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BRIEF ARTICLE

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Efficient Conversion of a Nonselective Norepinephrin Reuptake Inhibitor into a Dual Muscarinic Antagonist−β_2-Agonist for the Treatment of Chronic Obstructive Pulmonary Disease

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Supporting Information

ABSTRACT: Following interrogation of a wide-ligand profile database, a nonselective norepinephrin reuptake inhibitor was converted into a novel muscarinic antagonist using two medicinal chemistry transformations (M3/NRI selectivity of >1000). Conjugation to a β_2 agonist motif furnished a molecule with balanced dual pharmacology, as demonstrated in a guinea pig trachea tissue model of bronchoconstriction. This approach provides new starting points for the treatment of chronic obstructive pulmonary disease and illustrates the potential for building selectivity into GPCR modulators that possess intrinsic promiscuity or reverse selectivity.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) will become the third largest cause of death by 2030 and is characterized by chronic bronchitis and emphysema, mainly caused by cigarette smoking.[1] Inhaled adrenergic (β_2) agonists and muscarinic (M3) antagonists are used as bronchodilators to treat the symptoms of the disease. More recently, long acting muscarinic antagonists (LAMAs) and long acting β_2 agonists (LABAs) have been developed to enable once-daily dosing regimens.[2]

Triple therapy for COPD, via the synergistic combination of a LABA, LAMA, and an inhaled corticosteroid (ICS), would significantly enhance the treatment of the disease,[3] but the combination of three drugs into a dry powder inhalation device is extremely challenging. As a result, we and others have recently reported the concept of dual pharmacology muscarinic antagonists−β_2-agonists (MABAs) that should facilitate the combination with an ICS in a single device to enable the triple therapy paradigm.[4]

We have reported conjugation of a known muscarinic antagonist tolterodine **1** with the known quinolinone β_2 agonist trigger to create MABAs such as **2** that demonstrate balanced potency and pharmacological duration, incorporating the principles of "inhalation by design".[4c] Theravance published a similar strategy using the known biarylcarbamoylpiperidine antimuscarinic motif to furnish dual pharmacology molecules such as **3**.[4d] Although a mix-and-match approach to novel MABA creation is possible using known β_2 and M3 fragments, the available intellectual property space, and the opportunity to construct something truly innovative, soon becomes limited. Therefore, a need exists to create novel single pharmacology agents to broaden the palette that can enable dual pharmacology MABAs and subsequently the triple therapeutic approach.

We felt that the subtle structure−activity relationships (SARs) that trigger β_2 agonism may significantly hinder the creation of novel headgroup templates (avoiding β_1 activity is also far from trivial), and our efforts were therefore focused on the muscarinic antagonist motif. The caveat to this approach is that it is particularly difficult to design selective G-protein-coupled receptor (GPCR) modulators, and the antimuscarinic field is extremely well studied, thus making the creation of completely novel and selective scaffolds challenging.

Rather than modification of existing selective antimuscarinic templates, our strategy involved searching the Pfizer Bioprint (Cerep SA)[5] wide-ligand profile (WLP) database for molecules that possessed muscarinic antagonism but in a template not necessarily classed or claimed in the literature as specifically "antimuscarinic". Any "hits'" would likely have been designed for a different purpose, i.e., where the primary and desired pharmacology results from targeting an alternative protein. M3 SARs would then be harnessed to efficiently enhance antimuscarinic activity while switching selectivity, ultimately yielding novel muscarinic antagonists and dual pharmacology MABAs.

RESULTS

Following a search of our wide-ligand profile database for muscarinic antagonists, we discovered indazole **4** (Scheme 1), a known norepinephrin reuptake inhibitor (NRI, assessed through the inhibition of the norepinephrin transporter, NET)[6] that possessed moderate M3 activity (Table 1). We hoped that eventually, the presence of the piperidine moiety would provide an opportunity for conjugation to a β_2 pharmacophore through the nitrogen atom in a manner similar to that described previously by us and Theravance (e.g., MABA **3**). Not surprisingly, this rather lipophilic, basic compound is a promiscuous binder of many proteins[7] (see WLP in the Supporting Information).

It is noticeable that **4** lacks a hydrogen bonding group which is present in other selective antimuscarinics, such as the phenol

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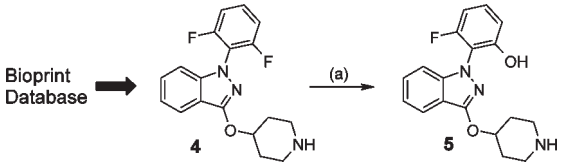
Scheme 1[a]



[a] Reagents and conditions: (a) ^{t}BuOK, THF, reflux, then HCl in dioxane.

Table 1. Antimuscarinic M_3 Potency, Human Liver Microsome (HLM) Metabolic Stability and Lipophilicity (log *D*) for Compounds 1, 4, 5, 11[a]

	1	4	5	11
M3 K_i (nM)[c]	3.6	49	10	2.5
NET K_i (nM)[d]	15% at 10 μM	6.8	nd[b]	2900
log *D*	1.8	1.4	0.6	0.3
HLM ((μL/min)/mg)	<8	<8	<8	<8

[a] All data are geometric mean values with 95% CI, n = 4. [b] nd = not determined. [c] Binding assay, human cloned M3 receptor using [^{3}H]*N*-methylscopolamine. [d] Binding assay, human cloned NET receptor using [^{3}H]nisoxetine.

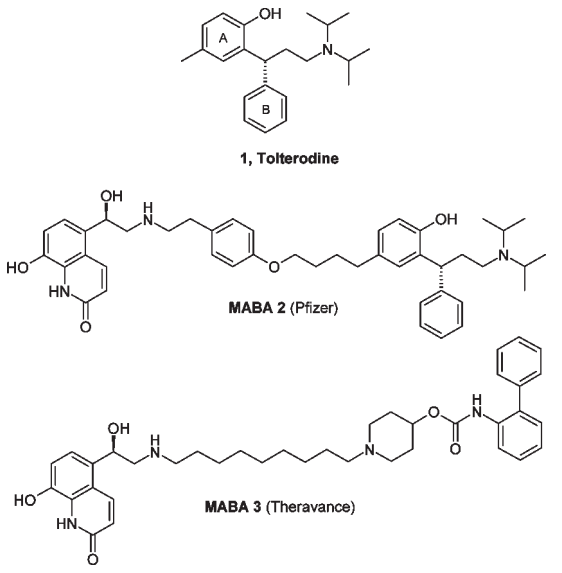


Figure 1. Structures of tolterodine **1** and MABAs **2** and **3**.

−OH present in tolterodine (Figure 1; see also the M3 dock with azoniabicyclo[2.2.2]octane muscarinic antagonists by Lainé et al. suggesting a key hydrogen bond between the ligand-OH and Ser$^{7:46}$).[8] A molecular overlay of the small molecule crystal structure of tolterodine[9] with an energy minimized structure of **4**[10] suggested that replacing one of the fluorine atoms with a hydroxyl group may improve affinity (Figure 2). In fact, treatment of **4** with potassium *tert*-butoxide and acidic workup provided the more potent analogue **5** (Table 1). In a single synthetic transformation, the M3 antimuscarinic potency was improved

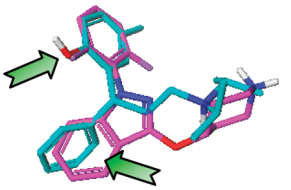
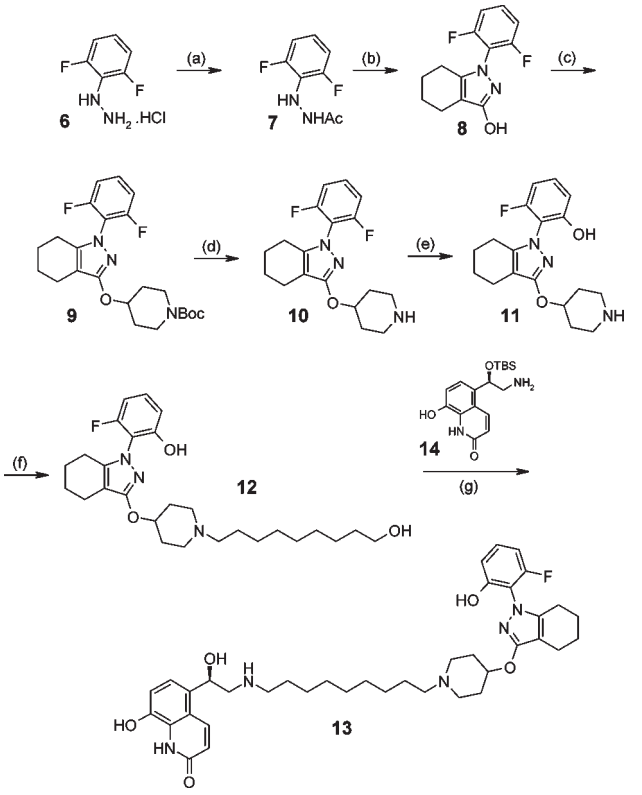


Figure 2. Molecular overlay of tolterodine **1** (blue, from small molecule crystal structure)[9] and energy minimized indazole **4** (pink). Arrows indicate design focus.

Scheme 2[a]



[a] Reagents and conditions: (a) Ac_2O, toluene; (b) 2-oxocyclohexanecarboxylic acid methyl ester, PCl_3, MeCN; (c) PPh_3, DIAD, THF; (d) HCl, dioxane; (e) ^{t}BuOK, dioxane, reflux, then HCl in dioxane; (f) 9-bromononanol, $NaHCO_3$, MeCN; (g) (i) SO_3·pyridine, DMSO, NEt_3; (ii) **14**, EtOH, $NaB(OAc)_3H$, CH_2Cl_2; (iii) NEt_3·3HF, THF.

5-fold. Interestingly, this is the first report of a molecule containing the *N*-(*o*-hydroxyphenyl)pyrazole substructure.

Additionally, M3 SAR suggests that one of the benzene rings usually present in aminergic GPCR inhibitors can be saturated to a cyclic aliphatic system, resulting in an improvement in affinity.[11] There is limited precedence that ring B in tolterodine can be saturated in this manner,[12] and therefore, guided by the overlay in Figure 2, the benzene ring of the indazole was "reduced". Acetylation of commercially available hydrazine **6** provided **7** that was cyclized with the requisite keto ester yielding hydroxypyrazole **8** (Scheme 2). Mitsunobu alkylation to **9** and subsequent deprotection provided **10** that was converted to the phenol **11** using the conditions described above.

This transformation resulted in a further improvement in potency such that the two-step medicinal chemistry conversion

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Table 2. Pharmacology, Metabolism, and Membrane Flux of MABA 13[a]

M3 K_i[b]	2.2 nM
β_2 EC_{50}[c]	14 nM
β_1 EC_{50}	>10 μM
M3 offset[d]	<16 min
β_2 wash-off[e]	1.5-fold
GPT EFS EC_{50}[f]	28 nM
GPT EFS DoA[f]	>16 h
GPT (+propranolol) EC_{50}[g]	27 nM
GPT (+propranolol) DoA[g]	>16 h
log *D*	2.4
cLogP	5.4
HLM	25 (μL/min)/mg
CACO-2 flux	$<1 \times 10^{-6}$ cm/s

[a] All data are geometric mean values with 95% CI, $n = 4$. [b] Potency of compounds at the human cloned M3 receptor using [^{3}H]NMS. [c] Ability to stimulate intracellular cAMP production in CHO-hβ_2 cells. [d] Offset kinetics from human cloned M3 receptor using [^{3}H]NMS (dilution method). [e] Fold-difference in β_2 potency following receptor wash. [f] GPT = guinea pig trachea. EFS = electric field stimulation. Potency and duration for β_2 and M3 components of bronchodilation. Submaximal concentration used for the duration is that which gives 70% inhibition of the EFS response. [g] Potency and duration for M3 component of bronchodilation.

of Bioprint hit **4** to pyrazole **11** improved M3 potency 20-fold, even though the molecule was 10-fold more polar (Table 1). As we predicted, the selectivity of **11** was significantly improved over starting point **4** and possessed considerably less activity as an NRI (>1000-fold selective over NET; see Table 1). Compound **11** also possessed excellent metabolic stability as assessed in human liver microsomes (HLM), suggesting that molecules from this class would be interesting to pursue as oral anticholinergic leads.

Conjugation of the selective antimuscarinic **11** to the quinolinone β_2 agonist headgroup is continued in Scheme 2. Installation of the C9-linker (previously proven optimal because of possible multivalent effects in the resultant MABA)[13] yielded **12**, and oxidation of the alcohol followed by reductive amination with the known amine **14**[4e] and desilylation furnished MABA **13**.

The pharmacological profile of **13** is shown in Table 2. MABA **13** possessed impressive primary biochemical pharmacology against M3 and β_2. The kinetics of pharmacological duration were assessed for β_2 agonism through a simple receptor wash-off assay as previously described,[14] and the small shift in potency following wash is indicative of long duration. Kinetics for the M3 component were assessed using a dilution-offset methodology whereby the offset was inferred from the on rate of coadministered ^{3}H-NMS (*N*-methylscopolamine) and is expressed as the time taken to reach 50% of total ^{3}H-NMS for solvent treated membranes.[15] The fast offset from the M3 receptor may be indicative of short pharmacological duration. We have previously suggested that cLogP > 5.5 for MABAs such as **2** is required for long offset kinetics.[4c]

We then assessed the potency of **13** in the guinea pig trachea (GPT) model using electric field stimulation (EFS) to release endogenous acetylcholine.[14] The drive for bronchoconstriction in this model operates through both β_2 and M3 mechanisms, and therefore, values in this assay reflect combined bronchodilatory effects. A "duration of action" (DoA) was defined as the time taken for the muscle tone at a submaximal concentration of the compound to recover by 50% of the inhibition induced. As expected, **13** is a potent bronchodilator in this model with impressive duration of action (Table 2). To delineate the M3 drive, the β_2 antagonist propranolol was added to the EFS model to block the β_2 effect. MABA **13** retained M3 potency as predicted from the in vitro experiment, but surprisingly, the DoA was significantly longer than that predicted by the offset assay. These results are significant, since generally there are good correlations between M3 offset and pharmacological duration in the GPT assay (suggesting that an alternative mechanism is at play and further work is warranted to explain this observation).

The relatively high clearance in human liver microsomes combined with poor membrane permeability (assessed as flux across the human colon carcinoma CACO-2 monolayer, Table 2) predicts that MABA **13** would have poor oral bioavailability, a necessary attribute for inhaled molecules where the risks of systemically driven side effects from the swallowed component can be ameliorated in this way.[4]

CONCLUSIONS

Introduction of hydrogen bonding potential and benzene ring saturation of a Bioprint hit resulted in the successful conversion of a nonselective NRI into a selective muscarinic antagonist. Subsequent conjugation to a quinolinone β_2 agonist motif efficiently furnished a MABA with balanced potency and pharmacological duration. Further exploration of this series includes a detailed in vivo pharmacological analysis and optimization of the synthetic chemistry to furnish additional molecules from this class. This research expands the opportunities for triple therapeutic approaches for the treatment of COPD. Additionally, this article provides a proof of concept that hits from a wide-ligand profile database can be converted into novel and selective GPCR modulators.

EXPERIMENTAL SECTION

General Methods. Unless otherwise stated, all reactions were carried out under a nitrogen atmosphere, using commercially available anhydrous solvents. Thin-layer chromatography was performed on glass-backed precoated Merck silica gel (60 F254) plates, and FCC (flash column chromatography) was carried out using 40−63 μm silica gel. NMR spectra were carried out on a Varian Mercury 400 spectrometer in the solvents specified. Mass and LCMS spectra were recorded on a Waters Micromass ZQ using electrospray chemical ionization (ESCI). Purities for all compounds were determined to be ≥95% by LCMS. Other abbreviations are used in conjunction with standard chemical practice.

3-Fluoro-2-[3-(piperidin-4-yloxy)indazol-1-yl]phenol (5). **4** (615 mg, 1.68 mmol) was dissolved in THF (30 mL). Potassium *tert*-butoxide (754 mg, 6.72 mmol) was added and the mixture heated to reflux for 16 h. HCl (aq, 4 M in dioxane, 20 mL) was added carefully and the solution refluxed for 2 h. The solvents were removed under reduced pressure and the mixture partitioned between EtOAc and saturated $NaHCO_3$ (aq). The organic layer was washed with water, then brine, separated, dried ($MgSO_4$), filtered, and concentrated. The mixture was triturated with diethyl ether, washed with cold methanol to yield **5**: 550 mg (75%).

Acetic Acid *N*′-(2,6-Difluorophenyl)hydrazide (7). **6** (5.0 g, 23 mmol) was partitioned between saturated $NaHCO_3$ (aq) and EtOAc. The organics were separated, dried ($MgSO_4$), filtered, and concentrated. The residue was dissolved in toluene (25 mL). Acetic anhydride (2.40 mL, 25.3 mmol) was added. After 1 h, the solvent was removed

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under reduced pressure and the residue triturated with diethyl ether to yield **7**: 2.90 g (68%).

1-(2,6-Difluorophenyl)-4,5,6,7-tetrahydro-1*H*-indazol-3-ol (8). **7** (2.90 g, 15.6 mmol) and 2-oxocyclohexanecarboxylic acid methyl ester (2.43 g, 15.6 mmol) were dissolved in acetonitrile (10 mL). PCl_3 (1.63 mL, 18.7 mmol) was added carefully. The mixture was heated to 50 °C for 40 h, then cooled to rt, poured on ice, extracted with EtOAc, washed with water, then brine, separated, dried ($MgSO_4$), filtered, and concentrated. The residue was triturated with MeOH to provide **8**: 2.0 g (51%).

4-[1-(2,6-Difluorophenyl)-4,5,6,7-tetrahydro-1*H*-indazol-3-yloxy]piperidine-1-carboxylic Acid *tert*-Butyl Ester (9). **8** (5.0 g, 20 mmol) and 4-hydroxypiperidine-1-carboxylic acid *tert*-butyl ester (4.0 g, 20 mmol) were dissolved in THF (100 mL). PPh_3 (5.8 g, 22 mmol) and then DIAD (4.4 g, 22 mmol) were added. After 16 h, the mixture was diluted with EtOAc, washed with water, then brine, separated, dried ($MgSO_4$), filtered, and concentrated. The residue was purified by FCC (heptanes/EtOAc, 70/30) to provide **9**: 8.7 g (76%).

1-(2,6-Difluorophenyl)-3-(piperidin-4-yloxy)-4,5,6,7-tetrahydro-1*H*-indazole Hydrochloride (10). **9** (6.6 g, 15.2 mmol) was treated with HCl (4 M in dioxane, 20 mL, 80 mmol). After 1 h the solvent was removed under reduced pressure and the residue triturated with CH_2Cl_2 to provide **10**: 5.0 g (99%).

3-Fluoro-2-[3-(piperidin-4-yloxy)-4,5,6,7-tetrahydroindazol-1-yl]phenol (11). **10** (5.0 g, 15 mmol) was dissolved in dioxane (80 mL). Potassium *tert*-butoxide (6.8 g, 60 mmol) was added and the mixture heated to reflux for 16 h. HCl (aq, 4 M in dioxane, 100 mL) was added carefully and the solution refluxed for 2 h. The solvents were removed under reduced pressure and the mixture partitioned between EtOAc and saturated $NaHCO_3$ (aq). The organic layer was washed with water, then brine, separated, dried ($MgSO_4$), filtered, and concentrated. The mixture was triturated with diethyl ether, washed with cold methanol to yield **11**: 4.5 g (90%).

3-Fluoro-2-{3-[1-(9-hydroxynonyl)piperidin-4-yloxy]-4,5,6,7-tetrahydroindazol-1-yl}phenol (12). **11** (250 mg, 0.75 mmol), 9-bromononanol (168 mg, 0.75 mmol), and $NaHCO_3$ (190 mg, 2.26 mmol) were added to acetonitrile (20 mL) and heated at relux for 16 h. The solvents were removed under reduced pressure, and the mixture was partitioned between EtOAc and water. The organic layer was separated, dried ($MgSO_4$), filtered, and concentrated to provide **12**: 320 mg (90%).

5-[2-(9-{4-[1-(2-Fluoro-6-hydroxyphenyl)-4,5,6,7-tetrahydro-1*H*-indazol-3-yloxy]piperidin-1-yl}nonylamino)-1-hydroxyethyl]-8-hydroxy-1*H*-quinolin-2-one (13). **12** (160 mg, 0.34 mmol) was dissolved in DMSO (5 mL). Then NEt_3 (470 μL, 3.38 mmol) and sulfur trioxide−pyridine complex (215 mg, 1.35 mmol) were added. The mixture was stirred for 16 h. The mixture was partitioned between EtOAc and water. The organic layer was washed with brine, separated, dried ($MgSO_4$), filtered, and concentrated. The crude aldehyde was dissolved in CH_2Cl_2 (9 mL) and EtOH (1 mL), and the amine **14**[4e] was added (106 mg, 0.32 mmol). $NaB(OAc)_3H$ (201 mg, 0.95 mmol) was added and the mixture stirred for 16 h. The solvents were removed under reduced pressure, and the residue was partitioned between EtOAc and water. The organic layer was separated, dried ($MgSO_4$), filtered, and concentrated. The crude adduct was dissolved in THF (10 mL). $NEt_3 \cdot 3HF$ (255 mg, 1.58 mmol) was added and the mixture stirred for 16 h. The mixture was partitioned between EtOAc and water, and the organic layer was separated and concentrated. The residue was purified by FCC (CH_2Cl_2/MeOH/NH_3 (aq) 80/20/2) to provide **13**: 25 mg (11% over three steps). ^1H NMR (CD_3OD, 400 MHz) δ 1.20−1.45 (m, 10H), 1.47−1.64 (m, 4H), 1.65−1.91 (m, 6H), 2.05 (m, 2H), 2.28−2.46 (m, 8H), 2.78−2.90 (m, 4H), 3.00 (m, 2H), 4.58 (m, 1H), 5.27 (m, 1H), 6.63−6.74 (m, 2H), 6.78 (m, 1H), 6.97 (m, 1H), 7.20−7.29 (m, 2H), 8.37 (m, 1H); LCMS *m/z* 676 $(M + H)^+$, >95% pure.

■ ASSOCIATED CONTENT

Supporting Information. Spectroscopic details for **5**, **7**−**12**; selectivity data for **1**, **4**, and **11**. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

CHO, Chinese hamster ovary; COPD, chronic obstructive pulmonary disease; DIAD, diisopropyl azodicarboxylate; DMSO, dimethylsulfoxide; DoA, duration of action; EFS, electric field stimulationn; ESCI, electrospray chemical ionization; FCC, flash column chromatography; GPCR, G-protein-coupled receptor; GPT, guinea pig trachea; HLM, human liver microsome; LABA, long acting β-2 agonist; LAMA, long-acting muscarinic antagonist; LCMS, liquid chromatography−mass spectrometry; MABA, muscarinic antagonist−β_2-agonist; nd, not determined; NET, norepinephrin transporter; NMR, nuclear magnetic resonance; NMS, *N*-methylscopolamine; NRI, norepinephrin reuptake inhibitor; rt, room temperature; SAR, structure−activity relationship; THF, tetrahydrofuran; WLP, wide-ligand profile

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