Lett 380:316-321, 2005

47. Pranevicius M, Pranevicius O: Non-opioid anesthesia with esmolol avoids opioid-induced hyperalgesia and reduces fentanyl requirement after laparoscopy. Anesth Analg 108:1048, 2009

48. Ramachandran S, Kota P, Ding F, Dokholyan NV: Automated minimization of steric clashes in protein structures. Proteins 79:261-270, 2011

49. Rasmussen SG, DeVree BT, Zou Y, Kruse AC, Chung KY, Kobilka TS, Thian FS, Chae PS, Pardon E, Calinski D, Mathiesen JM, Shah ST, Lyons JA, Caffrey M, Gellman SH, Steyaert J, Skiniotis G, Weis WI, Sunahara RK, Kobilka BK: Crystal structure of the β2 adrenergic receptor-Gs protein complex. Nature 477:549-555, 2011

50. Rodriguez M, Carillon C, Coquerel A, Le Fur G, Ferrara P, Caput D, Shire D: Evidence for the presence of beta 3-adrenergic receptor mRNA in the human brain. Brain Res Mol Brain Res 29:369-375, 1995

51. Roy A, Kucukural A, Zhang Y: I-TASSER: a unified platform for automated protein structure and function prediction. Nat Protoc 5:725-738, 2010

52. Saeed MA, Pumariega AJ, Cinciripini PM: Psychopharmacological management of migraine in children and adolescents. J Child Adolesc Psychopharmacol 2:199-211, 1992

53. Schweinhardt P, Abulhasan YB, Koeva V, Balderi T, Kim DJ, Alhujairi M, Carli F: Effects of intravenous propranolol on heat pain sensitivity in healthy men. Eur J Pain 17: 704-713, 2013

54. Segall SK, Maixner W, Belfer I, Wiltshire T, Seltzer Z, Diatchenko L: Janus molecule I: dichotomous effects of COMT in neuropathic vs nociceptive pain modalities. CNS Neurol Disord Drug Targets 11:222-235, 2012

55. Skouen JS, Smith AJ, Warrington NM, O' Sullivan PB, McKenzie L, Pennell CE, Straker LM: Genetic variation in the β-2 adrenergic receptor is associated with chronic musculoskeletal complaints in adolescents. Eur J Pain 16: 1232-1242, 2012

56. Stoschitzky K, Lindner W, Rath M, Leitner C, Uray G, Zernig G, Moshammer T, Klein W: Stereoselective hemodynamic effects of (R)-and (S)-propranolol in man. Naunyn Schmiedebergs Arch Pharmacol 339:474-478, 1989

57. Tallarida R, Murray R: Manual of Pharmacologic Calculation. New York, Springer-Verlag, 1981

58. Tan KS, Nackley AG, Satterfield K, Maixner W, Diatchenko L, Flood PM: β2 adrenergic receptor activation stimulates pro-inflammatory cytokine production in macrophages via PKA- and NF-kappaB-independent mechanisms. Cell Signal 19:251-260, 2007

59. Tchivileva IE, Lim PF, Smith SB, Slade GD, Diatchenko L, McLean SA, Maixner W: Effect of catechol-O-methyltransferase polymorphism on response to propranolol therapy in chronic musculoskeletal pain: a randomized, double-blind, placebo-controlled, crossover pilot study. Pharmacogenet Genomics 20:239-248, 2010

60. Tchivileva IE, Tan KS, Gambarian M, Nackley AG, Medvedev AV, Romanov S, Flood PM, Maixner W, Makarov SS, Diatchenko L: Signaling pathways mediating β3-adrenergic receptor-induced production of interleukin-6 in adipocytes. Mol Immunol 46:2256-2266, 2009

61. Vargas-Alarcon G, Fragoso JM, Cruz-Robles D, Vargas A, Martinez A, Lao-Villadoniga JI, Garcia-Fructuoso F, Vallejo M, Martinez-Lavin M: Association of adrenergic receptor gene polymorphisms with different fibromyalgia syndrome domains. Arthritis Rheum 60:2169-2173, 2009

62. Wacker D, Fenalti G, Brown MA, Katritch V, Abagyan R, Cherezov V, Stevens RC: Conserved binding mode of human β2 adrenergic receptor inverse agonists and antagonist revealed by X-ray crystallography. J Am Chem Soc 132: 11443-11445, 2010

63. Wang S, Zhu HY, Jin Y, Zhou Y, Hu S, Liu T, Jiang X, Xu GY: Adrenergic signaling mediates mechanical hyperalgesia through activation of P2X3 receptors in primary sensory neurons of rats with chronic pancreatitis. Am J Physiol Gastrointest Liver Physiol 308:G710-G719, 2015

64. Warne T, Serrano-Vega MJ, Baker JG, Moukhametzianov R, Edwards PC, Henderson R, Leslie AG, Tate CG, Schertler GF: Structure of a β1-adrenergic G-protein-coupled receptor. Nature 454:486-491, 2008

65. Warren GL, Andrews CW, Capelli AM, Clarke B, LaLonde J, Lambert MH, Lindvall M, Nevins N, Semus SF, Senger S, Tedesco G, Wall ID, Woolven JM, Peishoff CE, Head MS: A critical assessment of docking programs and scoring functions. J Med Chem 49:5912-5931, 2006

66. White WB: β blockers with intrinsic sympathomimetic activity. Am Fam Physician 31:115-118, 1985

67. Yin S, Biedermannova L, Vondrasek J, Dokholyan NV: MedusaScore: an accurate force field-based scoring function for virtual drug screening. J Chem Inf Model 48:1656-1662, 2008

68. Zhang C, Rui YY, Zhou YY, Ju Z, Zhang HH, Hu CY, Xiao Y, Xu GY: Adrenergic β2-receptors mediates visceral hypersensitivity induced by heterotypic intermittent stress in rats. PLoS One 9:e94726, 2014

69. Zhang Y: I-TASSER server for protein 3D structure prediction. BMC Bioinformatics 9:40, 2008