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Evidence that the multiflorine-derived substituted quinazolidine 55P0251 augments insulin secretion and lowers blood glucose via antagonism at α2-adrenoceptors in mice

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Abstract

Aims: To investigate the mechanism of action of 55P0251, a novel multiflorine-derived substituted quinazolidine that augments insulin release and lowers blood glucose in rodents, but does not act via mechanisms addressed by any antidiabetic agent in clinical use.

Materials and Methods: Using male mice, we determined the effects of 55P0251 on glucose tolerance, insulin secretion from isolated islets and blood oxygen saturation, including head-to-head comparison of 55P0251 to its inverted enantiomer 55P0250, as well as to other anti-hyperglycaemic multiflorine derivatives discovered in our programme.

Results: 55P0251 was clearly superior to its inverted enantiomer in the glucose tolerance test (area under the curve: 11.3 mg/kg 55P0251, 1.19 ± 0.04 min*mol/L vs 55P0250, 1.80 ± 0.04 min*mol/L; $P < .0001$). For insulin release in vitro, this superiority became visible only under concomitant adrenergic background stimulation (glucose-stimulated insulin release, fmol*islet^{-1}*30 min^{-1}: without α2-adrenoceptor agonist: 500 μmol/L 55P0251, 390 ± 34, vs 55P0250, 459 ± 40, nonsignificant; with α2-adrenoceptor agonist: 250 μmol/L 55P0251, 138 ± 9, vs 55P0250, 21 ± 6; $P < .0001$). Since receptor binding assays suggested antagonism at α2A-adrenoceptors as a potential mechanism of action, we measured oxygen saturation in capillary blood from the tail as a surrogate of vasoconstriction, which supported α2-antagonistic action in vivo (90 mg/kg 55P0251, $83 \pm 3\%$, vs 55P0250, $57 \pm 3\%$; $P < .0001$). Lack of association between glucose-lowering activities and α2A-adrenoceptor binding affinity arising from comparison of multiflorine derivatives was attributed to differences in their pharmacokinetic properties.

Conclusions: Our findings suggest that 55P0251 and related multiflorine derivatives are to be categorized as α2-adrenoceptor antagonists with potential to lower blood glucose by blocking α2A-adrenoceptors on pancreatic β cells.

KEYWORDS
animal pharmacology, antidiabetic drug, drug development, drug mechanism, insulin secretion

1 | INTRODUCTION

The majority of novel drugs reaching the market are still directly or indirectly derived from natural products.[1,2] Although discovery strategies based on a predefined molecular target and/or mechanism of action are currently more popular, the search for novel structures in natural remedies, which are traditionally used and carry disease-relevant activity, remains a rational approach. In the Mediterranean area, lupins have traditionally been used for the treatment of diabetes mellitus.[3] They are rich in quinolizidine alkaloids, such as lupanines, sparteine and multiflorine, which among other actions have been associated with insulinotropic effects on isolated pancreatic islets as well as with lowering of blood glucose.[4-10]

Based on work from Kubo et al,[9,10] who described multiflorine derivatives with antidiabetic properties in mice, we used a molecular scaffold derived from (−)-multiflorine as the starting point for an extensive derivatization programme. In line with the idea of building on bioactivity in vivo, we used glucose tolerance tests in orally dosed mice for structural optimization and discovered a novel class of fully synthetic substituted quinazolidines with distinct anti-hyperglycaemic activity.[11,12] Structural development based on oral administration and a disease-relevant endpoint allows selection for distinctive acute side effects, solubility, formulation properties and bioavailability at the earliest possible stage. This approach requires parallel efforts for tracking down the initially unknown molecular and biochemical mechanism(s) at work.

Our prototype compound 55P0110 exhibited a promising pharmacological profile, with the acute effects of a single dose outmatching most established oral antidiabetic drugs in the mouse glucose tolerance test.[11] Encouraged by this outcome, we continued our efforts, resulting in the development of numerous active derivatives, from which 55P0251 was selected for detailed examination. 55P0251 is a further-refined structure, which carries superior efficacy in comparison to 55P0110 accompanied by attractive pharmacokinetic attributes.[12] In rodents, 55P0251 exhibited attractive anti-hyperglycaemic, but not hypoglycaemic action, which could be attributed to augmentation of glucose-stimulated insulin secretion via a molecular mechanism that differed from those addressed by drugs currently in use for the treatment of diabetes mellitus. No evidence was found for an extrapancreatic mechanism contributing to the lowering of blood glucose.[12] Based on several experimental approaches, including comparison to promising candidates that were discovered later than 55P0251, we here report further efforts to pin down the molecular target and the mechanisms responsible for blood glucose-lowering.

2 | MATERIALS AND METHODS

2.1 | Animals

Male C57BL/6J mice and db/db mice were purchased from Charles River Laboratories (Sulzfeld, Germany) and male Sprague–Dawley rats were from the Core Unit for Biomedical Research, Division for Laboratory Animal Science and Genetics, Medical University of Vienna (Himberg, Austria). All animals were housed under an artificial 12-hour dark/12-hour light cycle at room temperature and, where not stated otherwise, had free access to tap water and a conventional carbohydrate-rich chow diet (sniff R/M-H; sniff Spezialdiäten GmbH, Soest, Germany). All procedures and experiments were approved by the Austrian Federal Ministry of Science and Research (approvals # BMWF-66.009/0317-II/3b/2008, -/0074-II/10b/2008, -/0100-II/3b/2011, and -/0178-II/3b/2012).

2.2 | Test compounds

Substituted quinazolidines from our derivatization programme were synthesized at 55pharmas chemistry laboratories (Tulln, Austria). The compounds were formulated as the free base or as salts. Since an influence of the employed formulation on anti-hyperglycaemic activity has never been noted (documented for 55P0251 by Stadlbauer et al[12]), we always refer to the parent compound throughout the present report. Accordingly, all indicated doses and concentrations refer to the free base.

2.3 | Glucose tolerance tests in mice

Oral glucose tolerance tests (OGTTs) were performed in mice fasted for ~10 hours and were performed according to a standard protocol described previously.[11,12] In short, the tip of the tail was pricked and basal blood glucose was measured in duplicate with a portable glucose meter (OneTouch; LifeScan, Milpitas, California). Immediately thereafter, mice were fed by gavage the vehicle alone (0.5% sodium carboxy-methylcellulose; 5 mL/kg body weight) or the indicated dose of the respective compound. Forty-five minutes after this first gavage, glycaemia was again measured and the OGTT was started by gavage feeding of aqueous glucose solution (3 g/kg in 6 mL/kg body weight). The time interval of 45 minutes between compound administration and starting the OGTT appeared appropriate for compounds with clinically useful pharmacokinetics, for example, sufficiently fast absorption and no first pass effect. Duplicate measurements of blood glucose were made 30, 60, 90, 120 and 150 minutes after glucose feeding. Where stated, time intervals of >45 minutes were used between the administration of drug/vehicle and glucose feeding.

2.4 | Perifusion of mouse pancreatic islets

As described before,[13,14] mice in the fed state were deeply anaesthetized with sevoflurane and killed by cervical dislocation. The common bile duct was clamped at the papilla Vateri and 3 mL collagenase solution was injected via the bile duct, which caused distension of the pancreatic tissue (1 g/L Collagenase NB; Serva, Heidelberg, Germany; dissolved in ice-cold Krebs–Ringer buffer, supplemented with 0.2% bovine serum albumin and 5 mmol/L HEPES; pH adjusted with NaOH to 7.35; this solution is referred to hereafter as KRB). The distended pancreas was digested for 13 minutes at 37°C and then further disrupted by vigorous hand shaking. Digestion was stopped by two cycles of centrifugation and resuspension in ice-cold KRB containing the same glucose concentration

as provided during the initial equilibration period of the subsequent perifusion experiment. From the pancreatic digest, 50 intact islets were collected under the microscope, transferred to a custom-made perifusion chamber, and perifused for equilibration with KRB containing 0.1% DMSO (60 minutes; 37°C; 1 mL/min). The perifusion medium for equilibration contained 5 mmol/L glucose or, alternatively, 7 mmol/L glucose plus 1 μmol/L of the α2-adrenoceptor agonist UK14,304 (brimonidine). Effluent for the duplicate measurement of insulin (Sensitive Rat Insulin RIA Kit; Millipore, Billerica, Massachusetts) was collected at −20 minutes, −10 minutes and immediately before the equilibration period ended and the subsequent test period started. During the test period, a test compound was added to the medium at the indicated concentration (only the vehicle added in control experiments). Thirty minutes later, the islets were stimulated with high glucose for the remaining 30 minutes of the perifusion (10 mmol glucose in experiments without, and 20 mmol/L glucose in experiments with UK14,304). During the total 60 minutes of the test period, effluent was collected after 2.5, 5, 7.5, 10, 15, 20, 30, 32.5, 35, 37.5, 40, 45, 50 and 60 minutes.
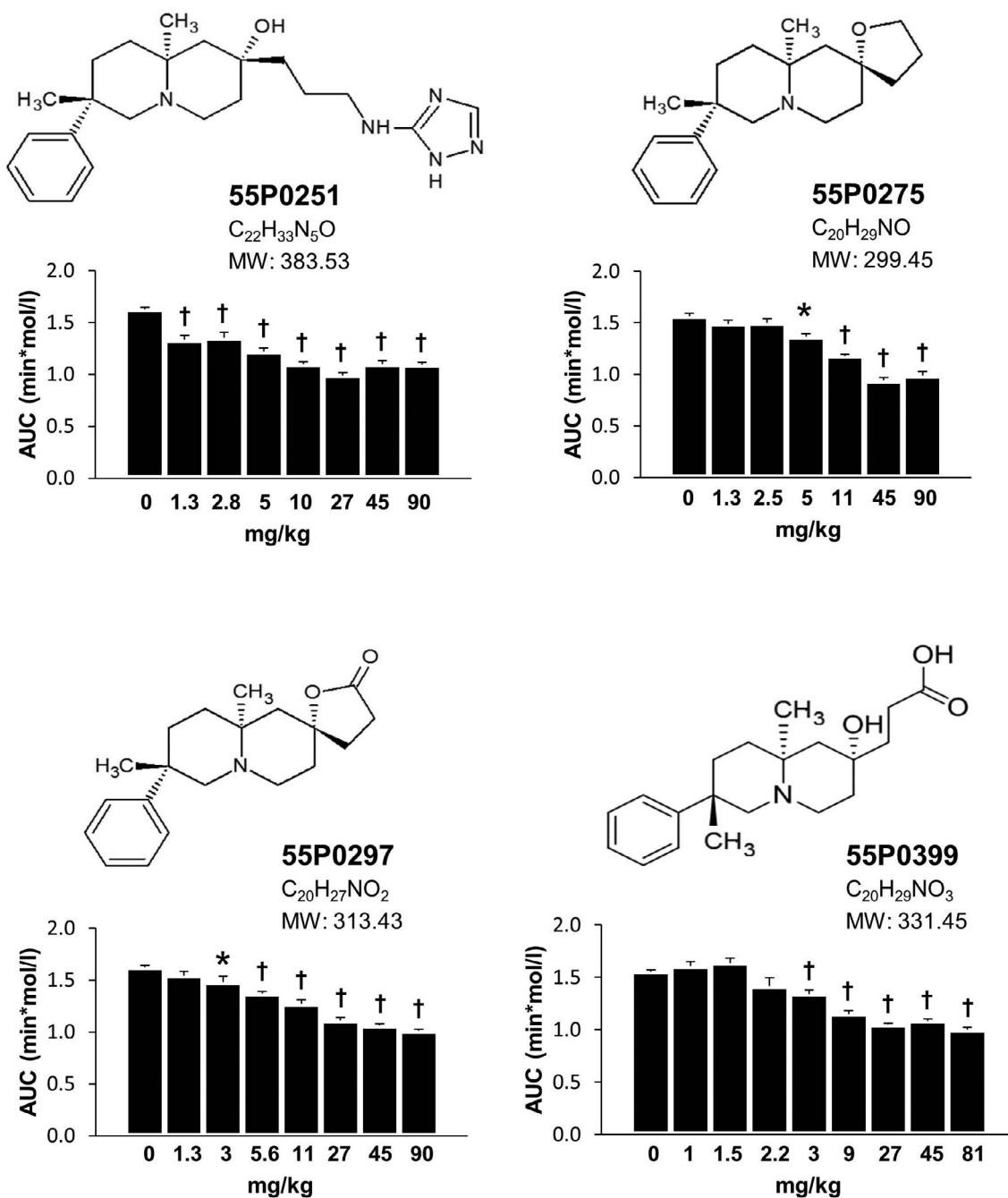

FIGURE 1 Structures, elemental formulas and molecular weights of substituted quinazolidines used in this study. Dose-dependent effects on areas under the glucose curves (AUC) are shown as obtained in oral glucose tolerance tests (OGTTs) performed in mice 45 minutes after a single oral dose of the respective compound. Means ± SEM; *$P < .01$; †$P < .0001$ versus control

2.5 | Pharmacokinetics

Mice were fasted for ~10 hours and received, by gavage, the indicated dose of compound 55P0399 (in 0.5% sodium carboxymethylcellulose, 5 mL/kg body weight). After a predefined time period, each mouse was deeply anaesthetized with sevoflurane and killed by bleeding via heart puncture. Blood was collected into chilled tubes containing EDTA and the plasma fraction was stored at −80°C for the later measurement of the concentration of 55P0399 and its metabolite

55P0297 by high-performance liquid chromatography at 55pharmas chemistry laboratories.

2.6 | Oxygen saturation in capillary blood of the tail

Mice or rats were fasted for ~10 hours, before they received, by gavage, a single oral dose of the indicated compound or the corresponding vehicle. Seventy-five mintues later, capillary blood was sampled from the tip of the
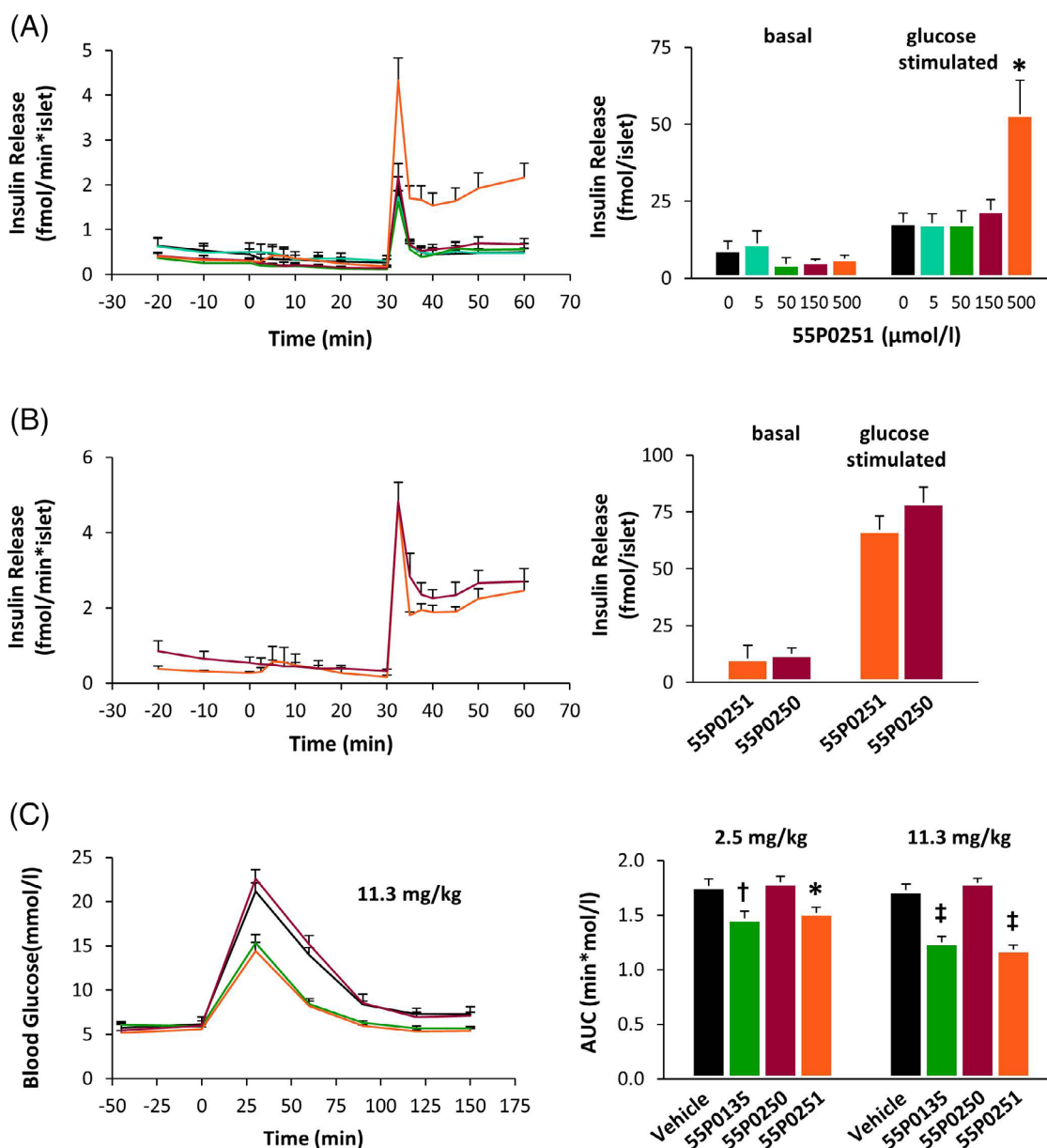

FIGURE 2 A, Isolated mouse islets were perifused for equilibration with medium containing 5 mmol/L glucose for 1 hour, before being exposed to different concentrations of 55P0251 from 0 minutes onwards. Thirty minutes later, islets were stimulated with 10 mmol/L glucose. Bar graphs show the cumulative amounts of insulin released under basal (0-30 minutes) and glucose-stimulated (30-60 minutes) conditions (n = 5-8 each). B, Using the same protocol as in (A), mouse islets were exposed to 500 μmol/L of 55P0251 or its inverted enantiomer 55P0250 (n = 4 each). C, Mice received a single oral dose of 2.5 mg/kg or 11.3 mg/kg 55P0135 (racemate), 55P250 or 55P0251 (pure enantiomers) 45 minutes before an oral glucose tolerance test (OGTT) (3 g/kg) was performed. Bar graphs show areas under the glucose curves (AUC; n = 8-9 each; full curves depicted for 11.3 mg/kg only). Means ± SEM; colour codes for line graphs are indicated by corresponding bar colours; bar graphs: *$P < .05$; †$P < .01$; ‡$P < .0001$ vs control
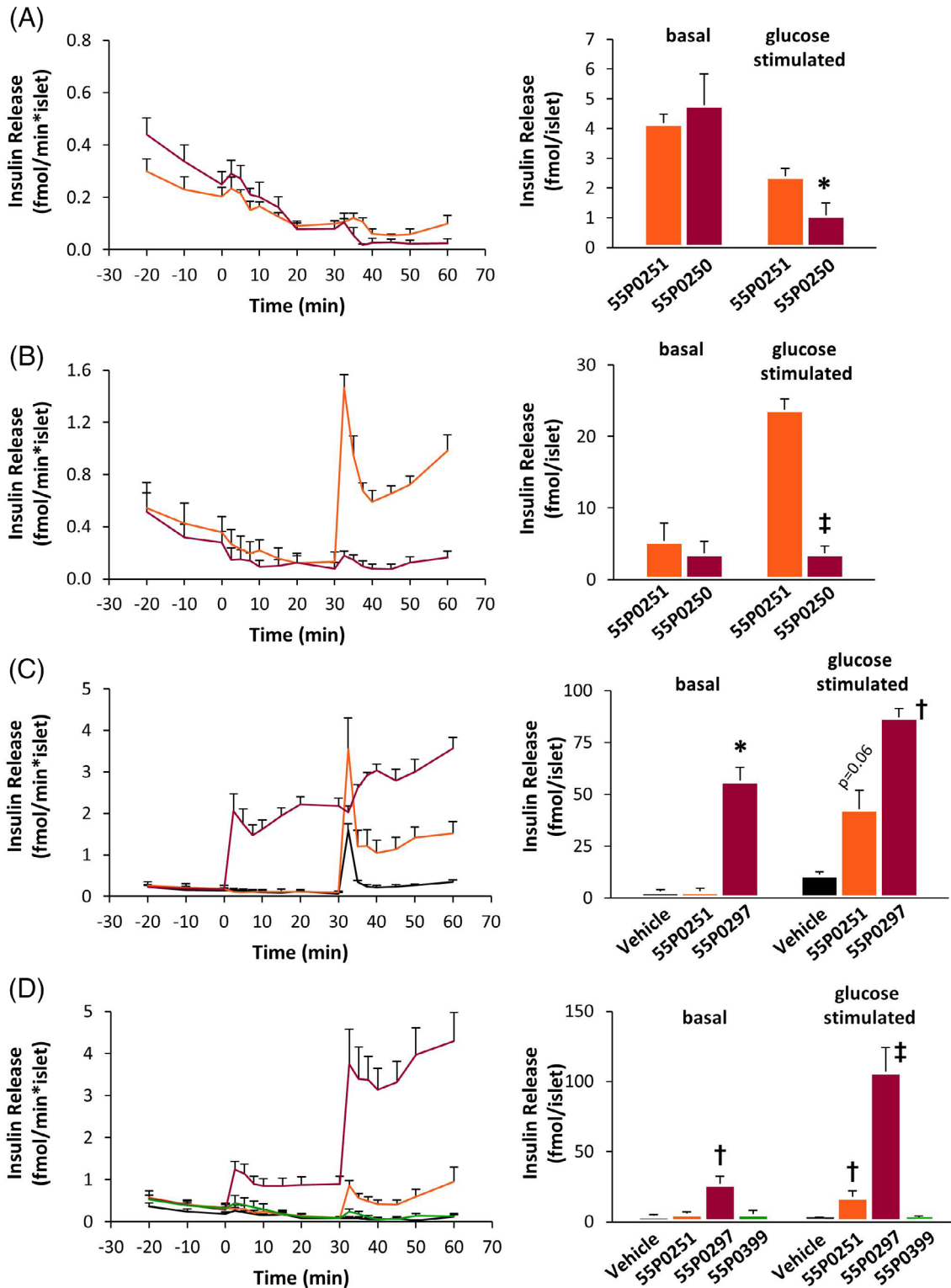
FIGURE 3 Isolated mouse islets were perifused for equilibration with medium containing 7 mmol/L glucose and 1 μmol/L of the α2-adrenoceptor agonist UK14,304 for 1 hour, before being exposed to A, 25 μmol/L or B, 250 μmol/L of 55P0251 or its inverted enantiomer 55P0250 from 0 minutes onwards. Thirty minutes later, islets were stimulated with 20 mmol/L glucose. C, Isolated mouse islets were perifused for equilibration with medium containing 5 mmol/L glucose for 1 hour, before being exposed to 500 μmol/L of 55P0251 or 55P0297 from 0 minutes onwards. Thirty minutes later, islets were stimulated with 10 mmol/L glucose. D, Isolated mouse islets were perifused under conditions as in (A) and (B), except that they were exposed to 250 μmol/L of 55P0251, 55P0297 or 55P0399 from 0 minutes onwards. Bar graphs show the total amounts of insulin released under basal (0-30 minutes) and glucose-stimulated (30-60 minutes) conditions; colour codes for lines are indicated by corresponding bar colours; means ± SEM; n = 3-6 each; bar graphs: $*P < .05$; $†P < .01$; $‡P < .001$ vs 55P0251 (A and B), or vs vehicle (C and D)

tail and the percentage of oxyhaemoglobin was determined with an OSM3 hemoximeter (Radiometer, Copenhagen, Denmark). In some experiments, where stated, glucose solution was fed by gavage 45 minutes after administration of the test compound/vehicle (as in the OGTT: 3 g/kg in 6 mL/kg body weight). Both oxygen saturation and blood glucose were determined in capillary blood from the tail 30 minutes thereafter.

2.7 | Pharmacologically induced hyperglycaemia

To study the interaction of compound 55P0251 with pharmacological inhibitors of insulin secretion, we exploited a protocol successfully applied before.[13,14] Endogenous hyperglycaemia was induced by the administration of a single dose of the K_{ATP} channel opener diazoxide (250 mg/kg) or the α_2-adrenoceptor agonist UK14,304 (100 µg/kg). Whereas diazoxide was admixed to the respective gavage solution containing vehicle or 55P0251, UK14,304 or its vehicle (saline containing 7% DMSO; 5 mL/kg) was injected intraperitoneally immediately after the gavage. Blood glucose was measured immediately before the administration of the respective compounds (0 minutes), as well as 30, 60, 90, 120, 150 and 180 minutes later.

2.8 | Direct interaction with adrenoceptors in vitro

Screening assays for the analysis of direct interaction of the test compounds with adrenoceptors in vitro were performed by a commercial provider, MDS Pharma Services (King of Prussia, Pennsylvania; later acquired by Ricerca). Assays with insect Sf9 cells expressing the human α_{2A}-adrenoceptor employed 1 nmol/L [3H]MK-912 as the radioligand and 10 µmol/L WB-4101 to define specific binding. Inhibition of binding (%) was first examined at a concentration of 10 µmol/L of the test compounds. Based on five serial dilutions of the test compound, IC_{50} values were determined by non-linear least squares regression analysis.

Yohimbine was used as a positive control, and binding affinity constants (Ki) were calculated using the equation presented by Cheng and Prusoff.[15]

Subsequent assays, performed at the Institute of Biomedicine, University of Turku (Finland), employed cell membranes prepared from Chinese hamster ovary (CHO) cells expressing the human α_{2A}-, α_{2B}- and α_{2C}-adrenoceptor subtypes, or the mouse α_{2A}-subtype. The competition binding reactions were performed as described[16] with the radioligand [3H] RS79948-197 (0.3 nmol/L) and eight serial dilutions of each test compound. Rauwolscine served as a positive control, and phentolamine (100 µM) was employed to determine specific binding. Ki was determined by non-linear regression analysis. A procedure for the determination of agonist-induced stimulation of [35S]GTPγS binding mediated by the human α_2-adrenoceptor subtypes was used as previously described; blocking effects of established α_2-antagonists have been confirmed in this setting.[17,18] Eight serial dilutions of the natural agonist (−)-noradrenaline (0.0001, 0.001, 0.01, 0.1, 1, 10, 100, and 300 µmol/L), 1 µmol/L of the respective test compound, and approximately 0.1 nmol/L [35S]GTPγS were incubated with 5 µg of CHO membrane protein. From this, functional antagonism was calculated, that is, the extent to which the concentration–response curve of the agonist (−)-noradrenaline was shifted to the right by the respective antagonist. In addition, possible agonist activity of the test compounds at each α_2-adrenoceptor subtype was determined in the [35S] GTPγS-binding assay using serial concentrations of the test compounds (0.1, 0.3, 1, 3 and 10 µmol/L) in the absence of an agonist. Results were compared with those obtained with the full agonist (−)-noradrenaline.

2.9 | Statistical procedures

Results are given as means ± SEM. P values were calculated in the context of an exploratory data analysis using two-tailed unpaired t-tests, with P values <.05 taken to indicate statistical significance.

TABLE 1 Binding characteristics and functional antagonism of 55P0251, 55P0297 and 55P0399 at α_2-adrenoceptors

Adrenoceptor subtype							
Inhibition of binding[a], %		55P0251	55P0297	55P0399	55P0250	(+)-Efaroxan	Reserpine
	Human α_{2A}	80	97	38	1	97	14
Ki[b], nmol/L		55P0251	55P0297	55P0399	Rauwolscine		
	Human α_{2A}	615	21	5677	0.43		
	Mouse α_{2A}	886	18	10 430	8.2		
	Human α_{2B}	71	2.6	742	0.96		
	Human α_{2C}	402	14	3841	0.21		
Functional antagonism[b,c], µmol/L		55P0251	55P0297	55P0399			
	Human α_{2A}	3.1	69	1.6			
	Human α_{2B}	16	382	3.8			
	Human α_{2C}	11	131	2.2			

Abbreviation: Ki, affinity constant.
[a]Tested at MDS Pharma Services, King of Prussia, PA, USA.
[b]Tested at Institute of Biomedicine, University of Turku, Finland.
[c]The functional antagonism gives an indication of the extent to which the concentration–response curve of the agonist (−)-noradrenaline is shifted to the right by 1 µmol/L of each test compound. These values are not true estimates of antagonist potency, since only one concentration of each test compound was examined. Tabular results from two independent runs are shown in Table S2B.

3 | RESULTS AND DISCUSSION

3.1 | Compounds

The present paper depicts results from compounds 55P0251, 55P0275, 55P0297 and 55P0399, which in the course of our derivatization programme were selected for deeper examination. Their structures and dose-dependent effects in the standard OGTT are shown in Figure 1.

3.2 | The effect of 55P0251 on insulin release depends on adrenergic background stimulation

Preceding efforts to track down the mechanism(s), which underlie the improvement of glucose tolerance induced by compounds from our derivatization programme, provided evidence for amplification of the glucose-induced rise in plasma insulin.[11,12] Glucose-stimulated insulin release was also enhanced in isolated mouse pancreatic islets exposed to 55P0251 in vitro, suggesting direct effects on islets.[12] Consistent with these previous results, a detailed examination of dose dependence in isolated islets confirmed that 55P0251 failed to affect insulin release as long as the glucose concentration remained basal (5 mmol/L), but boosted insulin release when stimulated with 10 mmol/L glucose (Figure 2A). However, the concentration of 55P0251 required for such amplification of insulin release in vitro (>150 µmol/L) ranged far above the plasma levels associated with glucose-lowering activity in vivo (<1 µmol/L).[12] Furthermore, comparison of 55P0251 to its inverted enantiomer 55P0250 revealed no difference between their actions in vitro (Figure 2B), which distinctly contrasted with marked superiority of 55P0251 (as well as of the racemate, 55P0135) with regard to improvement of glucose tolerance in vivo (Figure 2C). Such divergent patterns of action raised the question of whether the enantiomer-independent effect on isolated islets could be driven by an unspecific or even toxic mechanism that differs from the cause of glucose lowering in vivo.

Among the blood glucose-lowering mechanisms that are not exploited in the clinic, but are experimentally established, is antagonism of adrenergic inhibition of insulin secretion. Since this effect becomes apparent under adrenergic background stimulation only,[13,19,20] we examined the effects of 55P0251 and 55P0250 on isolated islets in the presence of the adrenergic α_2-agonist UK14,304, which is known to inhibit insulin release via α_{2A}-adrenoceptors located on β cells.[21,22] Under such conditions, superiority of 55P0251 over 55P0250, as observed in vivo (Figure 2C), was unmasked also in vitro, suggesting an enantiomer-specific potential to significantly counteract adrenergic inhibition of insulin release (Figures 3A,B). The concentration of 55P0251 required for this response in vitro (25 µmol/L) was still higher than effective plasma levels, which could relate to different binding affinities and concentrations of the α_2-adrenoceptor agonist(s) present in vitro versus in vivo (UK14,304 versus adrenaline and noradrenaline). As shown in Table 1, 55P0251 was a potent competitor for ligand binding at α_{2A}-adrenoceptors, the adrenoceptor subtype mediating inhibitory effects on pancreatic β cells,[22-24] with 80% inhibition of binding at 10 µmol/L 55P0251 compared to no inhibition by 10 µmol/L 55P0250. Notably, both agonist dependence and enantiomer specificity in vitro and in vivo were highly reminiscent of what we have previously found for the established α_{2A}-adrenoceptor antagonist efaroxan.[13]
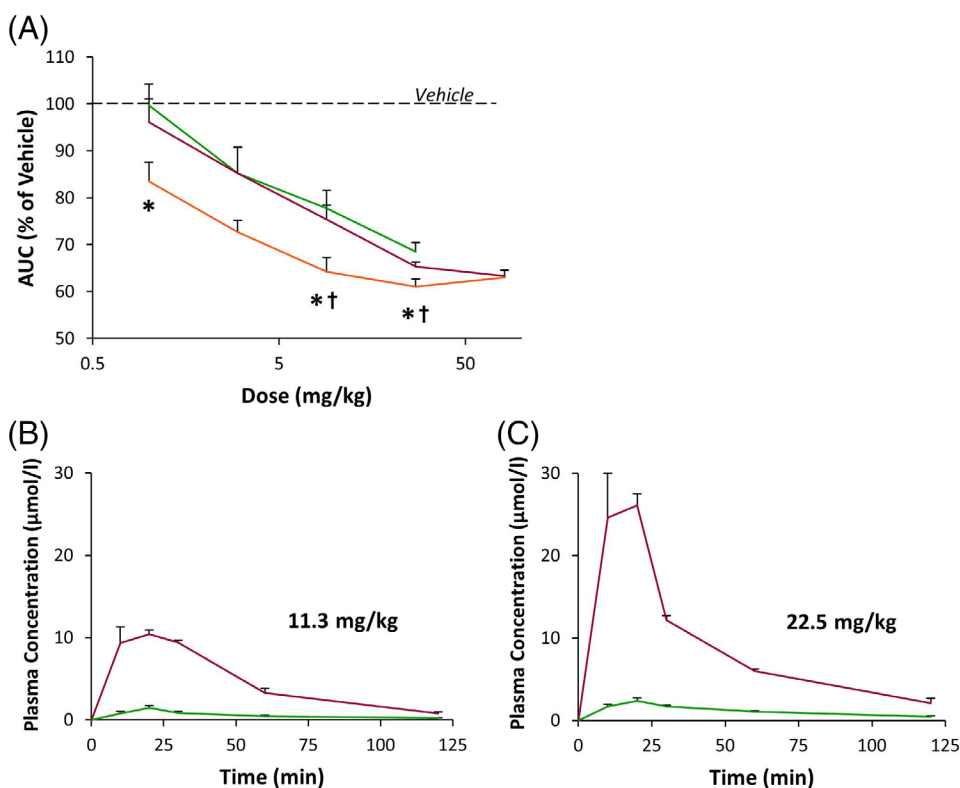

FIGURE 4 A, Dose-dependent effects of 55P0251 (orange), 55P0297 (green) and 55P0399 (red) on areas under the glucose curves (AUCs) from oral glucose tolerance tests (OGTTs) performed in mice 45 minutes after a single oral dose of the respective compound. Data given in % of the mean value obtained in vehicle-treated control mice. Means ± SEM; n = 8-9 each; *P < .05 vs 55P0297; †P < .05 vs 55P0399. B, Plasma concentrations in mice of 55P0297 (green) and 55P0399 (red) after an oral dose of 11.3 mg/kg (left graph) or 22.5 mg/kg 55P0399 (right graph). Means ± SEM; n = 3 each
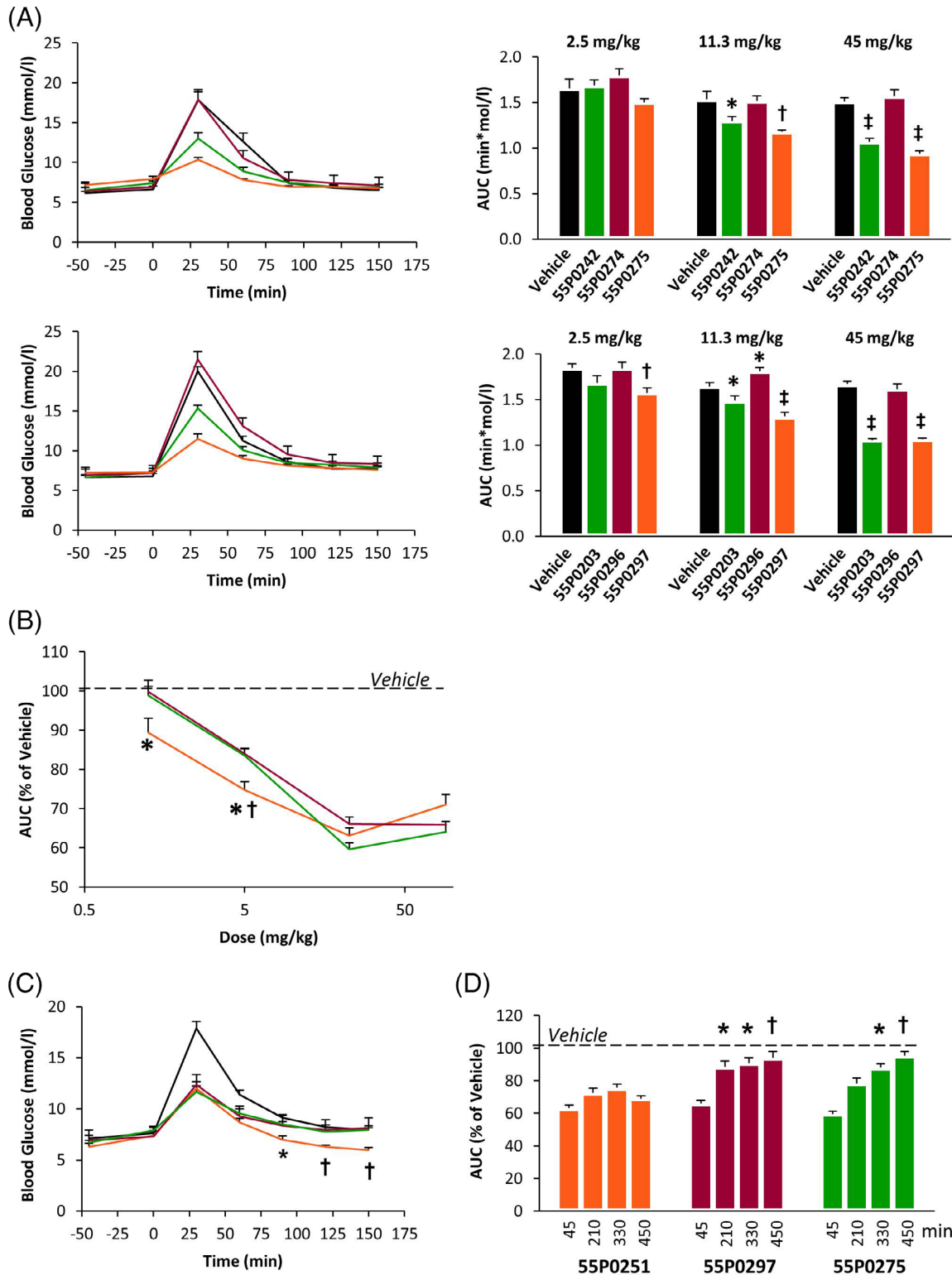
FIGURE 5 A, Mice received a single oral dose of 2.5, 11.3 or 45 mg/kg of racemates (55P0242; 55P0203; green) or their pure enantiomers (55P0274/55P0275; 55P0296/55P0297; red/orange) 45 minutes before an oral glucose tolerance test (OGTT) (3 g/kg) was performed. Bar graphs show areas under the glucose curves (AUCs). Means ± SEM; n = 8-9 each; colour codes for lines indicated by bar colours; bar graphs: *P < .05; †P < .01; ‡P < .0001 vs vehicle; full curves depicted for 11.3 mg/kg only. B, Dose-dependent effects of 55P0251 (orange), 55P0297 (red) and 55P0275 (green) on AUCs from OGTTs performed in mice 45 minutes after a single oral dose of the respective compound. Data given in % of mean of vehicle-treated mice. Means ± SEM; n = 7-9 each; *P < .05 vs 55P0297; †P < .05 vs 55P0275. C, Mice received a single oral dose of 5 mg/kg of 55P0251 (orange), 55P0297 (red), or 55P0275 (green) 45 minutes before an OGTT (3 g/kg) was performed. Means ± SEM; n = 8-9 each; *P < .001, †P < .001 for 55P0251 vs each other group. D, Effects of a single oral dose of 55P0251 (orange), 55P0297 (red) and 55P0275 (green) administered 45, 210, 330 or 450 minutes before an OGTT in mice. AUC, area under the glucose curve; data given in % of mean of vehicle-treated mice; means ± SEM; n = 7-9 each; *P < .001, †P < .0001 vs 55P0251

3.3 | Insulin release in vitro matches α₂ₐ-antagonistic activities of the individual derivatives

After evidence was obtained against the possibility that 55P0251 acts by modulating cAMP in islet cells (Table S1), we sought further evidence for or against α_{2A}-adrenoceptor antagonism as the cause of anti-hyperglycaemic action exerted by our novel structures. To this end, we compared the glucose-lowering activity of 55P0251 with that of other derivatives that had emerged from our derivatization programme. 55P0297 and 55P0399 exhibited similar minimal effective doses in the standard OGTT (Figure 1), but binding and functional antagonism assays with recombinant α_2-adrenoceptors in vitro showed that 55P0297 was clearly superior, whereas 55P0399 was clearly inferior to 55P0251 (Table 1). This hierarchy matched the effects of the individual compounds on insulin release from isolated islets (Figures 3C,D). Since no evidence was found of agonist activity of the test compounds at α_2-adrenoceptors (Table S2A), the results suggest that the direct actions on insulin release in vitro are at least mainly attributable to antagonism of α_2-adrenoceptors, with α_{2A} being the subtype located on pancreatic β cells and known to mediate regulation of insulin release.[22-24]

3.4 | Pharmacokinetics account for divergent activities in vivo versus in vitro

To obtain more precise information about relative potencies of the individual structures in the OGTT, direct head-to-head comparison within the same experimental run was performed in mice carefully matched for age and weight. While dose dependencies for 55P0297 and 55P0399 were very similar, 55P0251 was superior in the low dose range (Figure 4A). With regard to the hierarchy of individual compounds, direct effects on insulin release hence resembled α_{2A}-adrenoceptor binding, but deviated from anti-hyperglycaemic action. The presumptive explanation for such differences in vitro versus in vivo finally arose from examination of pharmacokinetics: whereas 55P0297 and 55P0399 were completely stable in solution for at least 1 day in the vial, roughly 10% of circulating 55P0399 was rapidly converted to 55P0297 after oral administration (Figure 4B). Despite the discrepant potencies of 55P0399 and 55P0297 as stimulators of insulin release in vitro as well as in the tests for binding and antagonism at α_{2A}-adrenoceptors (Table 1; Figure 3D), similar glucose-lowering activities in vivo (Figure 4A) thus seem to result from immediate interconversion, leading to a rapid equilibrium of plasma concentrations after oral administration. The information from a screening investigation of interactions of 55P0251, 55P0297 and 55P0399 with a broad panel of potential binding targets was compatible with this interpretation (Table S3).

Since comparison of the effects of 55P0297 and 55P0399 in vivo may therefore not be helpful, we selected another comparator, 55P0275 (Figure 1). 55P0275 and 55P0399 resembled 55P0251 in that their inverted enantiomers were largely inactive (Figure 5A; Figure 2C). Head-to-head comparison of AUC values from the standard OGTT revealed anti-hyperglycaemic effects of 55P0275, which in the low dose range were similar to those of 55P0399, but fell back somewhat versus those of 55P0251 (Figure 5B). At variance with this, 55P0275 was inferior to 55P0297 and superior to 55P0251 with respect to binding properties at the α_{2A}-adrenoceptor (Table 2). Juxtaposition of the individual structures therefore again revealed different hierarchies with regard to α_{2A}-adrenoceptor antagonism in vitro versus glucose-lowering activity in vivo.

Closer inspection of the OGTT curves revealed that 55P0251 was particularly more effective than its counterparts in the later course of the test (exemplified for the 5 mg/kg dose in Figure 5C). Aiming to pinpoint potential differences in time dependence, OGTTs were started at different time intervals after drug dosing. In this series of experiments, head-to-head comparison of the three compounds confirmed a higher durability of the effect of 55P0251 (Figure 5D), which fitted with evidence for a longer half-life of 55P0251 than 55P0297 (Figure 4B versus[12]).

In summary, the experiments based on compound comparison thus revealed dissociation of antagonistic properties at the α_{2A}-adrenoceptor in vitro from glucose-lowering activities in vivo. However, these seeming discrepancies could be plausibly explained by pharmacokinetic attributes. Hence, all results were ultimately compatible with the hypothesized role of α_{2A}-adrenoceptor antagonism in the actions of our novel compounds on insulin release and blood glucose.

3.5 | Evidence for effective α₂-adrenoceptor antagonism in vivo

While pharmacokinetics helped resolve several unclear matters, all the above evidence for α_{2A}-antagonism builds on in vitro experiments, including receptor binding and activation properties as well as insulin release from isolated islets. Hence, we sought evidence for α_2-adrenoceptor

TABLE 2 Displacement binding characteristics of 55P0251 (examined at 0.01, 0.1, 1 and 10 μmol/L), 55P0297 (examined at 0.3, 3, 30, 300 and 3000 nmol/L), 55P0275 (examined at 0.03, 0.1, 0.3, 1, and 3 μmol/L), yohimbine (examined at 0.3, 3, 30, 300 and 3000 nmol/L) and (+)-efaroxan (examined at 1, 3, 10, 30 and 100 nmol/L) at the human α_{2A}-adrenoceptor

		55P0251	55P0297	55P0275	(+)-Efaroxan[a]	Yohimbine
Ki, nmol/L	Human α_{2A}	1780	20	68	3.72	3.10
IC$_{50}$, nmol/L	Human α_{2A}	4750	54	183	9.91	8.40

Note: Tests were performed by MDS Pharma Services, King of Prussia, PA, USA.
Abbreviations: IC$_{50}$, half maximal inhibitory concentration; Ki, affinity constant.
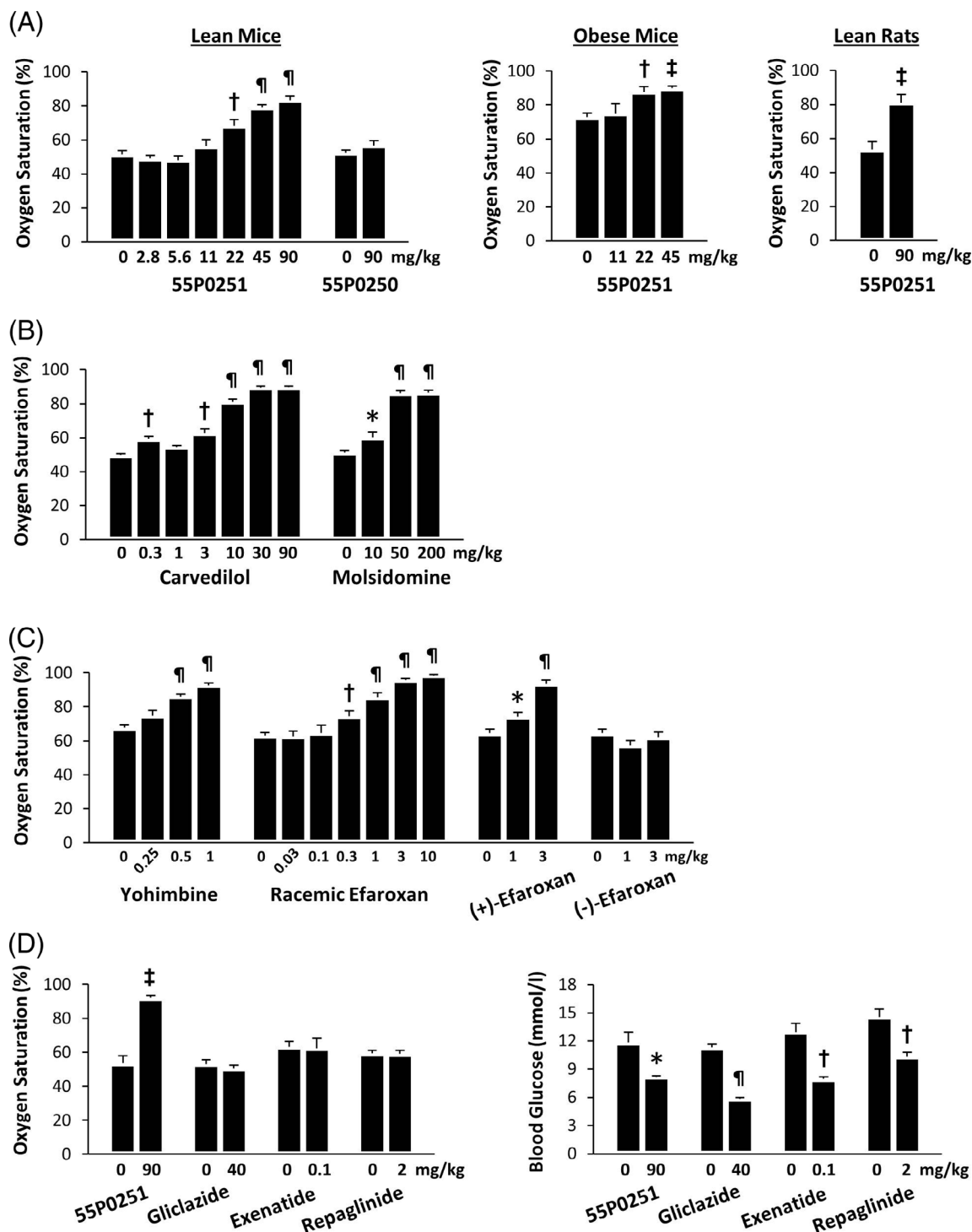[a]Data on (+)-efaroxan from Lehner et al[13] are included here for direct comparison.

FIGURE 6 Oxygen saturation in capillary blood from the tip of the tail 75 minutes after receiving an oral drug dose A, in lean mice, obese db/db mice and lean rats treated with 55P0251 or its inverted enantiomer 55P0250; B, in lean mice treated with the established vasodilators carvedilol and molsidomine; C, in lean mice treated with the established antagonists of the α_{2A}-adrenoceptor yohimbine and efaroxan, or with the purified enantiomers of efaroxan. D, Oxygen saturation in capillary blood from the tip of the tail in lean mice 75 minutes after receiving an oral dose of 55P0251 or of the insulin secretion stimulators gliclazide, exenatide and repaglinide, as followed by glucose feeding (3 g/kg) 45 minutes after drug administration (exenatide injected subcutaneously). Means ± SEM; $*P < .05$; $†P < .01$; $‡P < .001$; $¶P < .0001$ vs vehicle

antagonism in vivo. During the OGTTs, we noted that the anti-hyperglycaemic effects of our structures were accompanied by a brighter red colour of the capillary tail blood from our mice. This observation was confirmed by measurements of blood oxygen saturation, which was indeed dose-dependently increased 75 minutes after administration of 55P0251 in the tail blood of lean mice, lean rats and genetically obese db/db mice.
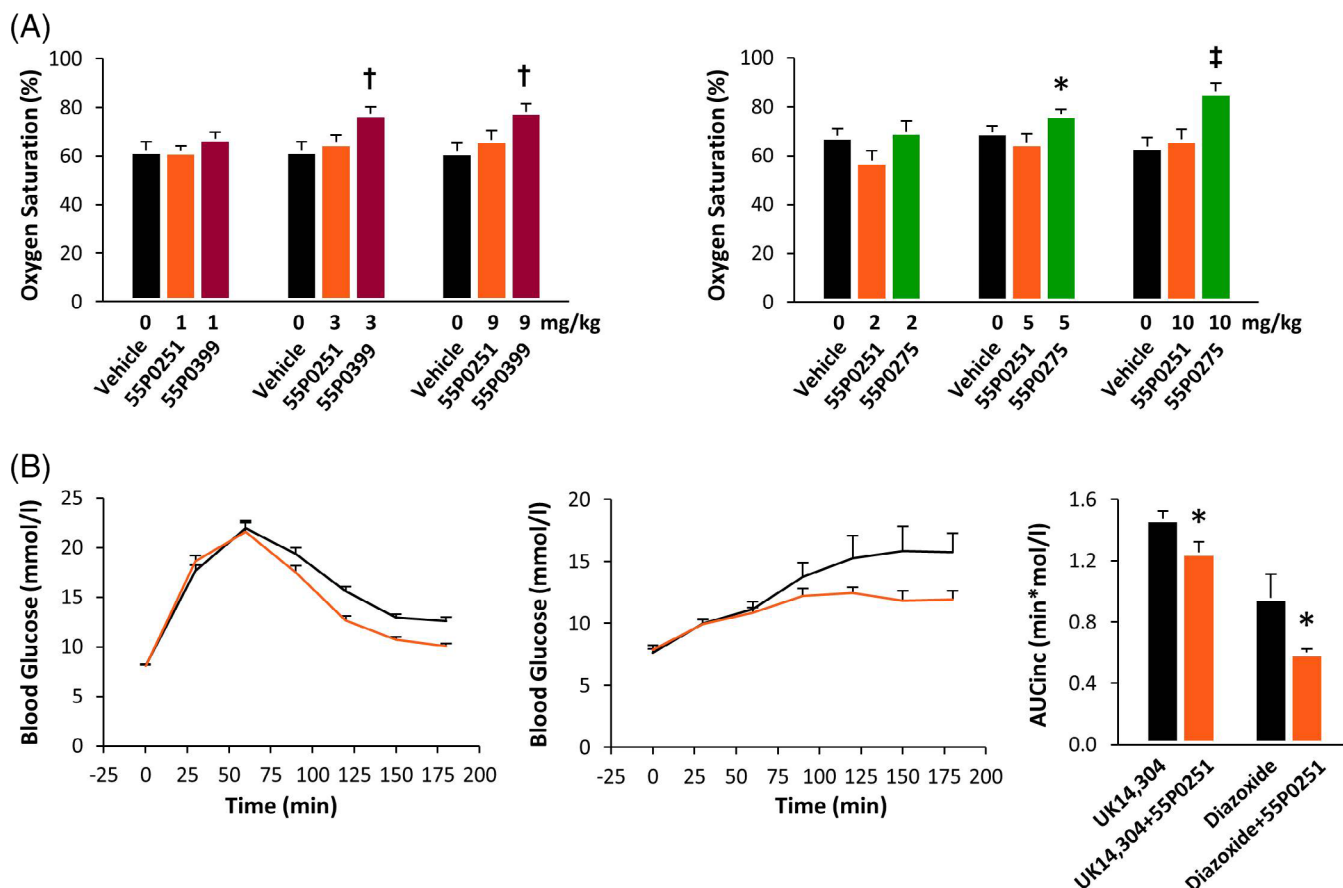
(A)

(B)

FIGURE 7 A, Oxygen saturation in capillary blood from the tip of the tail in lean mice 75 minutes after receiving an oral dose of 1 mg/kg 55P0251 (orange), 55P0399 (red) or 55P0275 (green). Means ± SEM; n = 7-15 each; *$P < .05$, †$P < .01$, ‡$P < .001$ vs vehicle. B, Effect of an oral dose of 55P0251 (1 mg/kg; orange) on hyperglycaemia as induced by intraperitoneal injection of the α_2-adrenoceptor agonist UK14,304 (100 μg/kg) or by an oral dose of the K_{ATP} opener diazoxide (250 mg/kg). All drugs were administered at 0 minutes; bar graphs depict the corresponding incremental areas under the glucose curves (AUCinc); means ± SEM; n = 8-24 each; *$P < .05$ vs without 55P0251

The inverted enantiomer 55P0250 lacked comparable effects not only on blood glucose and receptor binding, but also on oxygen saturation (Figure 6A). The α_2-adrenoceptor, including its α_{2A}-subtype, is known to be involved in thermoregulation by stimulating vasoconstriction in thermoregulatory tissues like human skin and, as relevant here, rodent tail.[25-28] Although a contribution of effects mediated via α_1-adrenoceptors cannot be excluded, increased oxygen saturation observed in tail blood from 55P0251-treated mice could reflect vasodilation due to α_2-adrenoceptor antagonism in vivo. Using agents that act as vasodilators via other molecular targets (carvedilol, molsidomine), we confirmed that increased oxygen saturation in tail blood is a typical attribute of vasodilation in our experimental setup (Figure 6B). We further demonstrated that such a response is triggered by established α_2-adrenoceptor antagonists (yohimbine, racemic efaroxan), but not by (−)-efaroxan, which lacks the α_{2A}-antagonistic potential of the (+)-enantiomer (Figure 6C).[13] This adds to the noteworthy parallels between 55P0251 and efaroxan mentioned above. Finally, we excluded the possibility that the increase in oxygen saturation occurred secondary to insulin secretion and/or glucose lowering rather than as a direct result of adrenoceptor antagonism. To this end, we used established antidiabetic drugs that increase insulin secretion (gliclazide, exenatide,

repaglinide). Although the administered doses caused similar amelioration of hyperglycaemia 30 minutes after glucose feeding, glucose lowering was accompanied by a rise in oxygen saturation of tail blood only in response to 55P0251 (Figure 6D). These findings provide additional evidence that 55P0251 antagonizes α_2-adrenoceptors not only in vitro, but also in vivo under the conditions prevailing during our OGTTs in live mice.

3.6 | Similarities and differences versus established α_{2A}-adrenoceptor antagonists

Comparison of the different derivatives showed somewhat more marked effects of 55P0399 and 55P0275 than 55P0251 on oxygen saturation (Figure 7A), which matches their superior α_{2A}-adrenoceptor binding properties (Tables 1 and 2) but differs from their relative efficacies in the OGTT (Figures 4A and 5B). This divergence could derive from pharmacokinetics. In addition, many α_{2A}-adrenoceptor antagonists are known to modulate insulin release not only via adrenoceptors, but also via one (or even more) non-adrenergic mechanism(s). While non-adrenergic action(s) obviously include direct interaction with K_{ATP} channels on β cells,[29-32] the potency of a given

α_{2A}-adrenoceptor antagonist as K_{ATP} channel blocker is not necessarily proportional to its potency at the adrenoceptor, which presumably contributes to variability among the pharmacological profiles of individual compounds.[13,14]

With regard to our novel structures, an adrenoceptor-independent mechanism could explain enantiomer-independent stimulation of insulin release in the absence of α_2-adrenoceptor agonists from isolated islets, as we observed it at high concentrations of 55P0251 and 55P0250 (Figure 2B). We then exploited a previously applied protocol for the dissection of contributions of adrenoceptor versus K_{ATP} channel-mediated effects on blood glucose in vivo.[13,14] In this setting, a low dose of 1 mg/kg 55P0251 potently counteracted hyperglycaemia induced by an α_2-adrenoceptor agonist as well as hyperglycaemia induced by a K_{ATP} channel-opener, suggesting that 55P0251, like other α_{2A}-adrenoceptor antagonists, addresses both mechanisms (Figure 7B). It is notable, however, that 55P0251 was relatively potent in counteracting hyperglycaemia caused by K_{ATP} channel-opening (reduction of incremental AUC diazoxide, $-37.4 \pm 2.7\%$, vs induced by UK14,304, $-14.8 \pm 4.7\%$; $P = .012$), which distinguished 55P0251 from other α_{2A}-adrenoceptor antagonists and extended previous observations of relevant variation in the molecular pharmacology of individual α_{2A}-adrenoceptor antagonists (Figure S1).[13,14]

In conclusion, without any postulation of a molecular mechanism at work we have discovered and developed a novel class of fully synthetic substituted quinazolidines, which shows distinct anti-hyperglycaemic activity in mice. Focusing on compound 55P0251, we made efforts to track down the glucose-lowering mechanism of action and, as previously reported, found that 55P0251 augments insulin secretion via a mechanism presently not exploited in the clinical treatment of type 2 diabetes.[12] Here, we provide evidence that 55P0251 is to be categorized as an α_2-adrenoceptor antagonist acting on insulin secretion via blocking α_{2A}-adrenoceptors located on pancreatic β cells. While further studies, for example in α_{2A}-adrenoceptor knockout mice, are required to test the hypothesis, this conclusion is backed by extensive and striking parallels with previously described effects of structurally different compounds, which augment insulin release via antagonism at α_{2A}-adrenoceptors on pancreatic islets (e.g., efaroxan[13]). These parallels include evidence for the additional involvement of a non-adrenergic mechanism, presumably via blockade of the K_{ATP} channel.[29-32] Although extensive efforts to develop α_2-adrenoceptor antagonists for clinical use in the treatment of type 2 diabetes have so far not been successful, this option gained new interest when a prevalent single nucleotide polymorphism causing overexpression of the α_{2A}-adrenoceptor was found to be associated with an elevated risk of type 2 diabetes.[33-36]

The results reported in the present study constitute a major step towards understanding the molecular mechanism(s) accounting for the anti-hyperglycaemic activity of 55P0251 and presumably of multiflorine derivatives in general. In addition, our work exemplifies a drug discovery strategy, where the identification and development of highly effective structures is based on disease-relevant activity in vivo and precedes the search for molecular mechanism(s).

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