Novel selective β₁-adrenoceptor antagonists for concomitant cardiovascular and respiratory disease


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ABSTRACT: β-Blockers reduce mortality and improve symptoms in people with heart disease; however, current clinically available β-blockers have poor selectivity for the cardiac β₁-adrenoceptor (AR) over the lung β₂-AR. Unwanted β₂-blockade risks causing life-threatening bronchospasm and reduced efficacy of β₂-agonist emergency rescue therapy. Thus, current life-prolonging β-blockers are contraindicated in patients with both heart disease and asthma. Here, we describe NDD-713 and -825, novel highly β₁-selective neutral antagonists with good pharmaceutical properties that can potentially overcome this limitation. Radioligand binding studies and functional assays that use human receptors expressed in Chinese hamster ovary cells demonstrate that NDD-713 and -825 have nanomolar β₁-AR affinity >500-fold β₁-AR vs. β₂-AR selectivity and no agonism. Studies in conscious rats demonstrate that these antagonists are orally bioavailable and cause pronounced β₁-mediated reduction of heart rate while showing no effect on β₂-mediated hindquarters vasodilatation. These compounds also have good disposition properties and show no adverse toxicologic effects. They potentially offer a truly cardioselective β-blocker therapy for the large number of patients with heart and respiratory or peripheral vascular comorbidities.—Baker, J. G., Gardiner, S. M., Woolard, J., Fromont, C., Jadhav, G. P., Mistry, S. N., Thompson, K. S. J., Kellam, B., Hill, S. J., Fischer, P. M. Novel selective β₁-adrenoceptor antagonists for concomitant cardiovascular and respiratory disease. FASEB J. 31, 3150–3166 (2017). www.fasebj.org

KEY WORDS: β-blocker · selectivity · heart disease · asthma

β-Adrenoceptor (AR) antagonists (β-blockers) block catecholamines from binding to β-AR, which reduces heart rate (HR) and the force of contraction, thereby reducing myocardial oxygen demand (1, 2). Randomized, placebo-controlled clinical trials demonstrate mortality reductions with β-blockers of 34 to 35% in patients with heart failure and of 36 to 39% in patients with myocardial infarction (3–7). β-Blockers are therefore recommended for all patients with heart failure or a recent myocardial infarction, as well as being first-line therapy for atrial arrhythmias and having important roles in hypertension, thyrotoxicosis, portal hypertension, migraine, glaucoma, and anxiety.

Bisoprolol (8) is one of the most β₁-selective β-blockers clinically available and, although often called cardioselective, has poor β₁- vs. β₂-selectivity (9–11). Unfortunately, this results in β₂-AR blockade in the airways that risks, in susceptible individuals, β₂-mediated bronchospasm and reduced effectiveness of life-saving β₂-agonist bronchodilatation therapy (12). A meta-analysis of randomized controlled trials of β-blockers in those with asthma reports a significant fall in lung function [forced expiratory volume in 1 s (FEV1)], an increase in symptoms, and attenuation of β₂-agonist rescue with cardioselective and nonselective β-blockers (13). Although the average fall in lung function was greater with nonselective agents, a >20% reduction in FEV1 still occurred in 1 in 8 patients who received cardioselective β-blockers. Other large, recent observational studies also report that both cardioselective and noncardioselective β-blockers result in reduced lung function in the general population [that recovers on cessation of β-blockers (14)] and in those with asthma when using β-blocker eye drops (15). A further observational study found that nonselective, but not cardioselective, β-blockers increase the risk of moderate and
severe asthma exacerbations (16). In addition, catastrophic outcomes have been also reported (17–23). Taken together, these studies suggest that \( \beta_1 \)-selective (cardioselective) \( \beta \)-blockers are better than nonselective \( \beta \)-blockers, but that current cardioselective agents still pose a risk to those with asthma. \( \beta \)-Blockers are therefore contraindicated in patients with asthma (24).

Patients with chronic obstructive pulmonary disease (COPD) have a high risk of heart disease: 40% of those who suffer from COPD also have heart disease (25) and they have high cardiovascular mortality (26, 27). As up to 50% of patients with COPD also have significant airway reversibility (improvement in lung function with \( \beta \)-agonists), they are at risk of bronchospasm from \( \beta \)-blockers (28, 29). \( \beta \)-Blockers reduce lung function in COPD, often by more than the 5 to 10% that is suggested by the American Thoracic Society/European Respiratory Society as important in COPD, but this is often well tolerated [for full details, see Baker and Wilcox (7)]. There are no randomized placebo-controlled studies of \( \beta \)-blockers in patients with heart disease and COPD, but currently, the number of observational studies with favorable or neutral outcomes outweigh those with detrimental outcomes (7, 30–32). Because of high cardiovascular mortality, current guidelines recommend trying \( \beta \)-blockers under careful medical supervision for those with heart disease and COPD, although this is not without patient risk and causes significant clinician anxiety (33, 34).

Highly \( \beta_1 \)-selective antagonists would overcome the problems that are associated with unwanted \( \beta_2 \)-blockade. CGP20712A and LK204-545 are the two most highly \( \beta_1 \)-selective \( \beta \)-blockers reported to date, but neither was developed into clinical drugs (35, 36).

Partial agonism [intrinsic sympathomimetic activity (ISA)] also varies significantly between \( \beta \)-blockers. This was once considered beneficial: Partial agonists would block HR surges from adrenaline but also provide increased basal tone in heart failure and reduce the chance of bronchospasm (12); however, partial agonism has been proven to be detrimental. In heart failure, \( \beta \)-blockers with little or no ISA prolonged life (3, 4), whereas those with higher ISA had no benefit or were detrimental (37, 38). Post–myocardial infarction, \( \beta \)-blockers without ISA reduced mortality (5, 6), whereas those with ISA did not (39, 40). Lowering HR is required for increased survival (41). LK204-545 has significant \( \beta_1 \)-partial agonism (42).

Thus, highly \( \beta_1 \)-AR–selective antagonists, devoid of partial agonism, could overcome unwanted \( \beta_2 \)-blockade, increasing the safety of \( \beta \)-blockers in those with COPD and peripheral vascular disease and potentially allowing treatment in those with asthma. This study aimed to develop highly \( \beta_1 \)-selective orally bioavailable \( \beta \)-blockers with no agonism or off-target effects that also had pharmacokinetic (PK) properties suitable for once daily oral dosing.

**MATERIALS AND METHODS**

**Study approval**

Cardiovascular monitoring procedures were carried out with approval of the University of Nottingham Ethical Review Committee under Home Office Project and Personal License Authority (to S.M.G. and J.W.).

**NDD compounds**

NDD-713 [4-[[2-[[25]-3-][[cyclopropylmethyl]methyl]-2,3-dihydro-1,4-benzodioxin-6-yl]oxyl]-2-hydroxypropyl] amino]ethoxy]benzamide; Chemical Abstracts Survey (CAS) registry no.1392488-35-5 and NDD-825 [5-[[2-[[25]-3-][[cyclopropylmethyl]methyl]-2,3-dihydro-1,4-benzodioxin-6-yl]oxyl]-2-hydroxypropyl]amino]ethoxy]-2,3-dihydro-1H-isodindol-1-one; CAS registry 1392488-42-4) were prepared and characterized as previously described (43).

**Cell culture**

Chinese hamster ovary (CHO) cells that stably express human \( \beta_1 \)-ARs (CHO-\( \beta_1 \): 1146 fmol/mg protein), human \( \beta_2 \)-ARs (CHO-\( \beta_2 \): 466 fmol/mg protein), or human \( \beta_3 \)-ARs (CHO-\( \beta_3 \): 790 fmol/mg protein) were grown as previously described (11). Human bronchial smooth muscle cells were obtained from Lonza (Basel, Switzerland) and grown in manufacturer media. Rat \( \beta \)-AR binding experiments (DNA from cDNA Resource Centre, Bloomsburg, PA, USA) were conducted by using transiently transfected CHO cells (44).

**3\(^{\text{H}}\)-CGP12177 whole-cell binding**

Confluent cells were examined (96-well plates, 2 h incubation at 37°C) as previously described (11) with \( ^{3\text{H}}\)-CGP12177 at 0.43–3.03 nM. Propranolol (10 \( \mu \)M) defined nonspecific binding. \( K_d \) values were derived from \( I_{\text{EC50}} \) values by using the Cheng-Prusoff equation as previously described (11). \( K_d \) values for \( ^{3\text{H}}\)-CGP12177, which were obtained from saturation binding in this study, were 0.49 ± 0.02 nM (\( n = 14 \)) for human \( \beta_1 \)-AR; 0.28 ± 0.01 nM (\( n = 14 \)) for human \( \beta_2 \)-AR; 0.24 ± 0.03 nM (\( n = 7 \)) for rat \( \beta_1 \)-AR, and 0.25 ± 0.04 nM (\( n = 7 \)) for rat \( \beta_2 \)-AR.

**3\(^{\text{H}}\)-cAMP accumulation**

Confluent cells were examined (24-well plates, 5 h incubation at 37°C) as described in Baker (45). Antagonist \( K_d \) values were calculated from a rightward shift of the agonist concentration response curve by using the Gaddum equation as previously described (46).

**cAMP response element–secreted placental alkaline phosphatase reporter gene assay**

cAMP response element (CRE)–secreted placental alkaline phosphatase (SPAP) production was measured in confluent cells (96-well plates, 5 h incubation at 37°C) as described in Baker (46). This assay is a sensitive measure of both cAMP and ERK1/2 MAPK pathways (47).

**Direct measurement of MAPK stimulation**

MAPK stimulation was examined by using the AlphaScreen Surefire ERK1/2 T202/Y204 assay kit from TGR Biosciences (PerkinElmer, Waltham, MA, USA), which gives a direct quantitative readout of ERK1/2 (p42/44) MAPK phosphorylation. Confluent cells (half-well 96-well plates) were serum starved for 24 h, then the assay was performed per manufacturer instructions (5- and 10-min incubations at 37°C: ligand concentrations of 3
and 10 μM. Phorbol 12,13-dibutyrate (1 μM) was used as a positive control.

**Duration of ligand binding**

A method that involved ligand washout was used as described (44). Cells were incubated with either competing ligand and ³H-CGP12177 for 2 h (control plate), or competing ligand alone for 2 h (duration plate). After 2 h, the control plate was processed while the duration plate was washed, and ³H-CGP12177 alone was added to wells and incubated for a second 2 h before processing. Long-acting ligands that do not dissociate from the receptor should result in similar ³H-CGP12177 binding to control. Shorter-acting ligands that were removed and/or that continued to dissociate during the 2-h ³H-CGP12177 incubation would result in more ³H-CGP12177 binding than control and, thus, an apparent rightward shift of the concentration response curve. Relative measures of ligand duration of binding are indicated by the degree of rightward shift of displacement curves (44).

**In vitro receptor selectivity profiling**

Receptor selectivity of reference compounds LK204-545 and CGP20712A (10 μM), and NDD-713 (4.8 μM) and NDD-825 (2.4 μM; concentrations approximately 400-fold greater than β₁ Kᵦ), were examined. Kᵦ (inhibition constant) was calculated by using the Cheng-Prusoff equation from the mean of duplicate determinations in cell-based receptor radioligand-binding assays. Cerep in vitro pharmacology profiling service was used (see the Supplemental Data for a list of the assays used).

**Aqueous solubility**

NDD-713.HCl and -825.HCl were added to either aqueous phosphate buffer (pH 6.5) or 0.01 M aqueous HCl (pH ~2) at final DMSO concentrations of 1%. Samples were analyzed by nephelometry to determine solubility ranges (48). Studies were carried out by the Centre for Drug Candidate Optimisation (CDCO; Monash University, Melbourne, VIC, Australia).

**Lipophilicity**

Octanol–water partition coefficient values (logD) of NDD-713 and -825 were estimated as described in Lombardo et al. (49) by correlation of the chromatographic retention properties of compounds against the characteristics of standard compounds with known partition coefficient values. Studies were carried out by CDCO.

**Permeability**

CaCo-2 cells were grown in Transwell permeable supports, and confluency was assessed by transepithelial electrical resistance measurements (>270 Ω/cm²) and by unidirectional permeability (apical-basolateral) of the low- and high-permeability reference compounds, ³¹C-mannitol and ³H-propranolol. NDD-713.HCl and -825.HCl (20 μM in HBSS that contained 20 mM HEPES, pH 7.4) were transferred to the donor compartment of Transwell plates and the acceptor compartments were filled with blank HBSS. Permeability was determined over 90 min by sampling both compartments. Quantitation of compound concentrations in donor and acceptor samples was by liquid chromatography–mass spectrometry (LC-MS; lower limit of quantification, 0.5–1.5 ng/ml), and for ¹⁴C-mannitol and ³H-propranolol, by liquid scintillation counting. Apparent permeability coefficients (P_app) were calculated as follows: P_app (cm/s) = dQ/dt × 1/(Cᵦ × A), where dQ/dt = apparent steady-state transport rate (μmol/s); Cᵦ = initial concentration in the chamber (μmol/cm³); and A = surface area of CaCo-2 monolayer (0.3cm²). Efflux ratios were calculated as the ratio of mean P_app values in B→A and A→B directions. Studies were carried out by CDCO.

**Metabolic stability**

NDD-713.HCl and -825.HCl were incubated (0.02, 0.1, or 0.5 μM at 37°C, 120 min) with human (XenoTech, Kansas City, KS, USA) or rat liver microsomes (BD Biosciences, Brea, CA, USA) at 0.4 mg/ml protein concentration. Metabolic reaction was initiated by addition of NADPH-regenerating system or NADPH-regenerating system with uridine diphosphate glucuronic acid. Samples were quenched at time points by acetonitrile addition. Control samples, which contained neither NADPH, nor uridine diphosphate glucuronic acid, were monitored for degradation. Compound concentrations were determined by ultraperformance LC-MS (UPLC-MS) relative to calibration standards that were prepared in quenched liver microsomes and fitted to exponential decay functions to determine first-order rate constants (k) for substrate depletion. These were used to calculate in vitro intrinsic clearance (CLᵦ) values: CLᵦ = k/μmol protein/ml. Metabolic stability assays that used rat liver cytosol (BD Biosciences), human cryopreserved hepatocytes, or rat hepatocytes were carried out similarly. Average viable cell concentrations were determined by Trypan Blue exclusion methods (in the absence of test compounds). Studies were carried out by CDCO.

**Metabolism**

NDD-713.HCl and -825.HCl (2 μM) and cytochrome P450 (P450) reference substrates (see Supplemental Data for full details) were incubated (37°C) with selected recombinant human P450 isoforms (CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6 CYP2E1, and CYP3A4) in heterologously expressed P450 Supersomes (BD Biosciences) that were suspended in 50 M Tris–HCl buffer (pH 7.4; for CYP2C9) or 100 mM phosphate buffer (pH 7.4; for the other isoforms) at final P450 concentrations of 85 pmol/ml. Reactions were initiated by additions of an NADPH-regenerating system and quenched at time points over 60 min (reference probes) or 120 min (test compounds) by acetonitrile. Quenched samples were centrifuged and supernatants were collected. NDD-713, -825, and probe metabolites were determined by UPLC-MS relative to calibration standards prepared in quenched matrix, and %substrate that remained was calculated relative to the initial sample concentration. The %substrate that remained vs. time was fitted to exponential decay functions to determine the pseudo first-order rate constant (k/minute) for substrate depletion. These were normalized to P450 concentrations (pmol/ml) to obtain in vitro CLᵦ values: CLᵦ, in vitro (μl/min/pmol P450) = [k (min⁻¹)]/P450 concentration (pmol/min) × 1000. Studies were carried out by CDCO.

**P450 inhibition**

Concentrations of NDD-713.HCl and -825.HCl (0.25–20 μM) were incubated in triplicate (37°C for 4–40 min) with
**TABLE 1. Affinity and selectivity of existing clinically used and novel β-AR ligands**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Human β₁-AR</th>
<th>Human β₂-AR</th>
<th>Human β₁ vs. β₂ selectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Log (K_d)</td>
<td>(n)</td>
<td>Log (K_d)</td>
</tr>
<tr>
<td>Bisoprolol</td>
<td>-7.96 ± 0.03</td>
<td>16</td>
<td>-6.33 ± 0.03</td>
</tr>
<tr>
<td>Carvedilol</td>
<td>-9.19 ± 0.04</td>
<td>10</td>
<td>-9.83 ± 0.06</td>
</tr>
<tr>
<td>CGP20712A</td>
<td>-8.76 ± 0.03</td>
<td>94</td>
<td>-5.62 ± 0.02</td>
</tr>
<tr>
<td>ICI118551</td>
<td>-6.66 ± 0.02</td>
<td>88</td>
<td>-9.17 ± 0.02</td>
</tr>
<tr>
<td>LK204-545</td>
<td>-8.17 ± 0.07</td>
<td>15</td>
<td>-5.30 ± 0.04</td>
</tr>
<tr>
<td>NDD-713</td>
<td>-7.82 ± 0.03</td>
<td>28</td>
<td>-5.05 ± 0.03</td>
</tr>
<tr>
<td>NDD-713.HCl</td>
<td>-7.00 ± 0.04</td>
<td>24</td>
<td>-5.01 ± 0.04</td>
</tr>
<tr>
<td>NDD-825</td>
<td>-8.28 ± 0.05</td>
<td>20</td>
<td>-5.27 ± 0.03</td>
</tr>
<tr>
<td>NDD-825.HCl</td>
<td>-8.17 ± 0.03</td>
<td>21</td>
<td>-5.34 ± 0.05</td>
</tr>
</tbody>
</table>

**TABLE 2. Affinity and selectivity of existing clinically used and novel β-AR ligands as determined from antagonism of cimaterol-induced \(^3\)H-cAMP accumulation in CHO-β₁, CHO-β₂, or CHO-β₃ cells**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Human β₁-AR</th>
<th>Human β₂-AR</th>
<th>Human β₃-AR</th>
<th>(β_1) vs. (β_2) selectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Log (K_d)</td>
<td>(n)</td>
<td>Log (K_d)</td>
<td>(K_{dβ2}/K_{dβ1})</td>
</tr>
<tr>
<td>Bisoprolol</td>
<td>-8.60 ± 0.03</td>
<td>15</td>
<td>-6.76 ± 0.03</td>
<td>13</td>
</tr>
<tr>
<td>Carvedilol</td>
<td>-9.75 ± 0.12</td>
<td>12</td>
<td>-10.62 ± 0.08</td>
<td>4</td>
</tr>
<tr>
<td>Nebivolol</td>
<td>-9.28 ± 0.08</td>
<td>22</td>
<td>-7.91 ± 0.09</td>
<td>11</td>
</tr>
<tr>
<td>CGP20712A</td>
<td>-9.64 ± 0.03</td>
<td>15</td>
<td>-6.02 ± 0.03</td>
<td>8</td>
</tr>
<tr>
<td>NDD-713</td>
<td>-8.52 ± 0.03</td>
<td>12</td>
<td>-5.44 ± 0.03</td>
<td>4</td>
</tr>
<tr>
<td>NDD-825</td>
<td>-8.99 ± 0.05</td>
<td>9</td>
<td>-5.75 ± 0.06</td>
<td>4</td>
</tr>
</tbody>
</table>

The Schild slope is that obtained in CHO-β₁ cells from the rightward shift of the cimaterol dose-response curve by 3 different concentrations of antagonist in the same experiment (e.g., Fig 3) where a value of 1 represents competitive antagonism. The affinity of most ligands at β₁-AR and β₂-AR was too low to allow a Schild plot to be constructed. Values shown are means ± SEM of \(n\) separate experiments.
incubated at 37°C for 1 h. Aliquots of the spiked matrix were spun (223,000 g, Beckman rotor type 42.2Ti; Beckman Coulter, Brea, CA, USA) for 4.2 h at 37°C to separate proteins. Noncentrifuged control samples were maintained at 37°C. After ultracentrifugation, supernatant was stored frozen, together with noncentrifuged samples at −20°C. NDD-713.HCl and -825.HCl concentrations of noncentrifuged matrix and protein-free supernatant were determined by UPLC-MS relative to calibration standards prepared in the respective matrices. Total concentration (C_total) was the average measured concentration in centrifuged samples maintained at 37°C, and unbound concentration (C_unbound) was the average measured concentration in protein-free supernatants from centrifuged samples (n = 3). The compound unbound fraction (f_u) was calculated: f_u = C_unbound / C_total. Compound degradation was assessed by comparing concentrations in noncentrifuged plasma samples that were incubated at 37°C with concentrations measured in the t = 0 noncentrifuged samples. Plasma protein binding values were estimated by correlation of their chromatographic retention properties on human albumin columns against those of standard compounds with known protein binding values as previously described (50). Studies were carried out by CDCO.

PK analysis

NDD-713.HCl and -825.HCl were intravenously administered after overnight food withdrawal to male Sprague-Dawley rats (weighing 241–289 g) at 2 or 6 mg/kg (formulated at 1 mg/ml in aqueous vehicle that contained 5% DMSO and 5% glucose) or 20 mg/kg (formulated at 5.3 mg/ml in aqueous vehicle that contained 5% DMSO in 0.2 M Captisol; Ligand Pharmaceuticals, Inc., San Diego, CA, USA) as a 10-min constant-rate infusion (1 ml/rat, 3 rats/dose level) and orally at 2, 10, or 20 mg/kg (formulated at 0.4–2.3 mg/ml in aqueous vehicle that contained 0.5% hydroxyethylcellulose, 0.5% benzyl alcohol, and 0.4% Tween-80) by gavage (1.0 ml/rat, 3 rats/compound). Arterial blood was collected for 24 h directly into borosilicate vials (at 4°C) that contained heparin, Complete (a protease inhibitor cocktail; Sigma-Aldrich, St. Louis, MO, USA), potassium fluoride, and EDTA to minimize potential ex vivo degradation of compounds in blood and plasma samples. Blood samples were centrifuged, supernatant plasma was removed, and plasma concentrations of compounds were determined by LC-MS. Bioavailability was calculated relative to the mean AUC value for each compound observed after intravenous dosing at 2 mg/kg. Studies were carried out by CDCO.

In vivo pharmacology

Adult male Sprague-Dawley rats, weighing 300–400 g, were chronically instrumented with pulsed Doppler flow probes and intravascular catheters for cardiovascular monitoring and drug administration in a 2-stage surgical protocol as described (51). Surgery was performed under general anesthesia (fentanyl and medetomidine, 300 μg/kg of each, i.p.), with reversal and postoperative analgesia provided by atipamezole (1 mg/kg, s.c.) and buprenorphine (0.02 mg/kg, s.c.). Experiments were performed with animals that were fully conscious and unrestrained in home cages and given free access to food and water. Cardiovascular variables were recorded by using a customized, computer-based system (Instrument Development Engineering Evaluation, Maastricht Instruments Bv, Maastricht, The Netherlands) with raw data sampled every 2 ms. Hindquarters vascular conductance (HQC) was calculated as Doppler shift (flow)/mean arterial pressure. Throughout, atropine methyl nitrate (1 mg/kg/h) was infused (0.4 ml/h) continuously to inhibit vagally induced HR changes.

Intravenous administration of β-blocker

Starting at least 1 h after atropine infusion began, rats were intravenously administered 3 doses of isoprenaline [12, 40, and 120 ng/kg/min, 3-min infusions (0.15 ml/min), 20–30 min between doses], before a β-blocker was administered as an intravenous bolus (2 mg/kg, 0.1 ml, except CGP20712A, which was 200 μg/kg; in saline) that was maintained by continuous infusion for 90 min (1 mg/kg/h, 0.4 ml/h, except CGP20712A, which was 100 μg/kg/h). Isoprenaline infusions were repeated during the
were grown in accordance with their guidelines by using DMEM. Cells at a density of 30,000 cells/well in 96-well plates were incubated overnight (37°C, 5% CO₂ atmosphere). Medium was then removed and replaced with DMEM that contained control or compounds (1, 10, and 100 μM; n = 2 per concentration) for 24 h before addition of 100 μl/well of CellTiter-Glo Reagent (Promega, Madison, WI, USA) for ATP measurement and shaken to induce cell lysis. Luminescence was quantified on an AnalystAD plate-reader (Molecular Devices, Sunnyvale, CA, USA). Thiouridine (known HepG2 cytotoxicity) was used as positive control. Study was carried out by BioFocus DPI (Safron Walden, United Kingdom).

**hERG inhibition**

Binding to the hERG potassium channel (recombinant protein from HEK293 cells) was examined by using ³H-astemizole radioligand binding assays [Cerep (53)] by measuring the displacement of radioligand binding from the channel by 10 μM NDD-713 and -825. In addition, the potential of the compound to inhibit hERG channels was tested by using an automated patch-clamp electrophysiological assay (BioFocus, Cambridge, United Kingdom; using the Precision hERG-HEK recombinant cell line from on an Ion Works Quattro automated electrophysiology platform; Millipore, Billerica, MA, USA). The antiarrhythmic research drug E4031 was used as positive control (IC₅₀ = 83 nM).

**Ames genotoxicity**

Ames fluctuation assays were performed by using *Salmonella typhimurium* strains TA98, TA100, TA1535, and TA1537 to detect frame-shifts and base substitutions that led to missense mutations in liquid culture in 384-well plates. Bacterial plates were incubated with serially diluted NDD-713 and -825 solutions for 96 h, and bacterial growth was measured spectrophotometrically by using a pH color-change indicator in response to bacterial growth acidification. Assays were conducted with or without metabolic activation via addition of rat liver SV fraction. Bacteriocidal or bacteriostatic effects were assessed by using a bacterial cytotoxicity assay that was conducted in parallel with Ames fluctuation assays. Reference compounds were 2-aminoanthracene, 9-aminoacridine, quercetin, streptozotocin (genotoxicity), and mitomycin C (bacterial cytotoxicity). Study was carried out by Cerep (Celle L’Evescault, France).

**Seven-day oral dose range rat toxicity study**

Male Cr:CD rats (9 wk old, weighing 316.8–349.4 g on d 1; Charles River) were administered 1 oral dose of NDD-825.HCl in water that contained 0.5% (w/v) hydroxypropylmethylcellulose K15M, 0.5% (v/v) benzyl alcohol, and 0.4% (v/v) Tween-80 at 20, 90, or 300 mg/kg at a dose volume of 10 ml/kg for 7 consecutive days (4 animals per group). Blood samples were collected before and at time points during the 24 h that followed for bioanalysis. Animals were monitored for adverse clinical signs and body weight. Terminal blood samples were taken for clinical chemistr, and organs were harvested for microscopic examination (see Supplemental Data for full details). Three additional male rats per group received NDD-825.HCl on d 1 only to collect blood samples for plasma bioanalysis of compound toxicokinetics. NDD-825 was quantifiable in plasma of all animals up to 8 h after dosing. Systemic exposure to NDD-825 in terms of Cmax increased in approximate proportion with increasing dose, whereas for the same dose increase, AUC₀₉ increased in a supraproportional
manner across the entire dose range. Overall, for a 15-fold increase in dose from 20 to 300 mg/kg/d, AUC0- and Cmax increased 31.4- and 12.4-fold, respectively. After repeat daily oral administration of NDD-825 for 7 d, systemic exposure in terms of both Cmax and AUC0- was comparable to that on d 1 at each respective dose level. Study was carried out by Aptuit (Verona, Italy).

Calculation of selectivity indices

Selectivity indices (SIs) for bisoprolol in terms of receptor binding (SI1; Supplemental Eq. 1) and receptor occupancy (SI2; Supplemental Eq. 2) were determined on the basis of the following parameters: Kd values at the human βARs from 3H-CGP12177 whole-cell binding (Table 1) for S11a; Kd,β values from antagonism of cimaterol-induced 3H-cAMP accumulation (Table 2) for S11b; and Cssf at 10 mg/d p.o.) = 87.3 nM (23). S2 values for NDD-713 and -825 were estimated similarly (using S11a data) and assuming that as the bisoprolol dose (10 mg/d, p.o.) required to lower exercise-induced HR is 35-fold over Kd (bisoprolol Cssf/Kd,β1AR = 35) (23), a similar 35-fold-higher dose of NDD-713 and -825 would also be required. In vivo pharmacologic responses in humans (Eβ-AR) were calculated for both β-ARs by using Supplemental Eq. 3, where Eβ1-AR and Eβ2-AR refer to effects on exercise HR and on FEV1 under steady-state conditions after drug administration, respectively. Supplemental Eq. 3 is based (23, 54) on a ternary complex model (Supplemental Eqs. 4–6) of agonist [A], receptor [R], transducer element [T], antagonist [B], total receptor [R0], and transducer [T0] concentrations are based on Supplemental Eqs. 7 and 8. The following values (23, 54) for β1-AR

Figure 3. 3H-cAMP accumulation in response to cimaterol in the absence or presence of bisoprolol (A), NDD-713 (B), and NDD-825 (C) in CHO-β1 and CHO-β2 cells. Bars represent basal 3H-cAMP accumulation and accumulation in response to 10 μM isoprenaline or β-blocker alone. Data points are means ± SEM of triplicate determinations, and these single experiments are representative of 5 (A), 4 (B), and 3 (C) separate experiments in each case.
TABLE 3. Affinity values obtained for ligands in human bronchial smooth muscle cells (that natively express β2-AR) and in CHO-β2 cells that stably express a transfected human β2-AR determined from antagonism of cimaterol-induced 3H-cAMP accumulation

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>Human bronchial smooth muscle cells</th>
<th>CHO-β2 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Log $K_d$ (nM)</td>
<td>n</td>
</tr>
<tr>
<td>CGP20712A</td>
<td>$-6.06 \pm 0.18$</td>
<td>4</td>
</tr>
<tr>
<td>ICI118551</td>
<td>$-6.06 \pm 0.13$</td>
<td>4</td>
</tr>
<tr>
<td>NDD-713</td>
<td>$-5.47 \pm 0.05$</td>
<td>4</td>
</tr>
<tr>
<td>NDD-825</td>
<td>$-5.87 \pm 0.11$</td>
<td>4</td>
</tr>
</tbody>
</table>

Log $K_d$ values shown are means ± SEM of n separate experiments.

RESULTS

As LK204-545 is a β1-selective ligand, we started with des-cyano analogs of LK204-545 (55), and after many iterations of multiparameter lead optimization, NDD-713 and -825 were the two compounds with the best overall performance (Fig. 1) (43).

Molecular pharmacology of NDD-713 and -825

Affinity and duration of action

Ligand affinity for human β1- and β2-AR was assessed first by radioligand binding (Fig. 2). CGP20712A was a highly β1-selective ligand (1380-fold β1-selective) and ICI118551 a highly β2-selective ligand (333-fold β2-selective), whereas current clinically used β-blockers had relatively poor selectivity in comparison (Fig. 2 and Table 1). NDD-713 and -825 both had high β1-affinity but poor β2-affinity (Fig. 2 and Table 1). At human β3-AR, NDD-713 and -825 affinity was so low that accurate measurements were not possible. Affinities of compounds for rat β1- and β2-AR were also determined by using transiently transfected cells (Table 1).

Ligand affinity was also assessed in a functional assay by examining the ability of ligands to inhibit cimaterol-induced 3H-cAMP accumulation responses (Fig. 3 and Table 2). Cimaterol is a stable β-agonist—less susceptible to degradation over 5 h at 37°C than adrenaline or noradrenaline—that is active across all 3 β-AR subtypes and that activates high-affinity catecholamine conformation of the β1-AR (42, 55). NDD-713 and -825 readily inhibited β1-mediated cimaterol-induced responses but not β2- or β3-mediated responses, which confirmed high human β1-affinity and high β1-selectivity over β2- and β3-ARs.

Finally, the ligand affinity for human β2-AR was also determined in the more native environment of human bronchial smooth muscle cells. Here, cimaterol-induced 3H-cAMP was readily inhibited with ICI118551 but poorly inhibited by CGP20712A, NDD-713, and -825 (Table 3). Thus, NDD-713 and -825 had poor affinity for native β2-ARs in these airway cells.

To assess whether β1-AR—agonist interactions were competitive, Schild plots (dose ratio 1 vs. ligand were used for the analysis: $E_{\beta_1 AR, max} = 35\%$, $K_{DA} = 2 \mu M$, and $[A] = 0.22 \mu M$. For β2-AR, we used $E_{\beta_2 AR, max} = 50\%$, $K_{DA} = 3 \mu M$, and $[A] = 0.01 \mu M$. The values (23, 54) for the analysis of exercise HR were $K_{DAR} = 0.64 \mu M$, $[R] = 0.29 \mu M$, and $[T_0] = 700 \mu M$. The corresponding values (23, 54) for the analysis of FEV1 were $K_{DAR} = 2.6 \mu M$, $[R_0] = 1.1 \mu M$, and $[T_0] = 2.6 \mu M$, respectively. $S$ values in terms of in vivo human pharmacologic response were calculated using Supplemental Eq. 9.

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Inhibition of specific 3H-CGP12177 whole-cell binding in CHO-β1 cells in response to bisoprolol (A), NDD-713 (B), and -825 (C), with the control curve (solid circles) and after washout of the competing β-blocker (open circles) as described in Materials and Methods. Nonspecific binding was determined by 10 μM propranolol. Data points are means ± SEM of triplicate determinations, and the concentrations of 3H-CGP12177 present in these experiments were 0.81 nM (A), 0.96 nM (B), and 0.74 nM (C). These single experiments are representative of 4 (A), 9 (B), and 8 (C) separate experiments.
concentration) (56) were constructed from experiments with 3 different concentrations of antagonists (Fig. 3). A slope value of 1 represents competitive antagonism and this was observed with all antagonists (Table 2). The affinity of NDD-713 and -825 was too low for this to be determined at $b_2$- and $b_3$-ARs.

An indication of duration of receptor occupancy was sought by using a washout technique (44) where ligand residence time is indicated by a rightward shift of the washout curve relative to control (Fig. 4 and Table 4). Carvedilol had a long residence time (control and washout curves are similar; log shift of 0.3 = 2-fold), which indicated no $b_1$-AR dissociation. In contrast, bisoprolol was readily washed out (rightward log shift of 2.7; 501-fold), which indicated significant ligand dissociation. NDD-713 and -825 had intermediate residence times (Fig. 4 and Table 4).

**Intrinsic efficacy**

Ligand efficacy was initially assessed at the primary Gs-cAMP pathway. Carvedilol and nebivolol were weak partial $b_1$-AR agonists, stimulating maximum responses of $15.2 \pm 1.0\%$ ($n = 20$) and $5.0 \pm 0.8\%$ ($n = 4$), respectively, of that with isoprenaline (similar to previous reports) (51). LK204-545 stimulated a response that was $70.1 \pm 2.5\%$ ($n = 3$) that of isoprenaline. No $^3$H-cAMP responses were observed with bisoprolol, CGP20712A, NDD-713, or -825 (Fig. 5A). In other signaling pathways, carvedilol and LK204-545 stimulated CRE-SPAP production (a sensitive readout of both cAMP and ERK1/2 MAPK) that was $31.2 \pm 2.8$ and $79.3 \pm 2.7\%$ ($n = 5$), respectively of the isoprenaline maximum, whereas no responses were observed with bisoprolol, CGP20712A, nebivolol, NDD-713, or -825 (Fig. 5B). Similarly, no agonist responses were observed when directly measuring ERK1/2 phosphorylation (Fig. 5C).

Previous studies that examined responses in the parent cell line CHO-CRE-SPAP cells (i.e., cells without the transfected receptor) have demonstrated that there is no endogenous $\beta$-AR present in these cells. There were no

<table>
<thead>
<tr>
<th>Compound</th>
<th>Log shift</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bisoprolol</td>
<td>2.70 ± 0.18</td>
<td>4</td>
</tr>
<tr>
<td>Carvedilol</td>
<td>0.31 ± 0.06</td>
<td>5</td>
</tr>
<tr>
<td>CGP20712A</td>
<td>1.35 ± 0.04</td>
<td>28</td>
</tr>
<tr>
<td>ICI118551</td>
<td>1.64 ± 0.07</td>
<td>24</td>
</tr>
<tr>
<td>Nebivolol</td>
<td>0.26 ± 0.07</td>
<td>3</td>
</tr>
<tr>
<td>LK204-545</td>
<td>2.06 ± 0.08</td>
<td>6</td>
</tr>
<tr>
<td>NDD-713</td>
<td>1.75 ± 0.08</td>
<td>9</td>
</tr>
<tr>
<td>NDD-825</td>
<td>1.62 ± 0.11</td>
<td>8</td>
</tr>
</tbody>
</table>

The log shift given is the measure of the rightward shift of the washout curve compared with the control curve examined in parallel in each experiment as seen in Fig. 4. A greater shift represents shorter duration ligands, and smaller shifts represent longer duration ligands. Values shown are means ± SEM of $n$ separate experiments ($n$).

![Figure 5](https://example.com/fig5.png)

**Figure 5.** A) $^3$H-cAMP accumulation in CHO-$\beta_1$ cells in response to NDD-713 and -825. Bars represent basal $^3$H-cAMP accumulation, in response to 10 $\mu$M isoprenaline alone, and in response to 10 $\mu$M carvedilol alone. Data points are means ± SEM of replicate determinations, and these single experiments are representative of 8 separate experiments in each case. B) CRE-SPAP production in CHO-$\beta_1$ cells in response to NDD-713 and -825. Bars represent basal CRE-SPAP production, in response to 10 $\mu$M isoprenaline alone, and in response to 10 $\mu$M carvedilol alone. Data points are means ± SEM of replicate determinations and are representative of 5 separate experiments in each case. C) Basal and ERK1/2 MAPK activation in CHO-$\beta_1$ cells in response to phorbol ester, carvedilol, NDD-713, and -825 (5 min incubation). Bars are means ± SEM of replicate determinations. This single experiment is representative of 4 separate experiments.
3H-cAMP accumulation or CRE-SPAP gene transcription responses to a wide range of different ligands (45, 47).

**Other GPCR and ion channel effects**

To assess potential off-target effects, CGP20712A, LK204-545, NDD-713, and -825 binding was assessed by using the Cerep *in vitro* pharmacology profiling service in a diverse panel of 80 receptors and ion channels (Fig. 6). Considerably more binding to other targets (off-target effects) was observed with CGP20712A and LK204-545 compared with NDD-713 or -825. Where NDD-713 or -825 receptor binding inhibition exceeded 50%, concentration-response experiments were conducted. $K_i$ values obtained were as follows: NDD-713: $K_i$ (5-HT2A),

![Figure 6](https://www.fasebj.org)

**Figure 6.** Receptor selectivity of CGP20712A (at 10 μM; A), LK204-545 (at 10 μM; B), NDD-713 (at 4.8 μM; C), and -825 (at 2.4 μM; D). The compounds were assayed against the receptors listed in duplicate and average values are shown. Values for β-ARs are shown as black circles, those for receptors with >50% inhibition as red circles, those in the range of 30–50% inhibition as orange circles, and those with <30% inhibition as open circles. GPCRs: adenosine (A1, A2, A3), adrenoceptor (α1, α2, β1, β2), angiotensin (AT1, AT2), benzodiazepine, central and peripheral (cBZD, pBZD), bombesin (BB), bradykinin (B2), chemokine (CGRP), CXC, CCR1, cannabinoid (CB1), cholecystokinin (CCK1, CCK2), cytokine (TNF-α), dopamine (D1, D1S, D3, D4, D5), endothelin (ETA, ETB), GABA, galanin (GAL1, GAL2), histamine (H1, H2), melancortin (MC4), motilin (MT1), muscarinic acetylcholine (M1, M2, M3, M4, M5), neurokinin (NK1, NK2, NK3), neuropeptide Y (Y1, Y2), neurotensin (NTS1), nociception (NOP), opioid (DOP, KOP, MOP), prostaglandin (EP2, EP4, IP), pituitary adenylate cyclase-activating polypeptide (PAC1), purinergic (P2X, P2Y), serotonergic (5-HT1A, 5-HT1B, 5-HT2A, 5-HT2B, 5-HT2C, 5-HT3, 5-HT5A, 5-HT-6), σ (σ, SCP), somatostatin (SST), thromboxane (TP), vasoactive intestinal polypeptide (VIPC1), vasopressin (VIA). Ion channels: calcium channel L verapamil site (Ca2+ Ch), potassium channel (KV Ch), sodium channels (SKCa Ch, hERG membrane preparation, Na+ Ch site 2), chloride channel (Cl– Ch GABA-gated), Tyr kinase receptor (PDGF). Nuclear hormone receptors: glucocorticoid (GR), peroxisome proliferator (PPARy). Monoamine transporter: noradrenaline (NE Tr), dopamine (D Tr), serotonin (5-HT Tr).
In vitro absorption, distribution, metabolism and elimination of NDD-713 and -825

In vitro disposition properties of NDD-713 and -825 were assessed in terms of solubility, lipophilicity, protein binding, cell permeability, metabolic stability, and P450 metabolism (Table 5). Both compounds are basic, display good pH-dependent aqueous solubility, and have comparatively low lipophilicity, plasma binding, and intrinsic clearance in both rat and human liver microsome and hepatocyte preparations. NDD-713 showed high and NDD-825 intermediate apical-to-basolateral membrane permeability in the bidirectional CaCo-2 assay, without pronounced efflux of either compound. NDD-713 was metabolized predominantly by CYP3A4 and CYP2A6 isoforms, whereas NDD-825 was metabolized mainly by CYP3A4 and CYP2D6 isoforms. Neither compound inhibited the activity of a panel of P450 enzymes.

In vivo pharmacology of NDD-713 and -825

PK analysis

As the in vitro properties of NDD-713 and -825 (receptor affinity and disposition properties) were similar to those of bisoprolol (57), similar dosing levels were administered to rats. After a single dose of 20 mg/kg, p.o., the plasma \( f_u \) level of bisoprolol exceeded the \( \beta_2 \)-AR \( K_d \) value for >5 h, whereas those of NDD-713 and -825 never reached the \( \beta_2 \)-AR \( K_d \) (Fig. 7). PK analysis showed that bisoprolol was a short-acting agent, whereas NDD-825, in particular, revealed slow oral absorption and elimination alongside a large volume of distribution and, thus, was present at therapeutic concentrations for considerably longer (Fig. 7 and Tables 5 and 6).

In vivo \( \beta_1 \)- vs. \( \beta_2 \)-selectivity—intravenous studies

To assess \( \beta_1 \)-selectivity in vivo, compounds were administered to conscious rats, and HR (\( \beta_1 \)) and HQC (\( \beta_2 \)) were monitored (51). Infusion of increasing concentrations of the nonselective \( \beta \)-AR agonist isoprenaline resulted in dose-dependent increases of HR and HQC (Fig. 8). Infusion of CGP20712A suppressed both basal and isoprenaline-stimulated HR, with no significant reduction of HQC, whereas ICI18551 suppressed a isoprenaline-induced increase in HQC but not HR (Fig. 8). Bisoprolol (moderate \( \beta_1 \)-selectivity at rat \( \beta \)-ARs and low \( \beta_1 \)-selectivity at human \( \beta \)-ARs; Table 1) reduced basal and isoprenaline-induced HR responses but also suppressed the HQC response. NDD-713 and -825 suppressed basal and isoprenaline-induced HR but had no effect on HQC responses, which confirmed their high \( \beta_1 \)-selectivity. These effects were still visible at 4.0–5.5 h (3 h after i.v. infusion finished) and suppression of basal activity was also observed at 24–25 h (23–24 h after intravenous infusion finished), which was in keeping with the longer PK clearance of NDD-713 and -825.

In vivo \( \beta_1 \)- vs. \( \beta_2 \)-selectivity—oral studies

NDD-825 was administered by oral gavage (Fig. 9). At 1 mg/kg, NDD-825 caused a small but significant lowering of basal HR. This was more pronounced on

### Table 5. In vitro disposition properties of NDD-713 and -825

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Assay</th>
<th>Units</th>
<th>NDD-713</th>
<th>NDD-825</th>
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<tr>
<td>Lipophilicity</td>
<td>Distribution coefficient, pH 3</td>
<td>gLogD&lt;sub&gt;pH 3&lt;/sub&gt;</td>
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<td>1.5</td>
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<td>Distribution coefficient, pH 7.4</td>
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<td>Solubility&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>&gt;100</td>
<td>&gt;100</td>
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<tr>
<td></td>
<td>Aqueous, at pH 6.5</td>
<td>S&lt;sub&gt;aq&lt;/sub&gt; (μg/ml)</td>
<td>25–50</td>
<td>50–100</td>
</tr>
<tr>
<td>Permeability&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Bidirectional CaCo-2</td>
<td>P&lt;sub&gt;aq, A-B&lt;/sub&gt; (10&lt;sup&gt;−6&lt;/sup&gt; cm/s)</td>
<td>25 ± 4</td>
<td>6.5 ± 0.5</td>
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<tr>
<td></td>
<td>Mass balance</td>
<td>A-B (%)</td>
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<td>79 ± 11</td>
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<td>Protein binding</td>
<td>Plasma protein</td>
<td>PPB (%)</td>
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<td>Human plasma</td>
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<tr>
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<td>Rat plasma</td>
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<td>0.16</td>
<td>0.11</td>
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<td>Human liver microsomes</td>
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<td>0.67</td>
<td>0.63</td>
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<td>Metabolic stability</td>
<td>Human liver microsomes&lt;sup&gt;b&lt;/sup&gt;</td>
<td>CL&lt;sub&gt;rat&lt;/sub&gt; (μl/min/mg)</td>
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<td></td>
<td>Rat liver microsomes&lt;sup&gt;b&lt;/sup&gt;</td>
<td>CL&lt;sub&gt;rat&lt;/sub&gt; (μl/min/mg)</td>
<td>&lt;0.1</td>
<td>0.18 ± 0.2</td>
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<td>Human hepatocytes&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>&lt;6</td>
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<td></td>
<td>Rat hepatocytes&lt;sup&gt;b&lt;/sup&gt;</td>
<td>CL&lt;sub&gt;rat&lt;/sub&gt; (μl/min/10&lt;sup&gt;6&lt;/sup&gt; cells)</td>
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<td>Rat liver cytosolic fraction&lt;sup&gt;b&lt;/sup&gt;</td>
<td>CL&lt;sub&gt;rat&lt;/sub&gt; (μl/min/mg)</td>
<td>&lt;1.4</td>
<td>&lt;1.4–1.7</td>
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<td>Metabolism&lt;sup&gt;a&lt;/sup&gt;</td>
<td>P450 in vitro clearance&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>P450 inhibition&lt;sup&gt;b&lt;/sup&gt;</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; (μM)</td>
<td>&gt;20</td>
<td>&gt;20</td>
</tr>
</tbody>
</table>

Values and ranges quoted are averages of 2 (or 3 where means ± SD are shown) separate determinations. *The hydrochloride salts of NDD-713 and -825 were used. **In the presence of NAD<sup>+</sup>, NADPH, or in the absence of cofactors. *Assayed: CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP2A6, CYP3A4, CYP2D6, and CYP2E1. **NDD-713 and -825 were not observed to be metabolized by other P450 isoforms assayed. *CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP3A4/5.
subsequent days (at doses of 3 and 10 mg/kg), as was the reduction in isoprenaline-stimulated HR. No significant changes in HQC responses to isoprenaline were recorded at any dose level.

Safety pharmacology and 7-d toxicology study

NDD-713 and -825 showed no cytotoxic effects in HepG2 cell viability assays even at the limits of aqueous solubility. They had no affinity for the hERG channel (at concentrations >3000-fold β1-AR affinity) and only blocked the hERG potassium current at high concentrations (IC50 > 10 μM). Neither compound displayed genotoxicity at concentrations of ≤10 μM in Ames tests. In the 7-d repeat-dose oral rat toxicology study, NDD-825 was well tolerated at the highest dose tested (300 mg/kg once daily) without any macro- or microscopic clinical changes at autopsy. The most significant change was a dose-related increase in triglycerides at the top dose, which is a well-recognized effect of β-blockers (58); however, the maximum tolerated dose was not achieved and is therefore >300 mg/kg daily.

DISCUSSION

β-Blockers are important for reducing mortality in cardiovascular disease. Although clinical studies have suggested that β-blockers are associated with a reduction in lung function in the general population (14), β-blockers are well tolerated by most people. However, current clinical β-blockers are poorly β1-selective and risk bronchospasm and the loss of effectiveness of β2-agonist rescue (13), and, thus, are contraindicated in patients with asthma (25). This poor selectivity also underlies safety concerns about β-blockers in two other cardiovascular high-risk groups: patients with COPD (bronchospasm) and those with peripheral vascular disease (β2-mediated vasoconstriction).

CGP20712A and LK204-545 are highly β1-selective β-blockers, but neither were developed into clinical drugs. CGP20712A interacts with many other receptors (Fig. 6), and LK204-545 has significant partial agonism, which increases HR in rats (42). As there are currently no highly β1-selective ligands without these problematic properties, we aimed to develop these novel ligands.

NDD-713 and -825 had high affinity for human β1-AR and low affinity for human β2- and β3-ARs in two direct measures of affinity, which gave high β1-selectivity compared with clinical β-blockers. NDD-713 and -825 also had longer durations of action at β1-AR than did bisoprolol, although these were shorter than those of nebivolol and carvedilol. CGP20712A and LK204-545 bound to a significant number of other targets, whereas NDD-713 and -825 had poor affinity for most other GPCRs and ion channels. The highest off-target affinity was for the 5-HT2A GPCR for which β1-selectivity over the 5-HT2A receptor was 510-fold for NDD-713 and 1115-fold for NDD-825. Unlike NDD-825, -713 also had some affinity for the noradrenaline transporter (β1-AR vs. noradrenaline transporter selectivity was 185-fold) and the dopamine transporter (β1-AR vs. dopamine transporter selectivity was 53-fold); therefore, NDD-825 has a clean off-target profile.

Having established high β1-selectivity in vitro, we investigated in vivo selectivity by using the conscious, freely moving rat model that was previously used to distinguish β1- and β2-selective compounds (51). Here, HR responses are purely β1 mediated, whereas HQC responses are purely β2 mediated. Intravenous NDD-713 and -825 both reduced basal and isoprenaline-stimulated HR but...
did not reduce HQC (Fig. 8), which suggested no blockade of rat vascular β2-ARs. As this may be considered a surrogate marker for β2-AR interaction in airways, NDD-713 and -825 affinity was also determined in human bronchial smooth muscle cells, the main target of β2-agonist therapy in asthma and COPD. Here, NDD-713 and -825 affinity for native human β2-AR was poor. Overall, these studies confirmed the highly β1-selective natures of NDD-713 and -825.

Previous studies have shown that the degree of partial agonism observed in CHO-β1 cells directly reflects the amount of β1-mediated partial agonism (increase in HR) observed in rats (51). Thus, xamoterol and bucindolol, with substantial β1-mediated partial agonism, stimulate HR, but carvedilol (lower partial agonism in CHO-β1 cells) and bisoprolol (no partial agonism) reduce HR (51). Of the 4 β-blockers that similarly reduce mortality in heart failure (7), carvedilol has the highest degree of partial agonism, and bisoprolol and metoprolol have no agonism or bias agonism at all. Thus, intrinsic activity that is low, equal to, or (ideally) below that of carvedilol is required, but the presence or absence of bias is not important. In CHO-β1 cells, NDD-713 and -825 did not stimulate any agonist response at cAMP, CRE-gene transcription (a sensitive readout of both cAMP and ERK1/2 MAPK), or by directly measuring ERK1/2 MAPK, which suggests no agonism or biased agonism. In rats, NDD-713 and -825 reduced both basal and isoprenaline-stimulated HR, which once again confirmed no partial agonism (ISA). Both in vitro and in vivo studies, therefore, demonstrate that NDD-825 and -713 are neutral antagonists and, thus, have an efficacy profile similar to that of bisoprolol, metoprolol, and CGP20712A, rather than carvedilol.

To assess pharmacologic activity by the oral route, NDD-713, -825 and bisoprolol were administered by oral gavage to lightly sedated rats and measurements taken once rats were fully recovered. All 3 compounds caused a reduction of basal and isoprenaline-stimulated HR, which confirmed good oral bioavailability. For NDD-825, where multiple different doses were administered, the reduction in basal and isoprenaline-stimulated HR was dose dependent, and a reduction in basal HR was observed the following day, which suggested a long duration of action.

This longer duration of action (compared with bisoprolol) was also observed in rat PK studies. After 20 mg/kg, p.o., plasma t1/2, of bisoprolol exceeded the β2-AR KD value for >5 h (Fig. 7). Bisoprolol-mediated β2-AR blockade would likely be manifest for 5 h and the β1-AR-mediated effects would probably last for ~8 h. PK analysis of NDD-713 and -825 (20 mg/kg p.o.; Tables 5 and 6) revealed slow oral absorption and elimination and large volumes of distribution; therefore, they are likely to block β1-AR for much longer periods (i.e., compare 10 h for NDD-713 and >24 h for NDD-825). Of importance, unbound plasma level for both compounds never reached the respective β2-AR KD values at any time, and even at Cmax were at least 5-fold below β2-AR KD, which suggests that β2-AR blockade by NDD-713 or -825 is unlikely.

Drug metabolism and PK properties suggest that NDD-713 and -825 have excellent characteristics that lead to extensive exposure at comparatively low doses, similar to those of bisoprolol but with significantly slower clearance and elimination. In addition, no safety problems were identified, with no cytotoxic, genotoxic, or hERG binding identified, and no problematic clinical chemistry, hematology, microscopic, or macroscopy organ changes identified (7-d dosing, up to 300 mg/kg/d). NDD-713 and -825, therefore, seem to be safe to administer to rats, even at high doses.

Finally, to understand why such a high degree of β1-α vs. β2-selectivity is required to discriminate effects on

### TABLE 6. PK properties of NDD-713 and -825 in rat after single bolus i.v. or p.o. administration (hydrochloride salt forms)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>2 mg/kg NDD-713</th>
<th>2 mg/kg NDD-825</th>
<th>6 mg/kg NDD-713</th>
<th>6 mg/kg NDD-825</th>
<th>20 mg/kg NDD-713</th>
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<tbody>
<tr>
<td>t1/2_app (h)</td>
<td>1.7 ± 0.2</td>
<td>5.3 ± 0.3</td>
<td>1.6 ± 0.1</td>
<td>7.3 ± 0.1</td>
<td>2.0 ± 0</td>
<td>7.1 ± 1.3</td>
</tr>
<tr>
<td>Plasma CLtotal (ml/min/kg)</td>
<td>62 ± 4</td>
<td>54 ± 2</td>
<td>43 ± 1</td>
<td>59 ± 1</td>
<td>51 ± 2</td>
<td>55 ± 1</td>
</tr>
<tr>
<td>Blood CLtotal (ml/min/kg)</td>
<td>51 ± 3</td>
<td>39 ± 1</td>
<td>36 ± 1</td>
<td>42 ± 1</td>
<td>42 ± 1</td>
<td>39 ± 1</td>
</tr>
<tr>
<td>Vss (L/kg)</td>
<td>4.3 ± 0.4</td>
<td>6.0 ± 0.5</td>
<td>3.6 ± 0.2</td>
<td>5.0 ± 0</td>
<td>4.2 ± 0.2</td>
<td>4.7 ± 0</td>
</tr>
<tr>
<td>AUC0-t (μM·h)</td>
<td>1.2 ± 0.1</td>
<td>1.4 ± 0</td>
<td>4.6 ± 0.3</td>
<td>3.8 ± 0.2</td>
<td>14.7 ± 1.0</td>
<td>12.1 ± 0.3</td>
</tr>
<tr>
<td>AUC0-t/dose [μM·h/(mg/kg)]</td>
<td>0.6 ± 0.1</td>
<td>0.6 ± 0</td>
<td>0.8 ± 0</td>
<td>0.6 ± 0</td>
<td>0.7 ± 0</td>
<td>0.6 ± 0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameter</th>
<th>2 mg/kg NDD-713</th>
<th>2 mg/kg NDD-825</th>
<th>10 mg/kg NDD-713</th>
<th>10 mg/kg NDD-825</th>
<th>20 mg/kg NDD-713</th>
<th>20 mg/kg NDD-825</th>
</tr>
</thead>
<tbody>
<tr>
<td>t1/2_app (h)</td>
<td>n.d.</td>
<td>9.8 ± 0.2</td>
<td>1.5 ± 0</td>
<td>4.6 ± 0.1</td>
<td>2.1 ± 0</td>
<td>3.3 ± 0.2</td>
</tr>
<tr>
<td>Cmax (μM)</td>
<td>0.2 ± 0.1</td>
<td>0.1 ± 0</td>
<td>2.1 ± 0.6</td>
<td>0.8 ± 0.1</td>
<td>2.0 ± 0.1</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>tmax (min)</td>
<td>30 ± 0</td>
<td>83 ± 70</td>
<td>45 ± 15</td>
<td>45 ± 25</td>
<td>45 ± 15</td>
<td>186 ± 64</td>
</tr>
<tr>
<td>AUC0-t (μM·h)</td>
<td>0.6 ± 0.2</td>
<td>0.4 ± 0.1</td>
<td>5.1 ± 0.2</td>
<td>3.0 ± 0.4</td>
<td>6.3 ± 0.3</td>
<td>11.9 ± 3.0</td>
</tr>
<tr>
<td>AUC0-t/dose [μM·h/(mg/kg)]</td>
<td>0.4 ± 0.1</td>
<td>0.2 ± 0</td>
<td>0.5 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>0.3 ± 0</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>F (%)</td>
<td>50 ± 11</td>
<td>32 ± 5</td>
<td>77 ± 3</td>
<td>45 ± 6</td>
<td>44 ± 1</td>
<td>89 ± 24</td>
</tr>
</tbody>
</table>

Values shown are means ± sd (n = 3). N.d., not determined.
Figure 8. Selectivity of various β-blockers in vivo. Responses to isoprenaline before and after administration of intravenous saline or β-blockers in conscious, atropine-treated, freely moving rats. Absolute values for HR and HQC were measured before (first value of each linked series) and at the end of 3-min infusions of isoprenaline (12, 40, and 120 ng/kg/min; remaining 3 values in each linked series). Isoprenaline was administered before (control, solid circles), during (open circles), 3–4 h after (solid triangles), and 23–24 h after (open triangles) the intravenous infusion of saline (0.1 ml/kg bolus; 0.4 ml/kg/h infusion; A), CGP20712A (200 μg/kg bolus; 100 μg/kg/h infusion; B), ICI118551 (2 mg/kg bolus; 1 mg/kg/h infusion; C), and bisoprolol (2 mg/kg bolus; 1 mg/kg/h infusion; D), NDD-713 (2 mg/kg bolus; 1 mg/kg/h infusion; E), and NDD-825 HCl (2 mg/kg bolus; 1 mg/kg/h infusion; F). Values are means ± SEM (n = 4). *P < 0.05 vs. baseline before β-blocker; #P < 0.05 vs. isoprenaline maximum value before β-blocker (Quade test).
cardiovascular and respiratory function, we calculated the SIs of NDD-713 and NDD-825 and compared these with those of bisoprolol. NDD-713 and NDD-825 have high β1-AR binding selectivity at the receptor level (SI1; Table 7). We assessed the relative in vivo receptor occupancies (Φ) of heart β1-AR and lung β2-AR as a function of unbound drug levels in plasma for bisoprolol. Bisoprolol plasma concentrations, measured in humans after 10 mg/d bisoprolol, p.o., were obtained from published data (59, 60) to yield an SI (SI2; Table 7) that takes into account relative receptor occupancies and fhu of drug in plasma at steady state (Css). For bisoprolol, with 43- to 69-fold β1-AR selectivity at the receptor level (SI1), the apparent β1-AR selectivity is reduced to 6-fold (SI2) when taking into account in vivo receptor occupancy.

A PK–pharmacodynamic model (23, 54) was then applied that considers relative β-AR affinity, receptor occupancy, and relative receptor numbers in target organs (61) and how receptor responses are actually transduced to the pharmacologic responses [i.e., reduction of exercise-induced HR for β1-AR (62) and reduction in lung function (FEV1) for β2-AR (63)]. We calculated a third SI (SI3; Table 7) that expresses selectivity in terms of in vivo pharmacologic responses (E) according to a ternary complex model (23, 54). We thus determined an SI3 value of 4 for bisoprolol. It is clear that with such a low in vivo pharmacologic selectivity, bisoprolol could cause bronchospasm in susceptible individuals.

We used the known ratio between bisoprolol exposure (Cwss) at an efficacious dose (10 mg/d, p.o., which lowers exercise-induced HR in humans) and receptor affinity (Kd,β1-AR) to estimate SIs for NDD-713 and NDD-825 (Table 7). Much higher indices (67- to 81-fold) were obtained for NDD compounds, which suggests greater in vivo selectivity than bisoprolol. The results demonstrate that to obtain significant separation of in vivo pharmacologic responses with respect to cardiac and respiratory function, high selectivity with regard to binding to β1-AR vs. β2-AR is required.

**CONCLUSIONS**

NDD-713 and, in particular, NDD-825 are high-affinity, β1-selective ligands that are devoid of ISA, off-target effects, and toxicology issues, but with good PK–pharmacodynamic and absorption, distribution, metabolism and elimination properties that maintain β1-selectivity in conscious animals. These ligands are therefore promising candidates for the development of β-blockers devoid of β2-AR–mediated adverse effects of bronchospasm and vasoconstriction and, thus, may prove beneficial in patients with concomitant cardiovascular and respiratory disease or limb ischemia (peripheral vascular disease).

**TABLE 7. Comparison SIs in terms or receptor binding (SI1), receptor occupancy (SI2), and pharmacological response (SI3) for bisoprolol, NDD-713, and -825**

<table>
<thead>
<tr>
<th>β-Blocker</th>
<th>SI1a (in vitro binding)</th>
<th>SI1b (in vitro inhibition of functional response)</th>
<th>SI2 (in vivo receptor occupancy)</th>
<th>SI3 (in vivo pharmacologic response)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDD-713</td>
<td>589</td>
<td>1202</td>
<td>67</td>
<td>67</td>
</tr>
<tr>
<td>NDD-825</td>
<td>1023</td>
<td>1738</td>
<td>115</td>
<td>81</td>
</tr>
<tr>
<td>Bisoprolol</td>
<td>43</td>
<td>69</td>
<td>6</td>
<td>4</td>
</tr>
</tbody>
</table>

*SI1a = K_d,β2/K_d,β1 (K_d values from 3H-CGP12177 whole-cell binding; Table 1). SI1b = K_d,β2/K_d,β1 (K_d values from antagonism of cimaterol-induced 3H-CAMP accumulation; Table 2). SI2 = Φ/Φβ2 = (K_d,β2 + Csat) / (K_d,β1 + Csat), where Φ is the receptor occupancy at the βARS, and Csat values refer to unbound drug levels in plasma at steady state upon once-daily oral dosing (K_d values from Table 1). Φc = E0/E02, where E01 and E02 refer to effects on exercise HR and on FEV1 under steady-state conditions after drug administration, respectively.**
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AUTHOR CONTRIBUTIONS

J. G. Baker, S. M. Gardiner, and J. Woolard performed the research; C. Fromont, G. P. Jadhav, S. N. Mistry, B. Kellam, and P. M. Fisher designed and synthesized the NDD compounds. J. G. Baker, S. M. Gardiner, J. Woolard, K. J. Thompson, B. Kellam, S. J. Hill, and P. M. Fisher designed the research study; J. G. Baker, S. M. Gardiner, J. Woolard, S. J. Hill, and P. M. Fisher analyzed the data; and J. G. Baker, S. J. Hill, and P. M. Fischer wrote the paper.

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