Comparison of the β-Adrenergic Receptor Antagonists Landiolol and Esmolol: Receptor Selectivity, Partial Agonism, and Pharmacochaperoning Actions

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ABSTRACT

Blockage of β₁-adrenergic receptors is one of the most effective treatments in cardiovascular medicine. Esmolol was introduced some three decades ago as a short-acting β₁-selective antagonist. Landiolol is a more recent addition. Here we compared the two compounds for their selectivity for β₂-adrenergic receptors over β₂₁-adrenergic receptors, partial agonistic activity, signaling bias, and pharmacochaperoning action by using human embryonic kidney (HEK)293 cell lines, which heterologously express each human receptor subtype. The affinity of landiolol for β₁-adrenergic receptors and β₂₁-adrenergic receptors was higher and lower than that of esmolol, respectively, resulting in an improved selectivity (216-fold versus 30-fold). The principal metabolite of landiolol (M1) was also β₁-selective, but its affinity was very low. Both landiolol and esmolol caused a very modest rise in cAMP levels but a robust increase in the phosphorylation of extracellular signal-regulated kinases 1 and 2, indicating that the two drugs exerted partial agonist activity with a signaling bias. If cells were incubated for ≥24 hours in the presence of ≥1 μM esmolol, the levels of β₁-adrenergic—but not of β₂₁-adrenergic—receptors increased. This effect was contingent on export of the β₁-receptor from endoplasmic reticulum and was not seen in the presence of landiolol. On the basis of these observations, we conclude that landiolol offers the advantage of: 1) improved selectivity and 2) the absence of pharmacochaperoning activity, which sensitizes cells to rebound effects upon drug discontinuation.

Introduction

Landiolol is a short-acting β₁-adrenergic receptor antagonist that has a half-life (3–4 minutes) shorter than the reference compound esmolol (9 minutes) (Plosker, 2013). Cleavage of an ester bond generates the major landiolol metabolite, M1, which has a half-life (1.8 hours) substantially longer than the parent compound (Murakami et al., 2005). During short-term infusion, M1 reaches peak concentrations in the range of 3.24 μM (Murakami et al., 2005). Because of its long half-life, the concentrations of M1 are predicted to increase further upon prolonged infusion and to reach their steady state, with continuous intravenous infusion, after about 7 hours. In fact, the ratio of half-lives predicts that under steady-state conditions, the concentration of M1 may exceed that of landiolol by several fold. It was therefore of interest to document the affinity and selectivity of M1 for human β₁-adrenergic receptors and to compare it to that of the parent compound, for which the affinity is quoted in reviews, (e.g., Plosker, 2013), but for which the original data are inaccessible.

G protein-coupled receptor antagonists have been categorized traditionally according to receptor selectivity, binding affinity, and the pharmacokinetic properties of the compounds. Additional possible discriminators that have been appreciated more recently are their intrinsic activity and signaling bias (Kenakin, 2005) and their ability to act as pharmacochaperones (Tao and Conn, 2014):

1) G protein-coupled receptors elicit signals not only by recruiting their cognate G protein. They can activate additional signaling pathways in a manner independent of heterotrimeric G proteins. Among all receptors known, the β₂₁-adrenergic receptor has been most extensively investigated with respect to its ability to generate a second wave of intracellular signals: Agonist occupancy triggers phosphorylation of several serine and threonine residues in the C-terminus of the β₂₁-adrenergic receptor by G protein-coupled receptor kinases (Lefkowitz and Shenoy, 2005). This supports binding of β₂₁-antagonists, which serve as versatile adapters: They recruit the endocytic machinery and thus support the clathrin-dependent endocytosis of receptors. In addition, β₂₁-antagonists scaffold via their C-terminus the...
assembly of kinase cascades, most prominently the mitogen-activated protein (MAP) kinase cascade (Lefkowitz and Shenoy, 2005; Shenoy and Lefkowitz, 2011). Receptor ligands differ in their ability to stabilize the active conformations of the receptor. Full agonists are thought to stabilize all active conformations, pure antagonists are thought to trap the receptor in the inactive conformation. However, there is a continuum with respect to agonistic or antagonistic activity and with respect to the conformations, which are achieved upon binding of individual ligands (Kenakin, 2005). Accordingly, in the case of the β2-adrenergic receptor, antagonists have been found to act as β-arrestin-biased ligands, i.e., they block the canonical signaling pathway (i.e., Gα-dependent stimulation of adenyl cyclase), but they support the recruitment of β-arrestin and the resulting MAP kinase stimulation (Azzi et al., 2003; Whalen et al., 2011).

2) Continuous exposure to cell-permeable ligands can increase the surface levels of a receptor and result in exaggerated responses to endogenous agonists, if the treatment with an antagonist is suddenly stopped. In fact, this was first observed with the β-adrenergic antagonist propranolol (“propranolol withdrawal rebound”; Alderman et al., 1974; Miller et al., 1975) and linked to an increase in surface receptor levels (Aarons et al., 1980). This effect is currently thought to reflect pharmacochaperoning by cell-permeable antagonists, i.e., specific ligands can assist receptor folding in the ER (endoplasmic reticulum) by binding to and stabilizing conformational intermediates on the trajectory to the stable low-energy state of the mature receptor (Morello et al., 2000; Nanoff and Freimuth, 2012).

The underlying chemistry behind the development of the short-acting β-blocker landiolol resulted in a large molecular structure. Given the chemical difference with conventional antagonists, it is reasonable to assume that it occupies a larger surface area on the β-receptor than do conventional and smaller antagonist ligands. Experimental data to show that landiolol—and similarly, esmolol—act as competitive antagonists are not publicly available. A priori, it cannot be ruled out that landiolol also occupies an allosteric site and for this reason may stabilize a unique type of receptor conformation. We therefore explored whether landiolol elicits a biased signal reason may stabilize a unique type of receptor conformation. However, there is a continuum with respect to agonistic or antagonistic activity and with respect to the conformations, which are achieved upon binding of individual ligands. Kenakin (2005). Accordingly, in the case of the β2-adrenergic receptor, antagonists have been found to act as β-arrestin-biased ligands, i.e., they block the canonical signaling pathway (i.e., Gα-dependent stimulation of adenyl cyclase), but they support the recruitment of β-arrestin and the resulting MAP kinase stimulation (Azzi et al., 2003; Whalen et al., 2011).

Materials and Methods

Materials. Landiolol and its metabolite M1 were obtained from Mehta Api Pvt Ltd (Mumbai, India), esmolol from Amomed Pharma (Vienna, Austria), [3H]CYP (iodo-cyanopindolol, specific activity 2200 Ci/mmol) and [3H]adenine were from Perkin Elmer (Shelton, CT), (−)-isoproterenol and the M2-anti-FLAG antibody were from Sigma-Aldrich (St. Louis, MO). All other reagents were of analytical grade. Landiolol (−)-(S)-2,2-dimethyl-1,3-dioxolan-4-yl)methyl-3-(4-(S)-2-hydroxy-3-(2-morpholino-carbonylamino)ethylamino)propoxy)phenylpropionate), its principal metabolite M1 (3-(4-(S)-2-hydroxy-3-(2-morpholino-carbonylamino)ethylamino)propoxy)phenylpropionic acid), and esmolol were dissolved in water to yield stock solutions of 100, 31, and 33 mM, respectively. Isoproterenol (33 mM) was dissolved in 0.1 M HCl.

Cell Transfections, Cell Cultures, and Cell Membrane Preparations. Plasmids encoding human β1- and β2-adrenergic receptors, which were tagged on their N-termini with a FLAG-epitope, were a generous gift of Dr. Mark von Zastrow (University of California at San Francisco). Human embryonic kidney (HEK293 cells (a fibroblast cell line) were transfected using the polycationic TurboFect reagent (Fermentas/Thermo Fisher Scientific) as follows: The plasmid encoding the human β1- or the human β2-adrenergic receptor plasmids (3 μg) were diluted with empty carrier plasmid pDNA3 (7 μg) and 20 μl of the TurboFect reagent in 1 ml Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich). This mixture was incubated for 20 minutes at room temperature (22°C) to allow for complex formation between the DNA and the polycationic TurboFect reagent. Thereafter, it was pipetted dropwise onto the layer of HEK293 cells (80% confluent in a 10-cm dish). After 24 hours the medium was exchanged and the cells propagated in a humidified atmosphere (95% air/5% CO2) at 37°C in DMEM containing 10% fetal calf serum (FCS) and 0.7 mg/ml geneticin (G418) for selection of stable transfectants. In some instances, a plasmid (3 μg/2×106 cells) encoding a green fluorescent protein (GFP)-tagged bovine β-arrestin-2 (arrestin-3) or an empty control plasmid was subsequently introduced into stably transfected HEK293 cells. For membrane preparation, stably transfected cells were harvested from 15-cm dishes (80–90% confluent) as follows: The dishes were first rinsed with phosphate buffered saline (PBS) and then mechanically detached from the dish with a plastic scraper in 5 ml of ice-cold PBS containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF). Cells were recovered by centrifugation (400g for 10 minutes at 4°C). The cell pellet was resuspended in 1 ml hypotonic HME buffer (20 mM HEPES NaOH, pH 7.5, 1 mM EDTA, 2 mM MgCl2) containing 0.1 mM PMSF and the Complete Protease Inhibitor Cocktail (Roche Biochemical Reagents/ Sigma-Aldrich) and subsequently frozen in liquid nitrogen. After rapid thawing, the cells were further homogenized by ultrasonication (Sonifier cell disruptor B15, 12 pulses of 0.5 second duration at 50% intensity; Branson Ultrasonics, Danbury, CT). Membranes were recovered by centrifugation (15 minutes at 34,000g at 4°C) and resuspended in HME buffer. The protein concentration (about 5 mg/ml) was determined by dye binding (using the Coomassie Brilliant Blue kit provided by BioRad Laboratories, Hercules, CA). Membranes were frozen in liquid nitrogen and stored at −80°C.

Radioligand Binding. The binding reaction was conducted in a final volume of 0.2 ml containing buffer (20 mM HEPES-NaOH, pH 7.5, 1 mM EDTA, 2 mM MgCl2, 1 mM ascorbic acid), with membranes (0.4–0.7/assay and 0.22–0.4 μg/assay for β1- and β2-adrenergic receptor-expressing membranes, respectively), competing ligands at the concentrations indicated in Fig. 2, and [125I]CYP (in the range of 40 pM for competition experiments; concentrations as indicated in Fig. 1 for saturation experiments). The reaction was started by the addition of the radioligand. After an incubation for 90 minutes at 30°C, the reaction was stopped by rapid filtration over glass-fiber filter mats [Skatron FilterMAT 11731 (Molecular Devices, Sunnyvale, CA)]. The radioactivity trapped was counted by liquid scintillation counting at an efficiency of 75% (i.e., 3700 cpm/fmol). Nonspecific binding was defined as binding not displaced by 0.1 mM isoproterenol or 5 μM propranolol (which gave equivalent results). This nonspecific binding was below 20% of total binding at the highest radioligand concentration employed (400 pM) and <10% at the radioligand concentration employed in the competition experiments.

[cAMP Accumulation. Untransfected and transfected HEK293 cells (3×105 cells/well) were seeded in 6-well dishes incubated for 16 hours in DMEM containing 0.5 mg/l each of benzylpenicillin and streptomycin, and [1H]adenine (1 μCi/ml; PerkinElmer). The cells were then stimulated with the indicated compounds of

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Fig. 1. Saturation hyperbola for specific binding of $[^{125}\text{I}]\text{CYP}$ (A), competition by landiolol, its metabolite M1, and esmolol for binding of $[^{125}\text{I}]\text{CYP}$ to human $\beta_1$ and $\beta_2$-adrenergic receptors in membranes prepared from stably transfected HEK293 cells (B), and landiolol- and adrenergic receptors, respectively) were incubated with the indicated concentrations of $[^{125}\text{I}]\text{CYP}$ for 90 minutes at 30°C. The solid lines were drawn by fitting the data to a rectangular hyperbola. Data are from a representative experiment done in duplicate. A second experiment gave similar results. (B) Experiments were carried out as outlined under Materials and Methods. Specific binding was converted to percentage of control (i.e., specific binding in the absence of any competitor) to normalize for differences in absolute levels (fmol/assay) and thus to allow for comparison. Data are means from three independent experiments carried out in duplicate; error bars (*n* = 3, means ± S.E.M.). (C) Experiments were carried out as outlined under Materials and Methods. Data are means from three independent experiments carried out in triplicate; error bars represent S.E.M. As a control, cells were also incubated in the presence of 1 μM propranolol (prop 1, right hand panel, where the median and the interquartile range is indicated; whiskers represent the 90% confidence interval).
landiolol (1 and 10 \( \mu \text{M} \)) and propranolol (1 \( \mu \text{M} \)). Alternatively, stably transfected cells were incubated for 24 hours with propranolol, esmolol, or landiolol, at concentrations ranging from 0.1–100 \( \mu \text{mol/L} \). After the incubation, the cells were washed with PBS and detached using EDTA (0.02%) solution. The following procedures were performed at 4°C: Cells were collected by centrifugation (1000 rpm for 5 minutes) and taken up in PBS with 1% bovine serum albumin (BSA) in a volume of \(-0.3 \text{ ml} \). Cell-surface receptors were labeled with the monoclonal M2 anti-FLAG antibody (at a concentration of \(-0.3 \mu \text{g/100 \mu L} \)) and a secondary anti-mouse IgG conjugated to the fluorescent dye AlexaFluor 488 (0.25 \( \mu \text{g/100 \mu L} \) of cell suspension). Labeling was at 4°C for 20 minutes with the primary and an additional 20 minutes with the secondary antibody. An aliquot of cells was labeled only with secondary antibody. Cells were washed once, resuspended in PBS with 1% BSA, and subjected to measuring cell-bound AlexaFluor 488 fluorescence by flow cytometry. Flow cytometry was performed on a FACSCanto instrument (BD Biosciences San Jose, CA). Fluorescence was recorded at 4°C on samples diluted in PBS. For the analysis a cell fraction was represented cells expressing FLAG-tagged receptors on their surface.

An aliquot of cells was labeled only with secondary antibody. Cells stained positive for propidium iodide. Ten thousand events were recorded per gated sample. Receptor-specific fluorescence was determined after excluding events that overlapped the range of nonspecific fluorescence (assessed on cells labeled with secondary antibody alone). A range of 4–8000 (out of 10,000) events per sample represented cells expressing FLAG-tagged receptors on their surface. Analysis of flow cytometry recordings was performed with Flowing Software (Dr. Perttu Terho, Turku Centre for Biotechnology, Turku, Finland). Treatment-dependent change was quantified by comparing the histogram distribution of receptor-specific fluorescence events.

**Data Analysis.** Data from saturation experiments and from concentration-response curves were subjected to nonlinear least squares curve fitting using a Levenberg-Marquardt algorithm to the law of mass action. Inhibition curves were fitted in a manner similar to the equation for a monophasic displacement curve. IC\(_{50}\) values (i.e., concentrations resulting in 50% inhibition of radioligand binding) were converted into \( K_i \) values using the Cheng-Prusoff approximation (Cheng and Prusoff, 1973): \( K_i = IC_{50}(1 + L/K_D,L) \), where \( K_i \) is the dissociation constant of the inhibitor, \( L \) is the radioligand concentration, and \( K_D,L \) is the dissociation constant of the radioligand (determined from saturation experiments). Statistically significant differences were examined by Student’s \( t \) test for paired comparisons and by analysis of variance (ANOVA) or Kruskal-Wallis test followed by a post hoc test for multiple comparisons.

**Results**

**Competition by Landiolol, Its Metabolite M1, and Esmolol for Binding of \([^125\text{I}]\)ICYP to Heterologously Expressed Human \( \beta_1 \)- and \( \beta_2 \)-Adrenergic Receptors.** Stably transfected HEK293 cells were selected that expressed the individual receptors at high density: \( B_{\text{max}} \) values were 2.7 pmol/mg and 6.9 pmol/mg for \( \beta_1 \)- and \( \beta_2 \)-adrenergic receptor-carrying membranes, respectively (Fig. 1A). The radioligand \([^125\text{I}]\)ICYP had only a very modest—if any—selectivity for \( \beta_2 \)-adrenergic receptors; \( K_D \) values were 37 ± 8 pmM and 30 ± 5 pmM for \( \beta_1 \)- and \( \beta_2 \)-adrenergic receptors, respectively (mean ± S.D.; \( n = 2 \)). Landiolol was substantially more potent in displacing the radioligand \([^125\text{I}]\)ICYP from human \( \beta_1 \)-adrenergic receptors than from human \( \beta_2 \)-adrenergic receptors (open and closed circles in Fig. 1B and Table 1). The inhibitory potency of the metabolite M1 was some two orders of magnitude lower (triangles in Fig. 1B). Esmolol was also selective for human \( \beta_1 \)-adrenergic receptors albeit to a lower extent than landiolol (open and closed columns in Fig. 2 and Table 1).

**Effects of Landiolol and Esmolol on Forskolin-Stimulated cAMP Accumulation.** HEK293 cells expressing FLAG-tagged human \( \beta_1 \)- and \( \beta_2 \)-adrenergic receptors were incubated in the presence of \([^3\text{H}]\)adenine to metabolically label their adenine nucleotide pool. The cells were then stimulated with 20 \( \mu \text{M} \) forskolin to raise cAMP levels (Klinger et al., 2002). Forskolin sensitizes adenylyl cyclase to the action of activated \( G_{\text{q}} \) (Sunahara et al., 1996; Kudlacek et al., 2001). Hence, basal receptor activity is magnified in the presence of forskolin. Accordingly, it is possible to detect inverse agonism in the presence of forskolin, because antagonists with inverse activity lock the receptor in the inactive conformation and hence produce a decline of cAMP accumulation (Klinger et al., 2002). Conversely, in the presence of forskolin, the weak intrinsic activity of antagonists is also magnified and detected as an increase in cAMP, which would not be detected under basal conditions. In fact, in the absence of forskolin, we failed to observe any change in cAMP accumulation (data not shown). However, in the presence of forskolin, both esmolol and landiolol modestly increased cAMP levels in cells expressing human \( \beta_1 \)-adrenergic receptors (Fig. 1C). Concentrations of landiolol \( \geq 1 \mu \text{M} \) were required to observe a significant rise in cAMP. Under the conditions employed, where 10 \( \mu \text{M} \) landiolol and esmolol elevated cAMP levels by roughly 1.5- and 2-fold, 1 \( \mu \text{M} \) isoproterenol stimulated cAMP accumulation by 18.2 ± 1.9-fold (means ± S.D. \( n = 3 \)). Taken together, these findings suggest that esmolol and landiolol are not inverse agonists but very weak partial agonists at the human \( \beta_1 \)-adrenergic receptor. High receptor occupancy by landiolol is required to elicit this partial agonism via \( G_{\text{q}} \).

**Stimulation by Landiolol and Esmolol of MAP Kinase Phosphorylation.** The weak partial agonist activity of landiolol and esmolol suggested that these compounds induced an active conformation in the human \( \beta_1 \)- and \( \beta_2 \)-adrenergic receptors albeit at low probability. This activity may give rise to biased agonism (Azzi et al., 2003; Kenakin, 2005; Whalen et al., 2011). This possibility was explored by assessing the capacity of esmolol and landiolol to stimulate the phosphorylation of p42 and p44 MAP kinases (ERK2 and ERK1, respectively) in HEK293 cells expressing human \( \beta_1 \)- or \( \beta_2 \)-adrenergic receptors.

In cells expressing human \( \beta_1 \)-adrenergic receptors, esmolol and landiolol caused a time-dependent stimulation of MAP kinase phosphorylation (Fig. 2A). The time course showed the typical biphasic response, i.e., an initial peak followed by a

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**TABLE 1**

<table>
<thead>
<tr>
<th>Human ( \beta_1 )-Receptor</th>
<th>Human ( \beta_2 )-Receptor</th>
<th>Selectivity Ratio ((K_i, \beta_2/K_i, \beta_1))</th>
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<tbody>
<tr>
<td>Landiolol 90 ± 16 nM</td>
<td>19.4 ± 5.5 ( \mu \text{M} )</td>
<td>216 ( b )</td>
</tr>
<tr>
<td>M1 3.8 ± 1.2 ( \mu \text{M} )</td>
<td>224 ± 61 ( \mu \text{M} )</td>
<td>59</td>
</tr>
<tr>
<td>Esmolol 194 ± 7 ( \mu \text{M} )</td>
<td>5.8 ± 2.1 ( \mu \text{M} )</td>
<td>30</td>
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sustained phase. In contrast, even when tested at 10 μM, neither esmolol nor landiolol caused any appreciable stimulation of MAP kinase phosphorylation in HEK293 cells expressing the human β2-adrenergic receptor (Fig. 2B). Incidentally, this experiment also showed that in HEK293 cells expressing β1-adrenergic receptors, esmolol and landiolol stimulated MAP kinase phosphorylation via their cognate receptor rather than via an off-target effect. This was also independently verified by challenging untransfected HEK293 with 1 μM esmolol or 1 μM landiolol, which did not result in a statistically significant increase in MAP kinase phosphorylation (not shown).

The peak activation, which was observed in HEK293 cells expressing the human β1-adrenergic receptor stimulated with esmolol and landiolol (Fig. 2A), was sufficiently large in magnitude to allow for determination of the concentration-response relation: Both compounds stimulated MAP kinase phosphorylation over a comparable concentration range with a half-maximum effect seen at about 100 nM (Fig. 3). The effect of esmolol and landiolol was lower than the effect elicited by isoproterenol (right hand lane and column in Fig. 2A). This is consistent with partial agonism of the two compounds at the G protein-independent, β-arrestin-dependent pathway of the β1-adrenergic receptor (Azzi et al., 2003; Whalen et al., 2011). If this was the case, raising the cellular levels of β-arrestin ought to enhance the ability of esmolol and landiolol to stimulate phosphorylation of ERK1/2 via the β1-adrenergic receptor. This prediction was verified: We compared the response of control cells and cells over-expressing β-arrestin-2 (= arrestin-3) to esmolol, landiolol, and isoproterenol (right and left hand panel in Fig. 4A) and used the effect elicited by the phorbol ester PMA as an internal reference: The rationale for this approach is the fact that protein kinase C-dependent activation of the MAP kinase

Fig. 2. Time-dependent phosphorylation of ERK1/2 (p42 and p44 MAP kinases) in HEK293 cells stably expressing the human β1-adrenergic (A) and the β2-adrenergic receptor (B). HEK293 cells were seeded into six-well dishes, allowed to adhere in the presence of 10% FCS for 8 hours and incubated in medium containing 0.5% FCS for 16 hours. Thereafter, the medium was exchanged for medium containing 1 μM (A) and 10 μM (B) esmolol or 1 μM (A), and 10 μM landiolol (B) for up to 40 minutes. Stimulation with 1 μM isoproterenol was done for 5 minutes and was used as a positive control to allow for comparison between blots. Bar diagrams: The immunoblots were probed with an antibody recognizing the dually phosphorylated active form of p42 (ERK2) and p44 (ERK1) MAP kinases (upper blot) and with an antibody directed against all forms of the proteins (lower blot). Phosphorylation was quantified by correcting the recorded phospho-specific intensity for loading (as shown in the respective immunoblots) and plotted to visualize the time course. Bars show means ± S.E.M. from three independent experiments. In (A), the stimulation at 5 minutes differs from the control value in a statistically significant manner (P < 0.001, repeated-measures ANOVA followed by Bonferroni multiple comparisons).

Fig. 3. Concentration-response curve for esmolol- and landiolol-induced phosphorylation of ERK1/2 (p42 and p44 MAP kinases) in HEK293 cells stably expressing the human β1-adrenergic receptor. (A) Assay conditions were as outlined in the legend to Fig. 3. Cells were incubated for 5 minutes in the absence and presence of esmolol or landiolol at the indicated concentration. Stimulation with 1 μM isoproterenol (iso) was used as a positive control to allow for comparison between blots. Data are representative for two additional independent experiments. (B) The levels of phospho-MAP kinase were quantified as outlined in the legend to Fig. 2 and plotted as a concentration-response curve. Data are means ± S.E.M. Repeated measures ANOVA followed by Holm-Sidak multiple comparisons verified that there was a statistically significant difference (P < 0.02) between the phospho-ERK1/2 levels seen at saturating concentrations and that observed in the ascending limb (i.e., 0.01, 0.3 and 0.1 μM) of the concentration-response curve.
was detected by monitoring the fluorescence resulting from binding of a monoclonal antibody to the N-terminal FLAG-epitope (middle histogram in Fig. 5A; see also box plot in Fig. 5B). The effect was comparable in magnitude to that elicited by propranolol (Fig. 5A, left hand histogram, and Fig. 5B). In contrast, landiolol was ineffective at 1 μM, and even at 10 μM the effect was so modest (Fig. 5A) that it did not reach the threshold of statistical significance (Fig. 5B). Esmolol and landiolol were also tested at 0.1 μM, which did not produce any statistically significant effect on the surface levels of the β₁-adrenergic receptor (not shown). We also compared the effect of esmolol and of landiolol on the cell surface levels of the human β₂-adrenergic receptor to that of propranolol: Cells exposed to 1 μM propranolol for 24 hours showed a substantial increase in β₂-adrenergic receptors on their surface (left hand histogram in Fig. 6A) that was statistically significant (box plot in Fig. 6B). In contrast, neither an incubation in 10 μM esmolol nor in 10 μM landiolol resulted in an appreciable elevation of surface receptor levels (Fig. 6, A and B).

cascade occurs at the level of or downstream from RAF and is therefore independent of β-arrestin (Schönwasser et al., 1998). As can be seen from Fig. 4B, the overexpression of β-arrestin-2 enhanced the response to the β₁-adrenergic ligands in a statistically significant manner. Overexpression of β-arrestin-2 did not affect the levels of the β₁-adrenergic receptor (Fig. 4A).

Change in Receptor Surface Levels after Long-Term Incubation of Cells in the Presence of Esmolol, Landiolol, and Propranolol. Prolonged incubation of cells with antagonists can increase the levels of their cognate receptors by binding to and stabilizing folding intermediates in the ER; this action is referred to as pharmacological chaperoning, pharmacocochaperoning (Morello et al., 2000; Leidenheimer and Ryder, 2014), or pharmacoperoning (Tao and Conn, 2014). If cells were incubated for 24 hours in the presence of 1 μM esmolol, an increase in surface levels of the human β₁-adrenergic receptor
Pharmacochaperones exert their actions on folding intermediates. During their synthesis, like all other integral membrane proteins, G protein-coupled receptors are inserted into the ER membrane and they must reach their folded state prior to recruiting the COPII machinery to exit the ER (Nanoff and Freissmuth, 2012). Export of integral membrane proteins from the ER is contingent on their incorporation into the nascent COPII-coated vesicles; this is accomplished via the interaction with the SEC24-component, which acts as the cargo receptor (Gillon et al., 2012; Zanetti et al., 2011). In mammalian cells, there are four isoforms of SEC24 (termed SEC24A, SEC24B, SEC24C, and SEC24D). The isoform required for ER export of the human \( \beta_1 \)-adrenergic receptor is not known. Accordingly, HEK293 cells were depleted of all isoforms by a combination of siRNAs or the mixture of siRNAs directed against all SEC24 isoforms (SEC24A–D). This was previously shown to reduce their cellular levels by 80–90% and to decrease ER export of membrane proteins (Sucic et al., 2011, 2013). As expected, this siRNA-mediated knockdown of SEC24A–D also reduced the surface levels of the human \( \beta_1 \)-adrenergic receptor. More importantly, under these conditions, neither preincubation with esmolol nor with propranolol increased the cell surface levels of the human \( \beta_1 \)-adrenergic receptor (Fig. 7, A and B).

### Discussion

Of the more than 800 human G protein-coupled receptors, about 70 are targets for currently approved drugs (Gruber et al., 2010). Among these, the \( \beta_1 \)- and \( \beta_2 \)-adrenergic receptors are the ones with the richest pharmacology. There is a long list of ligands that have been explored for their agonistic and antagonistic properties. A surprisingly small number of antagonists are useful for intravenous administration. Because of their short half-lives, esmolol and landiolol are of particular interest in the prevention and management of cardiac arrhythmias. Our comparison revealed the following salient differences:

**Fig. 6.** Quantification of \( \beta_2 \)-adrenergic-receptor surface levels by flow cytometry using an anti-FLAG antibody. (A) HEK293 cells expressing stably \( \beta_2 \)-adrenergic receptors carrying a FLAG-epitope tag on the (extracellular) N-terminus were incubated in the absence (control, gray histogram) and presence (black histograms) of esmolol, landiolol and propranolol for 24 hours at the indicated concentrations. The blue area corresponds to the histogram for nonspecific fluorescence observed after incubation of the nontreated cells with the secondary antibody alone (no anti-Flag antibody). The level of receptors at the cell surface were quantified by detecting the FLAG-epitope as outlined under Materials and Methods. Data are representative for seven additional experiments. (B) Median fluorescence intensity was quantified from the histograms of eight independent experiments carried out as outlined in (A). The box plot shows the median values and the interquartile range, and whiskers indicate the observed maximum and minimum values. Statistically significant differences between drug-treated and untreated control cells were verified by a Kruskal-Wallis test followed by Dunn’s multiple comparison (*\( P < 0.01 \) for propranolol 1 \( \mu \)M versus control).

**Fig. 7.** The pharmacochaperoning effects of \( \beta_2 \)-adrenergic antagonists is absent in HEK293 cells pretreated with siRNAs directed against SEC24A–D. (A) HEK293 cells were transfected with scrambled control siRNAs or the mixture of siRNAs directed against all SEC24 isoforms (SEC24A–D). After 48 hours, the cells were transfected with a plasmid encoding the human \( \beta_1 \)-adrenergic receptors carrying a FLAG-epitope tag on the (extracellular) N-terminus and incubated in the absence and presence of esmolol, landiolol, and propranolol for 24 hours at the indicated concentrations. Shown are representative original histograms for the quantification of \( \beta_1 \)-adrenergic receptor surface levels by flow cytometry using an anti-FLAG antibody (as in Figs. 5 and 6). The blue area corresponds to the histogram for nonspecific fluorescence observed after incubation of the nontreated cells with the secondary antibody alone (no anti-FLAG antibody). The black lines indicated the quantification of the FLAG-specific immunoreactivity in cells transfected with the mixture of siRNAs directed against all SEC24 isoforms (SEC24A–D). The black lines correspond to the FLAG-specific immunoreactivity in cells transfected with the mixture of scrambled siRNAs. (B) The data from (A) was quantified by measuring the median fluorescence intensity of the specific area under the curve and plotted to visualize the effect of landiolol, esmolol, and propranolol on HEK293 cells expressing human \( \beta_1 \)-adrenergic receptors transfected with mixture of siRNAs directed against all SEC24 isoforms (SEC24A–D). Bars show means ± S.E.M. from three independent experiments.
The affinity of landiolol for human $\beta_1$-adrenergic receptors exceeded that of esmolol by about 2-fold. Esmolol had a substantially higher affinity (i.e., more than 3-fold higher) for human $\beta_2$-adrenergic receptors than landiolol. Accordingly, the selectivity of landiolol for human $\beta_1$-adrenergic receptors is superior to that of esmolol, i.e., 216–versus 30-fold, which translates into a roughly 7-fold increase in selectivity. Hydrolysis of both esmolol and landiolol generates an active metabolite. The affinity of the esmolol acid metabolite (generated by releasing methanol) was reported to have a 400-fold lower affinity than the parent compound esmolol and to lack selectivity for $\beta_1$–versus $\beta_2$-adrenergic receptors (Jahn et al., 1995). Here we show that the landiolol metabolite M1 still discriminated between human $\beta_1$- and $\beta_2$-adrenergic receptors with a selectivity ratio that exceeds that of esmolol. However, and more importantly, the affinity of the M1 metabolite for $\beta_1$-adrenergic receptors was substantially lower than that of the parent compound landiolol (by about 42-fold).

Esmolol and landiolol are not inverse agonists, but are very weak partial agonists at $\beta_1$-receptors, when examined for their ability to induce cAMP accumulation. Landiolol is more efficacious. However, the differences between the two compounds are modest and presumably of little clinical significance: Even in cells with heterologous overexpression of $\beta_1$-receptors, partial agonism of esmolol and landiolol was only detectable in the presence of a sensitizing concentration of forskolin. When tested, neither compound had any detectable intrinsic effect on basal cAMP accumulation. In contrast, esmolol and landiolol are robust partial agonists in the noncanonical signaling pathway, which links the $\beta_1$-adrenergic receptor to stimulation of ERK1/2 (Azzi et al., 2003; Whalen et al., 2011): Esmolol and landiolol elicited about 40% of the response observed in the presence of isoproterenol (Fig. 4). This suggests that esmolol and landiolol have a signaling bias at $\beta_1$-adrenergic receptors: In fact, landiolol stimulated ERK1/2 phosphorylation at lower $\beta_1$-receptor occupancy than it caused an accumulation of cAMP in the presence of forskolin (Figs. 3 and 1). Depending on the cell type, elevations of cAMP can result in both inhibition and stimulation of ERK1/2 phosphorylation (Stork and Schmitt, 2002). However, in HEK293 cells, cAMP generated in response to $G_s$-coupled receptors does not affect the MAP kinase cascade (Daaka et al., 1997; Seidel et al., 1999). In addition, esmolol and landiolol did not affect cAMP levels in the absence of forskolin. Thus, changes in cAMP levels probably do not confound the response of ERK1/2 to esmolol and landiolol. It is currently difficult to interpret the relevance of this biased partial agonism of landiolol and esmolol in particular because in the heart, $\beta_1$-adrenergic receptors are weakly susceptible to partial agonist activity, which is related to receptor baseline downregulation (Nanoff et al., 1989, 1990). Although currently considered of major interest for drug development (Azzi et al., 2003; Kenakin, 2005; Whalen et al., 2011), the clinical significance of biased agonism is poorly understood. It is however clear that activation of ERK is cardioprotective during ischemia (Lips et al., 2004; Rose et al., 2010) and contributes to the cardioprotective actions of $\beta$-adrenergic antagonists (Kovacs et al., 2009).

Esmolol acts as a pharmacochaperone: Long term exposure of cells to esmolol raised the surface levels of $\beta_1$-adrenergic receptors. This action was not shared by landiolol. Integral membrane proteins fold in the endoplasmic reticulum. Their folding is assisted by luminal and cytosolic proteinaceous chaperones. These engage folding intermediates and lower the energy barrier, which separates individual conformational states of the folding trajectory (Nanoff and Freissmuth, 2012). Their action can be phenocopied by small molecules, which act as chemical chaperones if they promote folding in a general manner [e.g., dimethylsulfoxide], or as pharmacochaperones, if they promote folding of their cognate target (Morello et al., 2000; Leidenheimer and Ryder, 2014; Tao and Conn, 2014). In fact, pharmacochaperoning of G protein-coupled receptors was shown to occur in the endoplasmic reticulum (Málaga-Díéguez et al., 2010). We verified that an intact ER export machinery was required to observe the increase in surface expression of $\beta_1$-adrenergic receptors, which was induced by esmolol and propranolol. Export of proteins from the ER relies on the COP24 coat, where SEC24 acts as the cargo receptor (Zanetti et al., 2011; Gillon et al., 2012). The combined knockdown of all the SEC24 isoforms abolished the ability of esmolol and of propranolol to raise the surface levels of $\beta_1$-adrenergic receptors. This observation proves that the pharmacochaperoning action of esmolol and propranolol was contingent on ER export.

Esmolol and landiolol differ in their ability to act as pharmacochaperones. This difference can be rationalized as follows: to act as a pharmacochaperone, a ligand must be able to permeate cell membranes. A commonly used metric to predict membrane permeation is the polar surface area of the molecule, e.g., a polar surface area $>90 \, \text{Å}^2$ correlates with weak permeability (Hitchcock and Pennington, 2006). The polar surface areas of landiolol and esmolol are 128 and 67.8 $\, \text{Å}^2$, respectively, and predict that esmolol distributes into the cell interior sufficiently to make it an effective pharmacochaperone, whereas landiolol is unlikely to reach effective intracellular concentrations. The volume of distribution—assessed in human subjects—reflects the difference in permeation. Its size was found to be more than an order of magnitude larger for esmolol ($V_D = 3.43 \, \text{l/kg}$; Sum et al., 1983) than for landiolol ($V_D=0.16–0.24 \, \text{l/kg}$; Murakami et al., 2005; Plosker, 2013).

It is worth pointing out that this difference in pharmacochaperoning activity is probably of clinical relevance: Continuous infusion of therapeutically relevant doses of landiolol results in concentrations in plasma of about 1–4 $\mu$M (Murakami et al., 2005; Plosker, 2013). Given its small apparent volume of distribution, landiolol is not enriched within the intracellular compartment. Landiolol is thus unlikely to have any pharmacochaperoning action in vivo because at concentrations up 10 $\mu$M it failed to increase surface levels of human $\beta_1$-adrenergic receptors. In contrast, approved doses of esmolol lead to steady-state plasma levels of esmolol in the range of 1.2–2.4 $\mu$g/ml (i.e., 4–8 $\mu$M, Menkhaus et al., 1985). As the volume of distribution of esmolol is about six times larger than total body water, intracellular concentration of esmolol must be higher than those in plasma. Within this clinically relevant concentration range (i.e., 1–10 $\mu$M), esmolol was found to be an effective pharmacochaperone.

The current observations predict that: 1) prolonged infusion of esmolol may be associated with a loss in $\beta_1$-receptor blocking activity and an exaggerated response to endogenous catecholamines upon cessation of therapy, but 2) that there should not be any loss of efficacy resulting from continuous administration of landiolol. In fact, it has been noted
previously that, after prolonged infusion of esmolol (at individual doses set to achieve a target heart rate), its effect faded gradually with time (to a pronounced extent after more than 48 hours) and that withdrawal of esmolol resulted in an increase in heart rate (> 33% over baseline) in the majority of patients (Harwood et al., 1999). The time course of diminishing beta-blocking activity is consistent with an enhanced sensitivity of the myocardium to beta-1-adrenergic receptor stimulation (Nanoff and Schütz, 1991) and the effect of stopping the infusion reminiscent of the propranolol withdrawal syndrome (Alderman et al., 1974; Miller et al., 1975; Aarons et al., 1980). Hence on the basis of our observations, we conclude that landiolol has three potentially interesting features: it is a biased antagonist, it is highly beta-1-selective, and it lacks phosphacochaperoning activity. The latter two features distinguish it from esmolol.

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