Inhibition of Eph/ephrin interaction with the small molecule UniPR500 improves glucose tolerance in healthy and insulin-resistant mice

Manuscript Details

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Abstract

Eph/ephrin interactions and their bidirectional signaling are integral part of the complex communication system between β-cells, essential for glucose homeostasis. Indeed, Eph/ephrin system was shown to be directly involved in the glucose-stimulated insulin secretion (GSIS) process occurring in the pancreatic islets. Here we tested the Eph antagonist UniPR500 as GSIS enhancer. UniPR500 was validated as EphA5-ephrin-A5 inhibitor in vitro and its efficacy as GSIS enhancer was assessed on EndoC-βH1 cells. The selectivity of UniPR500 was evaluated by testing this compound on a panel of well-known molecular targets responsible for the regulation of glucose homeostasis. Plasmatic levels of UniPR500 were measured by HPLC/MS approach after oral administration. Finally, UniPR500 was tested as hypoglycemic agent in healthy mice, in a non-genetic mouse model of insulin resistance (IR) and in a non-genetic mouse model of type 1 diabetes (T1D). The compound is an orally bioavailable and selective Eph antagonist, able to increase GSIS from EndoC-βH1 cells. When tested in vivo UniPR500 showed to improve glucose tolerance in healthy and IR mice. As expected by a GSIS enhancer acting on healthy β-cells, UniPR500 was ineffective when tested on a non-genetic mouse model of type 1 diabetes, where pancreatic function was severely compromised. In conclusion our findings suggest that Eph targeting is a new and valuable pharmacological strategy in the search of new hypoglycemic agents.

Keywords	Eph/ephrin; PPI-inhibitors; protein-protein; diabetes; insulin

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Dear Editor,

please consider the submission of the paper entitled "**Inhibition of Eph-ephrin interaction with the small molecule UniPR500 improves glucose tolerance in healthy and insulin-resistant mice**" by Giorgio C. et al., that we are submitting for publication in *Pharmacological research* on behalf of all authors.

Our research group in engaged from 10 years in the discovery and development of PPI-inhibitors targeting the Eph/ephrin system. This proteins are well known for their primary role during embryogenesis where they regulates cell segregation and tissue differentiation. These proteins are upregulated in the tumor environment where they regulate the angiogenesis, the replication of tumor propagating cell and the invasiveness of the cancer. Their expression in the healthy adult is almost missing but recent evidence showed their expression in the pancreas where they regulate Glucose Stimulated Insulin Secretion (Konstantinova et al., 2007). A proof-of-concept about the druggability of the system was offered by Jain et al (2013) by means kinase inhibitors. In the present paper we showed for the first time that the inhibition of Eph/ephrin interaction by using a small molecule can increase insulin release *in vitro* and in healthy and insulin-resistant mice *in vivo*, resulting in a better glucose tolerance.

This manuscript has been read and approved by all the authors. The criteria for authorship have been met. The manuscript or part of it has neither been published nor is currently under consideration for publication by any other journal. M. Tognolini, A. Lodola and M. Incerti patented the described compound (Italian patent 0001426534, no extensions).

Hoping that our manuscript will be eligible for publication, we convey our best regards.

Sincerely,

Massimiliano Tognolini

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Comments from the editors and reviewers:
-Reviewer 1

-

This paper by C. Giorgio et al. entitled “Inhibition of Eph/ephrin interaction with a small molecule UniPR500 improves glucose tolerance in healthy and insulin-resistant mice” dealt the generation of a novel Eph antagonist UniPR500 more selective for the Eph/ephrin system and featured by a fair oral bio-availability, the authors tested the Eph antagonist UniPR500 as GSIS enhancer. The study is well done; it can be considering for publication after addressing some concerns.

- The figure 3 should be put or as figure 2, or put the actual figure 2 before the figure 3.

This section was re-arranged following the suggests of both the referees. Figure 2 was removed and included in suppl material.

- In the figure 3 should be put the p value.

Done

- In the figure 11 C should be changed the label on y axis.

Done, thank you

- The authors should add the glucagon level in the data showed.

Sorry, we do not have residual plasma to accomplish the request

- This reference should be added:

The novel therapeutic effect of phosphoinositide 3-kinase-y inhibitor AS605240 in autoimmune diabetes. Azzi J et al. Diabetes. 2012 Jun;61(6):1509-18.

Included in the discussion

-Reviewer 2

-

In this manuscript, the authors show that targeting Eph-epherin axis with UniPR500 induced a substantial improvement of GSIS in vitro as when applied to a human beta cell line EndoC-BH1 enhanced insulin secretion 5 fold upon glucose stimulation. When tested in vivo, in an insulin resistant model particularly showed a slight improvement og glucose intolerance when compared to HFD-mice mainly at 60 and 120 min after glucose

stimulation. While the use of UniPR500 was ineffective in a streptozotocin treated model, as STZ-treated mice showed a severe glucose intolerance similar to HFD-mice.

Overall, the concepts in this manuscript are interesting, the general design of

experiments appropriate, and a tremendous amount of work was carried out. However,

there are many details particularly the first part describing/dealing with the design of the inhibitor UniPER500 should be cut to the half and summarized, some if not most the figures should be recalled in supplemental. Authors are also requested to add a sub-heading to every part as their results should be summarized in 3 main sections:

1-design of the inihibitor

2- effect of targeting Eph-epherin in vitro

3- effect of targeting Eph-epherin in vivo

Done

The manuscript is lacking an important section, discussion ,where the authors should compared their main findings to authors, authors are required to add this section to the manuscript.

An improved section of discussion was included

One comment regarding the supplemental section:

Showing the histological micrographs of only STZ-HFD mice only while lacking the micrographs related to other studied groups is no longer informative as the authors are required to show one representative micrograph from every studied group.

Pictures included as requested

Lastly it would be informative if the authors could validate their finding through the use of a KO mouse model may be EphA5-/- mouse?

We thank the reviewer to give us the opportunity to discuss this point.

Genetic manipulation was used by Konstantinova et al (2007) to assess for the first time the involvement of EphA5 and ephrin-A5 in glucose homeostasis. The employment of Ephrin-A5-/- mice demonstrated that ephrin-A5 activation induced insulin release since the lack of this protein resulted in poor or no insulin release upon glucose stimulation. Ephrin-A5 -/- mice showed a lower glucose tolerance as well.

A dominant negative EphA5 protein (DN-EphA5) lacking its cytoplasmic domain was expressed in MIN6 cells, by Konstantinova as well. DN-EphA5 increased basal and glucose-stimulated insulin secretion, whereas WT EphA5 overexpression decreased

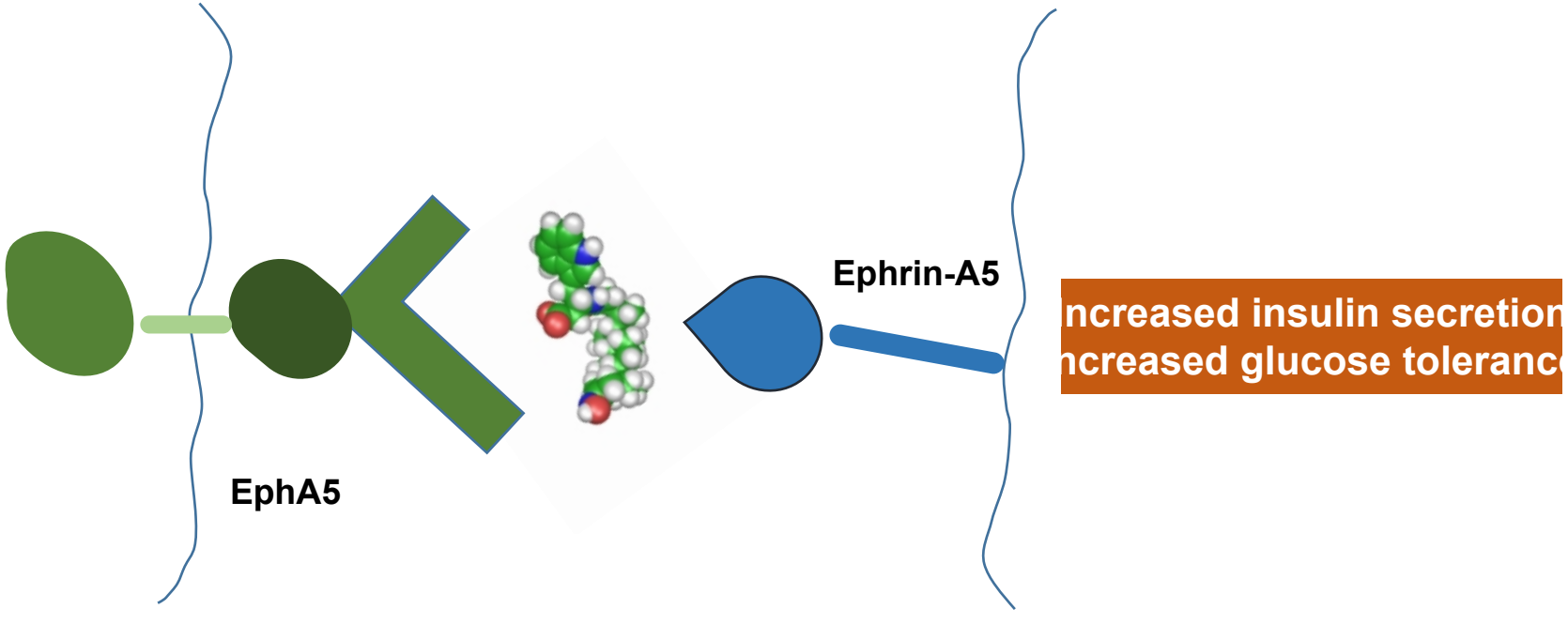
glucose stimulated insulin secretion in confluent MIN6 cells, supporting the idea that EphA forward signaling suppressed insulin secretion.

Ephrin-A5 KO mice were used also by Jain et al (2013) to validate the activity of some kinase inhibitors acting on the EphA5 receptor by measuring insulin levels before and after glucose administration. Ephrin-A5 mice showed an impaired insulin release accordingly to Konstantinova. The administration of the kinase inhibitor did not modify insulin release on ephrin-A5 -/- mice demonstrating that pharmacological inhibition of EphA5 is worthless without ephrin-A5.

EphA5-/- mice were never studied in this field however, since their behavior can not be related to the effect of DN-EphA5. In fact, you should consider that DN-EphA5 is unable to transduce forward signaling (since it lacks the intracellular domain) but it can activate ephrin-A5 through its extracellular domain and thus increase insulin secretion.

On the other hand EphA5 -/- will be unable to stimulate forward signaling, in accordance with DN-EphA5 but the lack of EphA5 will be unable to stimulate reverse signaling as well, and thus unable to stimulate insulin secretion. In conclusion the use of EphA5 -/- mice can give ambiguous results.

Finally, we tried to validate the activity of our compound not only by characterizing its activity on the Eph receptors but also checking its activity on other systems involved in glucose homeostasis such as TGR5, GLP1, PPAR-γ,PTP1B, DPP IV and K_{ATP} channel, where it showed no activity.


Ephrin-A5
EphA5
ncreased insulin secretion
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Inhibition of Eph/ephrin interaction with the small molecule UniPR500 improves glucose tolerance in healthy and insulin-resistant mice

Running title: Eph antagonists and diabetes

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ABSTRACT

Eph/ephrin interactions and their bidirectional signaling are integral part of the complex communication system between β-cells, essential for glucose homeostasis. Indeed, Eph/ephrin system was shown to be directly involved in the glucose-stimulated insulin secretion (GSIS) process occurring in the pancreatic islets. Here we tested the Eph antagonist UniPR500 as GSIS enhancer.

UniPR500 was validated as EphA5-ephrin-A5 inhibitor *in vitro* and its efficacy as GSIS enhancer was assessed on *EndoC-βH1* cells. The selectivity of UniPR500 was evaluated by testing this compound on a panel of well-known molecular targets responsible for the regulation of glucose homeostasis. Plasmatic levels of UniPR500 were measured by HPLC/MS approach after oral administration. Finally, UniPR500 was tested as hypoglycemic agent in healthy mice, in a non-genetic mouse model of insulin resistance (IR) and in a non-genetic mouse model of type 1 diabetes (T1D).

The compound is an orally bioavailable and selective Eph antagonist, able to increase GSIS from *EndoC-βH1* cells. When tested *in vivo* UniPR500 showed to improve glucose tolerance in healthy and IR mice. As expected by a GSIS enhancer acting on healthy β-cells, UniPR500 was ineffective when tested on a non-genetic mouse model of type 1 diabetes, where pancreatic function was severely compromised.

In conclusion our findings suggest that Eph targeting is a new and valuable pharmacological strategy in the search of new hypoglycemic agents.

INTRODUCTION

Over the last three decades, several studies concurred to demonstrate that the communication between β cells contributes to glucose homeostasis by inhibiting insulin secretion from pancreatic islets during fasting while enhancing it after food intake [1]. In 2007, Konstantinova and collaborators revealed that Eph/ephrin system participated to the β cells communication network and was directly involved in the glucose-stimulated insulin secretion (GSIS) process occurring in the pancreatic islets [2].

The Eph receptors (erythropoietin-producing hepatocellular carcinoma) represent the largest family of receptor tyrosine kinases (RTK) in humans. They are divided in 2 classes, EphA and EphB, based on sequence homology of extracellular domain and their affinity for ephrin ligands, proteins tethered to the cell membrane by either glycosylphosphatidyl-inositol (GPI) linkage (ephrin-As) or a transmembrane domain in turn linked to a cytoplasmic tail (ephrin-Bs)[3]. The unique characteristic of these RTK is that upon Eph/ephrin interaction a bidirectional signaling is generated: in fact not only the Eph receptors ("forward signaling"), but also the ephrins ("reverse signaling") are able to transduce signals into the cells [4]. EphA receptors and ephrin-A ligands are expressed in human and rodent pancreas [5] and pancreatic β cells use EphA/ephrin-A bidirectional signaling to modulate insulin secretion [2]. In particular, EphAs forward signals work as a brake for insulin release whilst ephrin-As reverse signals stimulate it. When mice are in a condition of satiety, EphAs forward signals are in part attenuated by phosphatases activated by glucose and consequently this cellular communication system is unbalanced toward ephrin-As reverse signals that prompt insulin secretion in the blood stream. Among all the cloned Eph receptors and ephrin proteins, EphA5 and its cognate ligand ephrin-A5, co-expressed in β cells, are critical for this process [6].

The involvement of the Eph/ephrin system and in particular of EphA5 receptor in the GSIS process has been unequivocally demonstrated through its pharmacological inhibition by means of RTK inhibitors featured by a 4-methyl-N-(3-(trifluoromethyl)phenyl)benzamide core, also present in the clinically approved bcr-abl inhibitor Nilotinib. Intraperitoneal administration of these benzamide derivatives enhanced glucose stimulated insulin secretion from mouse and human pancreatic islets *in vit*ro and increased plasma insulin and glucose tolerance *in vivo*. At the same time they did not affect glucose homeostasis in Efna5$^{-/-}$ mice providing the proof-of-concept that the modulation of the Eph/ephrin system represents a new strategy for the treatment of T2D.

An alternative way to inhibit the signaling transduced by the Eph/ephrin system is through the use of compounds able to bind their extracellular ligand binding domain and thus to prevent their associations to the ephrin-A ligands [7]. Among the classes of available small molecules targeting the Eph receptors , amino-acid conjugates of bile acids are emerging as promising agents, as they possess good potency towards Eph receptors in binding

and functional assays [8–11]. These compounds possess two key advantages over traditional RTK inhibitors: *i* they can block the activity of the Eph receptors without having to enter into the cells, *ii* they have the potential to be more selective than agents designed to mimic the structure of the ubiquitous ATP molecule. The most potent small-molecule antagonist direct to the extracellular domain of the Eph receptors is UniPR129, L- β-homotryptophan conjugate of lithocholic acid (LCA) [8]. While UniPR129 exhibited submicromolar inhibitory activity on Eph receptors, its *in vivo* use has been so far hampered by low bio-availability and off-target activity, which also includes proteins involved in the regulation of the glucose homeostasis, such as the well-known TGR5 receptor.

In the present work, starting from the structure of UniPR129 we provide evidence that following a structure-based drug design approach it is possible to generate a novel Eph antagonist more selective for the Eph/ephrin system and featured by a fair oral bio-availability. Thanks to this novel pharmacological tool, we will show that the antagonism at the extracellular domain of the EphA receptors is a viable strategy to control glucose levels in normal and obese mice. Our findings not only confirm the involvement of the Eph/ephrin system in glucose and insulin homeostasis but propose Eph antagonists as potential antidiabetic agents.

METHODS

ELISA assays and K_i/IC_{50} determination

ELISA assays were performed as previously described [8]. Briefly, 96-well ELISA high binding plates (Costar, Corning, New York, NY, USA #9018) were incubated overnight at 4 °C with 100µL/well of 1 µg mL^{-1} EphA5-Fc (R&D systems, Minneapolis, MN, USA, #541-A5) diluted in sterile phosphate buffered saline (PBS, 0.2 g L^{-1} KCl, 8.0 g L^{-1} NaCl, 0.2 g L^{-1} KH_2PO_4, 1.15 g L^{-1} Na_2HPO_4, pH 7.4). The day after wells were washed with washing buffer (PBS +0.05% tween20, pH 7.4) and blocked with blocking solution (PBS + 0.5% BSA) for 1 hour at 37 °C. Compounds were added to the wells at proper concentrations in 1% DMSO and incubated at 37 °C for 1 hour. Biotinylated ephrin-A5-Fc (R&D systems, Minneapolis, MN, USA, #BT374) was added at 37 °C for 4 hours at its K_D in displacement assays or in a range from 1 to 2000 ng mL^{-1} in saturation studies. Then wells were washed and incubated with 100µL/well Streptavidin-HRP (Sigma-Aldrich, Milan, Italy, #S5512) for 20 minutes at room temperature, washed again and finally incubated at room temperature with 0.1 mg mL^{-1} tetra-methylbenzidine (Sigma-Aldrich, Milan, Italy, #860336) reconstituted in stable peroxide buffer (11.3 g L^{-1} citric acid, 9.7 g L^{-1} sodium phosphate, pH 5.0) and 0.02% H_2O_2 (30% m/m in water), added immediately before use. The reaction was stopped with 3N HCl 100 µL/well and the absorbance was measured using an ELISA plate reader (Sunrise, TECAN, Switzerland) at 450 nm. IC_{50} values were determined using one-site competition non-linear regression analysis with Prism software (GraphPad Software Inc., La Jolla, CA, USA). To assess the selectivity of compounds, all EphA

(R&D systems, Minneapolis, MN, USA, #SMPK1) and EphB (R&D systems, Minneapolis, MN, USA, #SMPK2) receptors were incubated overnight similarly to EphA5-Fc as previously described; biotinylated ephrin-A1-Fc or biotinylated ephrin-B1-Fc (R&D systems, Minneapolis, MN, USA, #BT602 and #BT473, respectively) at their K_D were used towards EphAs or EphBs, respectively.

Surface plasmon resonance

Surface plasmon resonance (SPR) measurements were performed on a BIAcore X100 instrument (GE-Healthcare, Milwaukee, WI, USA), using research-grade CM4 carboxyl-methyl-dextran-coated sensorchips (GE-Healthcare, Milwaukee, WI, USA). SPR was exploited to measure changes in refractive index caused by the binding of UniPR500 to surface-immobilized EphA5 receptor. To this aim, the fusion protein EphA5-Fc (R&D systems, Minneapolis, MN, USA, #541-A5) or the Fc fragment alone (Merck Millipore, Darmstadt, Germany, #AG714) were dissolved at 20 µg/mL in 10 mM sodium acetate pH 4.0 and allowed to react with two separate flow cells of a CM4 sensorchip, pre-activated with 50 mL 0.2 M N-ethyl-N-(3-diethylaminopropyl) carbodiimide hydrochloride and 0.05 M N-hydroxysuccinimide, leading to the immobilization of 3000 and 950 RU for EphA5-Fc and Fc fragment, respectively. Increasing concentrations of UniPR500 in PBS, 0.05% surfactant P20 and 5% DMSO, pH 7.4 were injected over the EphA5 or Fc surfaces for 90 seconds and then washed until dissociation was observed. Binding parameters were calculated by the non-linear curve fitting software package BIA evaluation 3.2 using a single site model with correction for mass transfer (GE Healthcare Europe GmbH, Milan, Italy). Dissociation constant (Kd) was obtained with a steady state analysis, by fitting the bound RU at equilibrium versus the ligand concentration in solution with a proper form of the Scatchard equation.

UniPR500 Off-targets

Stimulant or inhibitory effects of UniPR500 towards GLP1 receptor and PPAR-γ receptor as well as the inhibitory effect versus protein tyrosine phosphatase 1B (PTP1B), dipeptidyl peptidase IV (DPPIV) and ATP-sensitive potassium channel (K_{ATP} channel) were performed at CEREP (Eurofins Cerep SA, Celle L'Evescault, France). Test codes are reported in Table 1. The activity of UniPR500 towards TGR5 receptor was performed at INTERCEPT (Intercept, London, UK).

EndoC-βH1

The genetically engineered human pancreatic β cell line EndoC-βH1, kindly provided us by Professor Ravassard [12], was grown in DMEM low glucose (1 g/L) (ThermoFisher Scientific, Waltham, MA, USA, #11885084), 2% BSA, 50 µM 2-mercaptoethanol (Sigma-Aldrich, Milan, Italy, #M3148), 10 mM nicotinamide (Sigma-Aldrich, Milan,

Italy, #N0636), 5.5 µg/mL transferrin (ThermoFisher Scientific, Waltham, MA, USA, #11107018), 6.7 ng/mL sodium selenite (Sigma-Aldrich, Milan, Italy, #S5261), 1% Pen/Strep.

Dynamic perifusion

A high-capacity, automated perifusion system (Bio rep® Perifusion V2.0.0) was used to dynamically measure insulin secretion from EndoC-βH1 cells. A low pulsatility peristaltic pump pushed HEPES-buffered solution (125 mM NaCl, 5.9 mM KCl, 2.56 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM HEPES, 0.1% BSA, pH 7.4) through a sample container harboring $7*10^5$ EndoC-βH1 cells. Cells were stabilized in the machine with a 200µl/min flow perifusion rate with low glucose (2mM) for 60 minutes. Combination of stimuli (2 and 20 mM Glucose, 10 µM UniPr500 and 0.1% DMSO as vehicle) were then added for 20 minutes at a flow rate of 200 µl/min, followed by another 20 minutes with low glucose (2 mM) to evaluate ability of cells to recover the basal insulin secretion. The perifusates were collected every minute in an automatic fraction collector designed for multiwell plate format. Cells and the perifusion solutions were kept at 37°C in a built-in temperature controlled chamber, and the perifusate in the collecting plate was kept at <15°C.

Animals.

All animal experiments were authorized by the local Animal Care Committee and by the Italian Ministry of Health. All procedures involving animals and their care were performed according to the Italian (DL 26/2014) and European (Directive 2010/63/EU) laws and policies on the protection of animals used for scientific purposes.

Non-genetic mouse model of Insulin-Resistance

C57BL/6J mice were fed *ad libitum* for 10 weeks with high-fat diet (HFD, 60% calories from fat, Mucedola, Milan, Italy) in order to induce insulin resistance and, as a consequence, impaired glucose tolerance [13]. After eight weeks of high fat diet, animals with an insulin level higher than 1.20ng/dl and plasma glucose higher than 190mg/dl, were randomly divided in two groups. UniPR500 (30 mg/kg) or methocel alone were orally administered for the next 14 days, from the 9^{th} to the 10^{th} week of HFD treatment. The last administration was done 30 minutes before performing glucose tolerance test. C57BL/6J mice fed with standard diet (Mucedola, Milan, Italy) were used as healthy control.

Mice weight, fasting blood glucose and cholesterol levels were evaluated right before the beginning of the pharmacological treatment and the day before the last administration, in order to evaluate the effect of drug on disease progression.

Non-genetic mouse model of Type 1 diabetes

C57BL/6J mice were injected with streptozotocin 40mg/kg i.p. for 5 consecutive days (days 1-5) and fed *ad libitum* for at least 6 weeks (days 6-50) with high-fat diet in order to induce metabolic characteristics of T1D such as increased fasting blood glucose levels and impaired glucose tolerance [14]. At day 37 mice were randomly divided in two group. UniPR500 (30 mg/kg) or methocel alone were orally administered for 14 days, from the day 37 to the day 50 and the last administration was done 30 minutes before performing glucose tolerance test. C57BL/6J mice fed with standard diet (Mucedola, Milan, Italy) were used as healthy control.

Intraperitoneal glucose tolerance test (IGTT)

Glucose tolerance test was performed on fasted male C57BL/6J mice. Healthy mice, insulin resistant mice (IR) and diabetic type 1 animals (T1D) were challenged by intraperitoneal injection of 2 g glucose/kg body weight. Glucose levels were measured in plasma by means of a colorimetric assay (Instrumentation Laboratory Kit, Karlsruhe, Germany, #0018259140).

Plasma insulin and cholesterol determination

Plasma insulin and cholesterol levels were determined by mean of an insulin ELISA assay (Merck Millipore, Darmstadt, Germany, #EZRMI-13K) and a colorimetric assay (Instrumentation Laboratory Kit, Karlsruhe, Germany, #0018250540), respectively.

Anatomo-pathological analysis

Anatomo-pathological analysis were performed on streptozotocin-HFD mice treated and untreated with UniPR500 30mg/kg os for 14 days. Liver, kidneys, pancreas were collected from each mice and samples were immediately fixed in 10% neutral buffered formalin. After paraffin embedding, 4-5 µm thick sections, were obtained with microtome (Leica), stained with Hematoxylin and Eosin (H&E) and Periodic acid-Schiff (PAS). Histological slides were examined with Nikon Eclipse E800 microscope (Nikon Corporation, Japan) using Nikon PLAN APO lenses. Sections were photographed at 4x, 10x, 20x and 40x (Nikon PLAN APO lenses) with Camera DIGITAL SIGHT DS-Fi1 (Nikon Corporation, made in Japan); pictures were acquired with DS Camera Control Unit DS-L2 (Nikon Corporation, Japan) and stored in USB device. Histological lesions were graded and classified based on the extension/distribution (focal, multifocal and diffuse) and severity (scant, mild, moderate, severe).

Data and statistical analysis

Results are expressed as mean ± SD or percentage ± SD change versus controls. Statistical significance was evaluated by one way-ANOVA or two-way ANOVA followed by Dunnett's or Bonferroni test, respectively. The

post hoc tests were run only if F achieved $p < 0.05$ and there was no significant variance inhomogeneity. A *p*-value <0.05 was considered significant. All statistical tests were performed on raw or normalized data (perifusion) using the Prism Graph Pad 5.01 (La Jolla, California, USA). Insulin release during perifusion was normalized to basal value. More details are reported in the legend of the pictures.

RESULTS

Design of the inhibitor

We previously identified UniPR129 as a tool inhibiting EphA2/ephrin-A1 binding *in vitro* [8] and we asked whether UniPR129 was able to interfere with EphA5/ephrin-A5 binding since they are involved in insulin release. We immobilized the EphA5-Fc ectodomain on ELISA plates and detected the binding of biotinylated ephrin-A5-Fc. UniPR129 dose-dependently reduced EphA5/ephrin-A5 interaction with an IC_{50} value of 1.2 μM (Figure 1A). Saturation curves of EphA5/ephrin-A5 were built in the presence of increasing UniPR129 concentrations allowing to calculate K_D and apparent K_D values. These values were used to draw a Schild plot [15] (Figure 1B and 1C) characterized by a well-interpolated regression line (r^2=0.98) with a slope of 0.91. The pKi resulting from the intersection of the interpolated line with the X-axis was 6.11±0.07 (corresponding to a Ki of 0.77 μM). The removal of UniPR129 from the displacement assay wells through a washing procedure restores ephrin-A5 binding to EphA5 (Figure 1D). Taken together, these data suggest a competitive and reversible antagonism of UniPR129 at the EphA5 receptor.

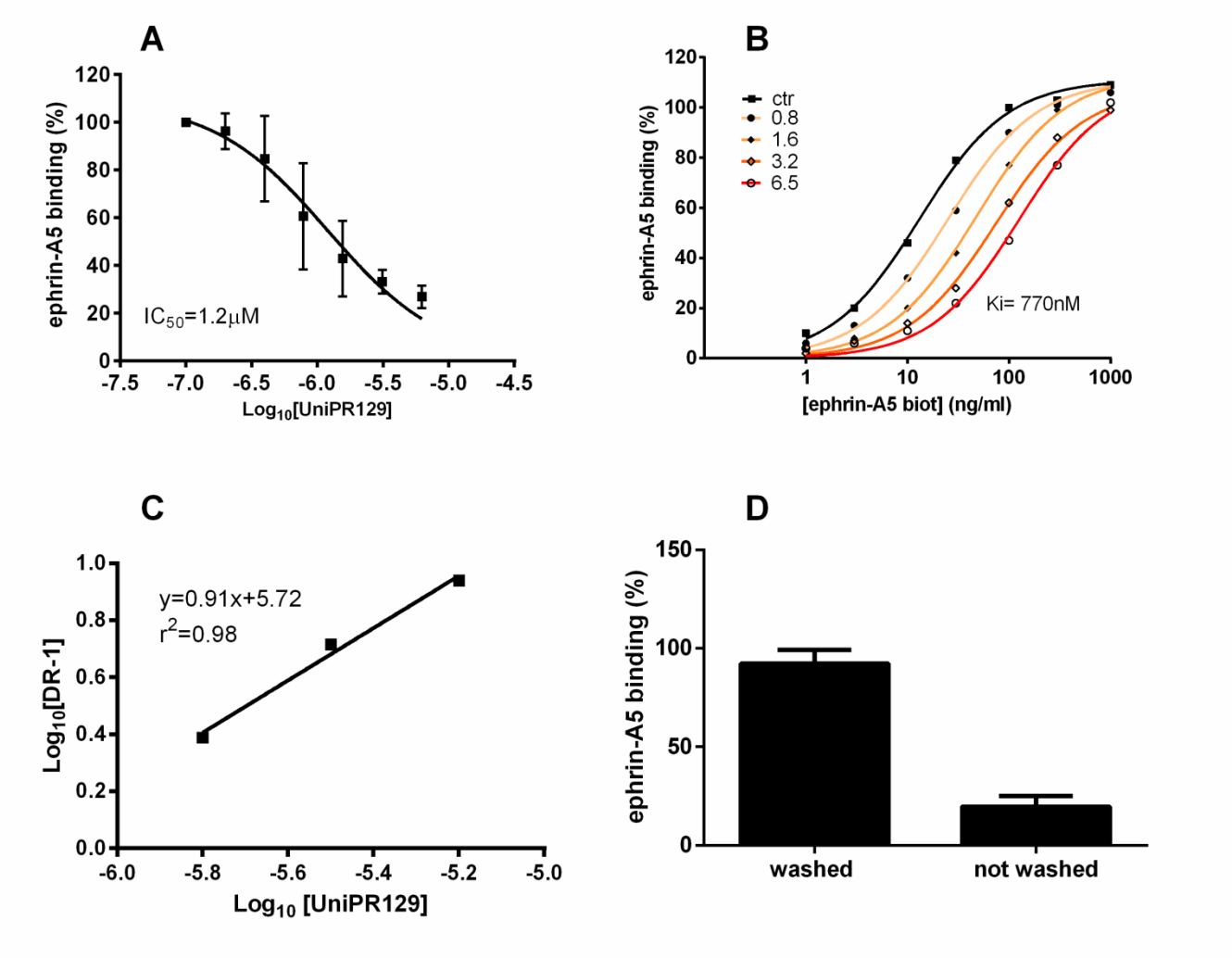

Figure1 ***A****, UniPR129 dose-dependently displaced the binding of biotinylated ephrin-A5-Fc from immobilized EphA5-Fc.* ***B****, binding of biotinylated ephrin-A5-Fc to immobilized EphA5-Fc ectodomain in the presence of different concentrations of UniPR129.* ***C****, the dissociation constants (K_D) from the previous plot were used to calculate Log_{10} (Dose-ratio - 1) and to graph the Schild plot. pKi of UniPR129 was estimated by the intersection of the interpolated line with the X-axis.* ***D****, EphA5-ephrin-A5 binding in the presence of 10 μM UniPR129 with or without washing three times with PBS before adding biotinylated ephrin-A5-Fc. Data are the means of at least four independent experiments ± st. dev.*

Amino acid conjugates of LCA have been reported to activate TGR5, a G-protein coupled receptor responsive to bile acids, in the micromolar range [16]. This is also the case of UniPR129 which activates TGR5 in CHO cells with an EC_{50} of 3.9 μM (figure 2). TGR5 agonists, such as the clinical candidate INT-777, have been shown to enhance glucose tolerance in obese mice [17]. Considering our willingness to assess the impact of an Eph antagonist on

glucose homeostasis, the availability of a molecule lacking of any activity on TGR5 receptor was a mandatory step of our research program. To this end, we built a homology model of TGR5 according to procedure reported by Macchiarulo and colleagues [18] to identify a likely binding mode for UniPR129. Docking simulations, performed as described in reference [19], identified a top-ranked model where UniPR129 interacted with Ser270 thanks to its carboxylate and with Asn93 by its 3α-hydroxyl group (Figure S1, left panel, supplementary material). Starting from this interaction model, we hypothesized that the introduction of a 3α substituent bulkier than a hydroxyl group, (i.e. not able any more to bind Asn93) would lead to a compound inactive on TGR5. Docking simulations confirmed our working hypothesis as in the top-ranked docking pose, the 3-hydroxyimino analogue UniPR500 failed to undertake polar interactions with the key asparagine Asn93 (Figure S1, right panel). We also evaluated *in silico* if the same chemical modification was tolerated by the EphA5 receptor. Docking simulations indicated that UniPR129 and UniPR500 bound EphA5 with the same orientation and that the H-bond undertaken by the 3α hydroxyl of UniPR129 with Glu88 could be also undertaken by the corresponding 3-hydroxyimino group of UniPR500 (Figure S2). This computational investigation suggested that UniPR500 may selectively bind EphA5 receptor.

In order to confirm computational data, the 3-hydroxyimino derivative UniPR500 was thus tested for its ability to activate the TGR5 receptor in comparison with UniPR129 and deoxycolic acid (DCA) in a functional assay developed by DiscoverX. In this experiment CHO cells were incubated with compounds and cAMP production was correlated to the activation of TGR5 receptor. As mentioned above and reported in figure 2 UniPR129 dose-dependently activated TGR5 with a EC_{50} of 3.4 μM and reached a maximal response of 80 % in comparison with DCA, used as positive control [20]. Conversely UniPR500 failed to activate TGR5 receptor showing a negligible response at the highest concentration tested (30 μM).

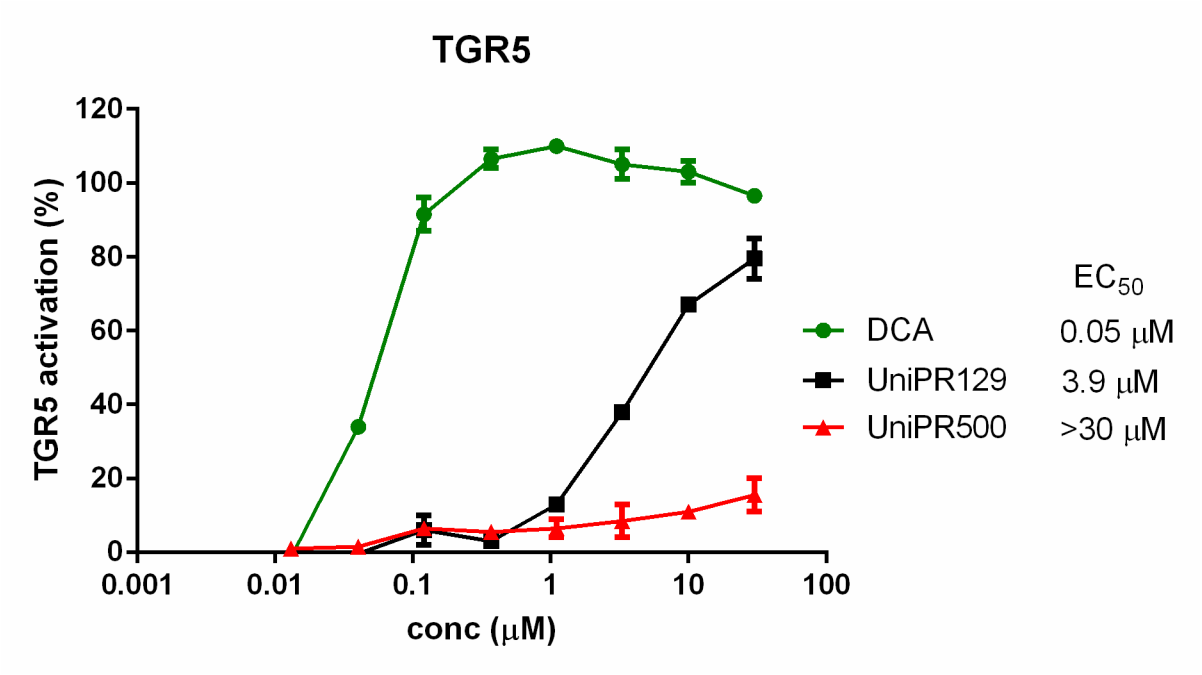

Figure 2. Dose response curve for TGR5 activation in CHO cells. Activation of the receptor was assessed measuring the production of cAMP level. Green line, deoxycholic acid; black line, UniPR129; red line UniPR500. Experiments are performed in duplicate.

UniPR500 was thus tested for its ability to disrupt EphA5-ephrin-A5 interaction by using the ELISA assay described above. The compound was still able to inhibit EphA5-ephrin-A5 interaction with an IC_{50} value of 3.7 μM (Figure 3A) comparable to UniPR129, indicating that the EphA5 receptor is significantly more tolerant versus chemical modification than TGR5 receptor.

Saturation curves of EphA5-ephrin-A5 binding in the presence of increasing UniPR500 concentrations allowed us to draw a Schild plot (Figure 3B and 3C). We obtained a well-interpolated regression line (r^2=0.92) with a slope of 0.84, consistent with a competitive antagonism. The resulting Ki was 1.7±0.2 μM. The removal of UniPR500 from the binding assay wells through washing, restores ephrin-A5 binding to EphA5 indicating that UniPR500 acts as a reversible antagonist (Figure 3D).

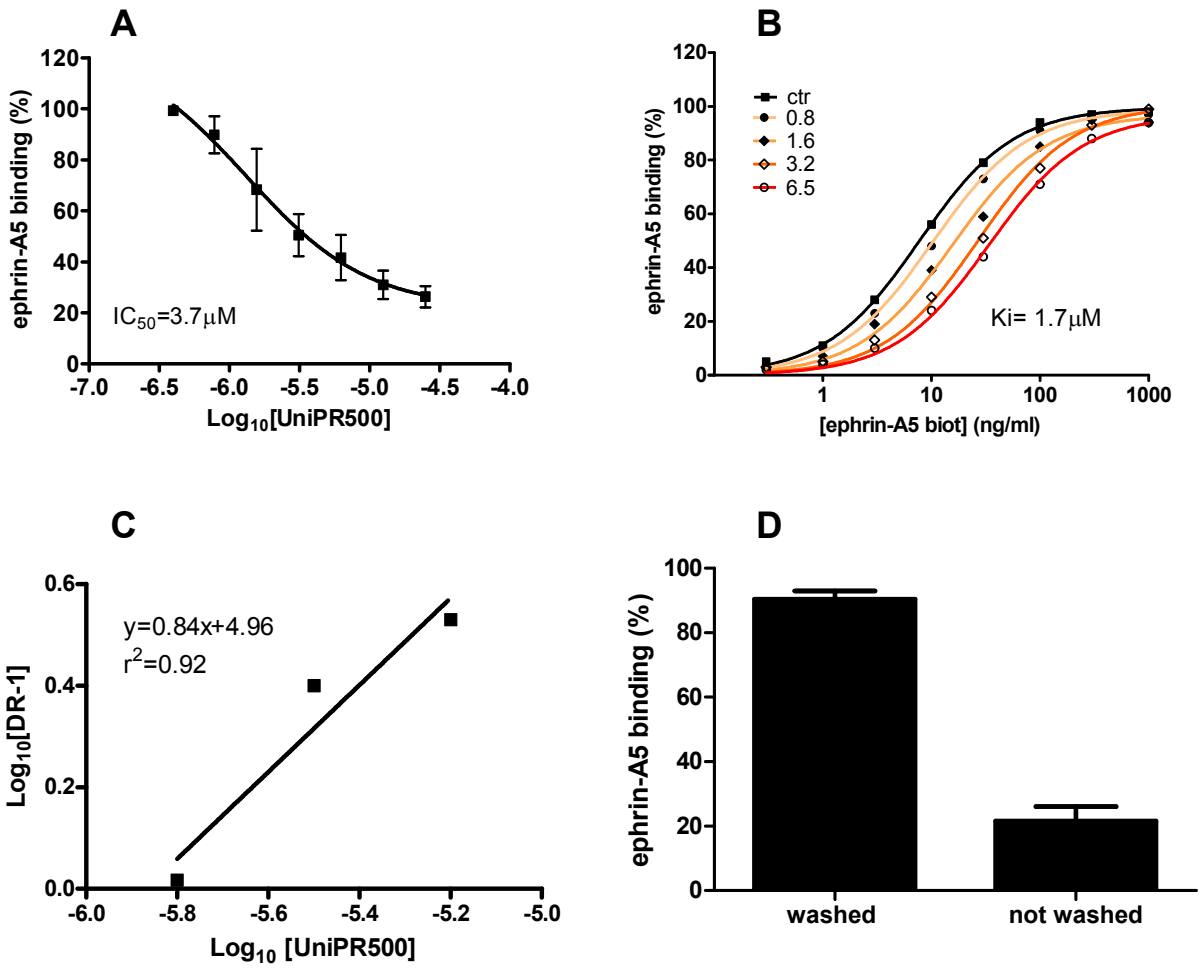

Figure 3 ***A****, UniPR500 dose-dependently displaced the binding of biotinylated ephrin-A5-Fc from immobilized EphA5-Fc.* ***B****, binding of biotinylated ephrin-A5-Fc to immobilized EphA5-Fc ectodomain in the presence of different concentrations of UniPR500.* ***C****, the dissociation constants (K_D) from the previous plot were used to calculate Log_{10} (Dose-ratio - 1) and to graph the Schild plot. pKi of UniPR500 was estimated by the intersection of the interpolated line with the X-axis.* ***D****, EphA5-ephrin-A5 binding in the presence of 10 μM UniPR500 with or without washing three times with PBS before adding biotinylated ephrin-A5-Fc. Data are the means of at least four independent experiments ± st. dev.*

UniPR500 binds EphA5 receptor

We next set a surface plasmon resonance (SPR) assay to characterize the mechanism of interaction between UniPR500 and EphA5 at a molecular level. UniPR500 binds surface-immobilized EphA5 in a concentration-dependent way with saturation binding reached at concentrations ranging between 60 and 80 μM (Figure 4).

The dissociation constant (K_D) measured from steady state analysis results equal to 22 μM.

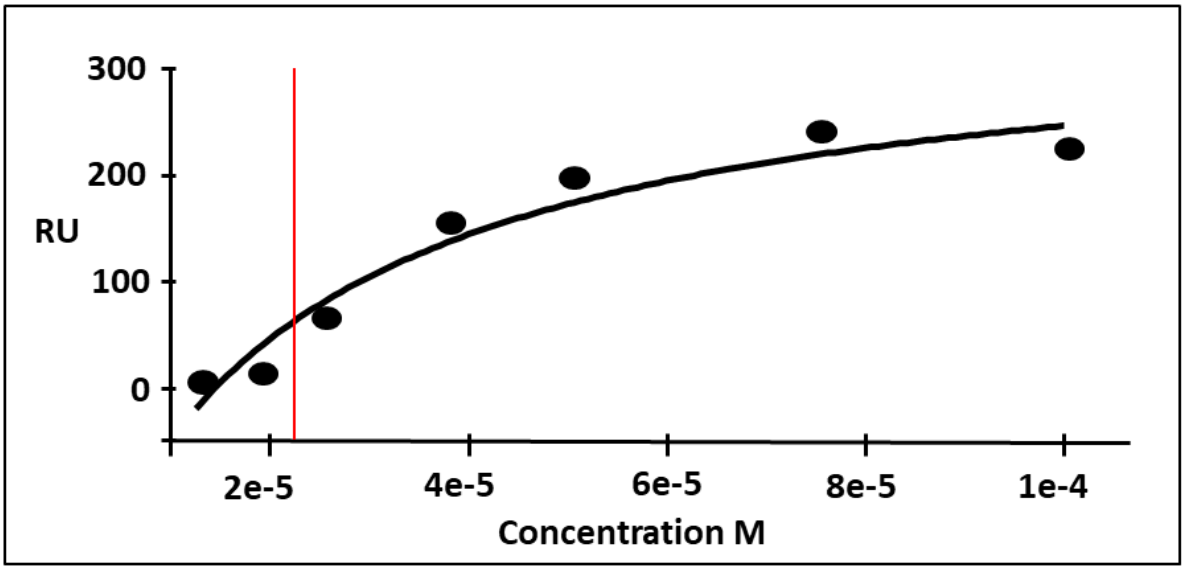

Figure 4. Steady state analysis of the interaction of UniPR500 with EphA5-Fc immobilized to a SPR sensorchip. The saturation curve is obtained by plotting the blank subtracted RU values at equilibrium resulting from the injection of increasing concentrations of UniPR500 onto the Eph5-Fc surface versus the UniPR500 concentration. Black line represents the fitted curve while the redline identifies the K_D value, as equal to the concentration yielding 50% of the maximum response.

UniPR500 is a pan Eph/ephrin inhibitor

The selectivity of UniPR500 interaction versus the different EphA and EphB receptors was studied using biotinylated ephrin-A1-Fc and biotinylated ephrin-B1-Fc, respectively, at their K_D concentrations. As showed in Figure 5 the compound inhibited the interaction of ephrin-A1-Fc or ephrin-B1-Fc with all Eph receptors. The IC_{50} values for EphA receptors ranged from 1.14 to 1.52 μM whilst for EphB receptors were in the 2.05-5.09 μM range (Figure 5). UniPR500 can thus be regarded as a pan-antagonist of the Eph receptors targeting their highly conserved ligand binding domain [19].

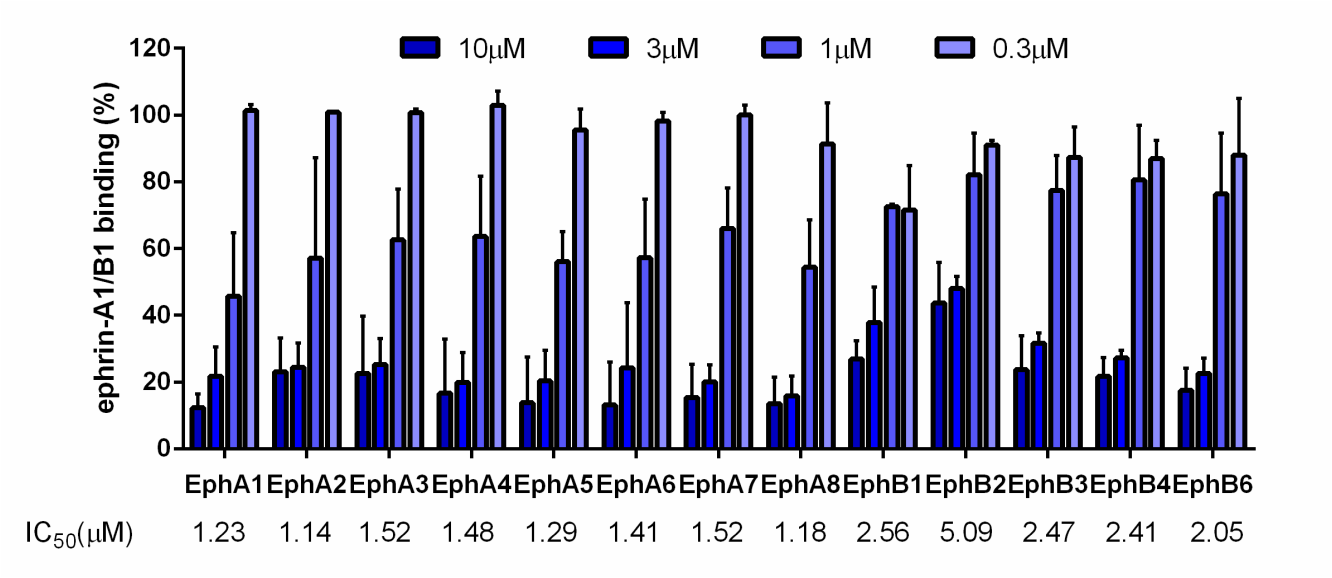

Figure 5. Binding of biotinylated ephrin-A1-Fc or ephrin-B1-Fc to EphA or EphB receptor ectodomains was dose-dependently inhibited by UniPR500. Data are the means of at least three independent experiments ± st. dev.. IC_{50} values are indicated at the bottom.

Effect of targeting Eph/ephrin *in vitro*

UniPR500 increases insulin release from EndoC-βH1 cells

It was previously demonstrated that the activation of EphA5 by means of ephrin-A5-Fc inhibited insulin secretion upon glucose stimulation on murine MIN6 cell line and on primary isolated islets of both mice and humans [2]. Here, using a high-capacity, automated perifusion system, we checked if the incubation with the Eph antagonist UniPR500 was able to increase insulin secretion from EndoC-βH1 cells, a valid model of human beta cells [21], upon stimulation with 20mM glucose. As expected glucose stimulation doubled the basal insulin concentrations and this effect was enhanced by the inhibition of Eph receptors by means of UniPR500. Indeed 10μM UniPR500 significantly enhanced the insulin release by 5-fold upon glucose stimulation, confirming that Eph antagonism is a valuble strategy to improve glucose stimulated insulin secretion (GSIS) (Figure 6).

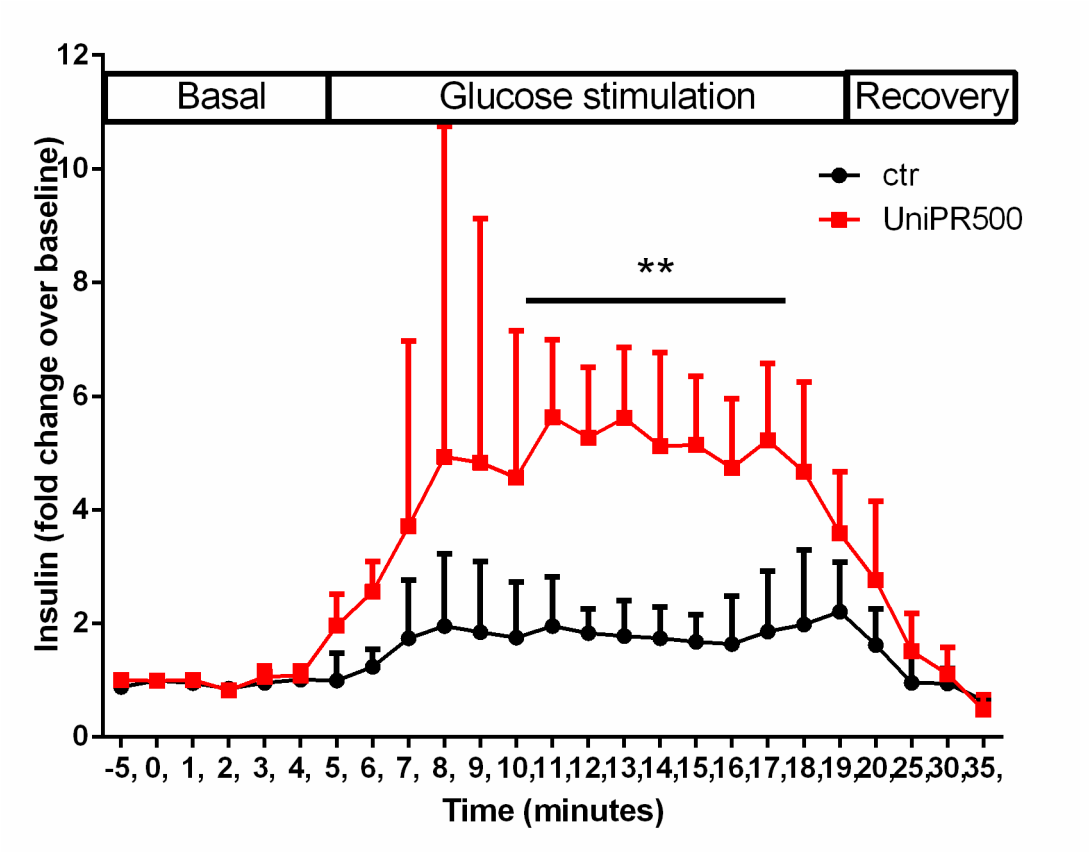

*Figure 6. Release of insulin from EndoC-βH1 perifusate. Glucose 20mM were added at minute 5 and removed at minute 25. Data are the means of at least three independent experiments ± st. dev.. Two-way ANOVA followed by Bonferroni's post-test. **, p<0.01.*

UniPR500 does not interfere with classical targets involved in regulations of blood glucose levels

In order to confirm UniPR500 as a GSIS enhancer acting through the inhibition of Eph activation the compound was tested at 10 μM on a panel of molecular targets known to be involved in the regulation of glucose levels (Table 1). UniPR500 resulted inactive both as an agonist or an antagonist on PPAR-γ (target of glitazones, i.e. pioglitazone and rosiglitazone) and GLP-1 (target of GLP-1 agonists, i.e. exenatide and liraglutide) receptors. Furthermore UniPR500 did not inhibit DDP-4 (target of the gliptins, i.e. sitagliptin and vildagliptin) or PTP1B enzyme (target of the clinical candidate trodusquemine [22] and of the natural EphA2 antagonist lithocholic acid [23]) nor it interfered with functioning of the K_{ATP} ion channel (target of sulfonylureas, i.e. tolbtamide and glipizide).

Table 1. Selectivity panel for the Eph/ephrin antagonist UniPR500. The compound was tested at single dose (10 μM) in triplicate. The biological activity of a reference standard for each target is also reported.

Target and test code	test	%inhibition or activation (±SD)	Standard IC_{50} or EC_{50}
GLP1, agonism 2181	Cellular functional assay GPCR/cAMP/HTRF	0.4±0.2	GLP-1 (7-37), 0.052nM
GLP1, antagonism 2182	Cellular functional assay GPCR/cAMP/HTRF	13±12	exendin-3(9-39), 5.7nM
PPAR-γ, agonism 2771	Nuclear functional assay Coactivator recruitment/alphascreen	0.0±0.0	Rosiglitazone, 190nM
PPAR-γ, antagonism 2772	Nuclear functional assay Coactivator recruitment/alphascreen	-2.4±6.8	GW9662, 91nM
PTP1B, inhibition 2593	Enzyme assay, fluorogenic substrate	-4.5±0.7	(NH4)6Mo7O24, 12nM
DPP IV, 2942	Enzyme assay, fluorogenic substrate	-3.3±3.5	K579, 3nM
K_{ATP} channel, 0165	Radioligand assay	7.0±9.8	Glibenclamide, 0.25nM

UniPR500 has a good physicochemical profile and it is orally available in mice

As our final aim was to administer UniPR500 *in vivo*, we next looked at key physicochemical properties of UniPR500 that could affect its oral bioavailability. We first evaluated physicochemical parameters affecting the absorption phase, such as lipophilicity (log D) and solubility (S), as well as the interaction with specific transporters that may enhance the uptake of UniPR500 in the bloodstream. As a second step, we focused our attention on the potential metabolic liability of UniPR500 by evaluating its stability in mouse plasma and liver. Table 2 summarizes the results of our *in vitro* ADMET profiling. UniPR500 showed log D (4.23) and kinetic solubility (52 μM) values suitable for an oral absorption [24]. Furthermore, *in vitro* binding experiments indicated that UniPR500 interacts with the apical sodium-dependent bile acid transporter (ASBT), a key transporter expressed on the enterocytes of the terminal ileum which drives bile acids into the portal vein [25]. Next we evaluated *in vitro* the metabolic stability of UniPR500 by following its disappearance after incubation in murine plasma or in murine liver. In plasma, about 95% of UniPR500 was recovered after 24 h of incubation indicating that neither the amide linkage nor the iminohydroxyl group were subject to a significant chemical or enzymatic

hydrolysis. In mouse liver S9 fraction, one of the available model to investigate oxidative metabolism, nearly 70 % of UniPR500 was recovered unmodified after 1 h of incubation.

Table 2. Physicochemical properties, ASBT binding and *in vitro* stability of UniPR500

Cpd	log$D_{oct,7.4}$	solubility[a](μM)	% ASBT binding [b]	% remaining compound in plasma[c]	% remaining compound in liver[d]
UniPR129	4.90 ± 0.15 [e]	31.8 ± 4.2 [e]	22.7 ± 2.4	98.3 ± 9.5 [e]	56 ± 5 [e]
UniPR500	4.23 ± 0.11 [e]	51.9 ± 4.4 [e]	92.6 ± 1.5	93.7 ± 11.3 [e]	70 ± 7 [e]

[a] From DMSO stock solution. [b] Percent bound at 10 μM [c] Percent remaining after 24h of incubation, 37 °C [d] Percent remaining after 1 h incubation in the presence of a NADPH-regenerating system in liver S9 fraction [e] data from [19]. Data are the means of at least three independent experiments.

Encouraged by these promising results we evaluated if UniPR500 was effectively bioavailable in mice by the oral route. After a single administration (30 mg/kg), the C_{max} of UniPR500 was 0.7 μM after 30 minutes, while its area under the curve (AUC_{0-t}), a measure of systemic exposure to the compound, was equal to 573.1 ng/mL·h, indicating that UniPR500 could be used as a pharmacological tool in mice (Figure 7).

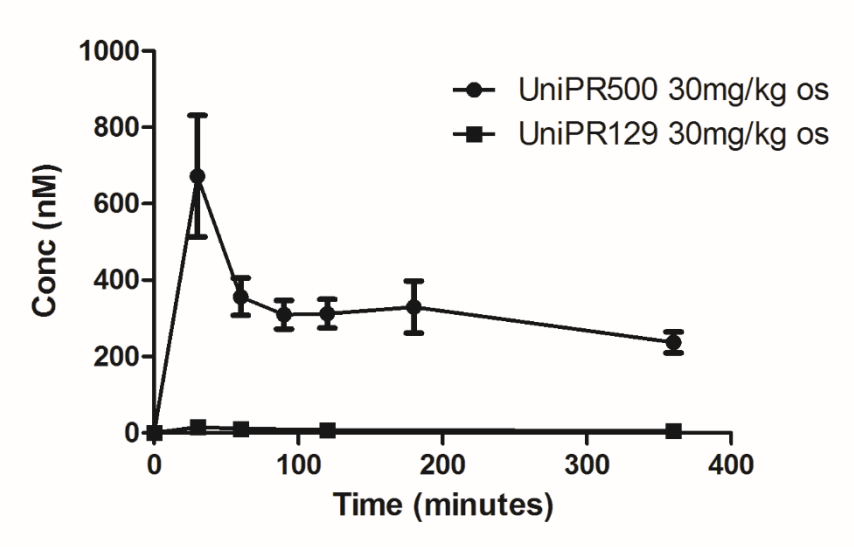

Figure 7 UniPR500 is orally bioavailable in mice. Plasma concentrations (nM) of UniPR500 over 6 hours time course after a single oral administration in male mice (30 mg/kg, os) are reported as obtained from HPLC/MS analysis. Data are the means of at least four independent experiments ± SEM.

Effect of targeting Eph/ephrin *in vivo*

UniPR500 facilitates glucose adsorption in healthy and insulin-resistant mice

The bioavailability of the compound allowed us to evaluate the ability of UniPR500 to improve the glucose tolerance in healthy C57BL/6J mice. The vehicle (methocel) or the compound (30mg/kg) were orally administered to mice 30 minutes before challenge with glucose (2g/kg i.p.). In these conditions, UniPR500 significantly improved overall glucose tolerance and significantly reduced plasma glucose levels 60 minutes after glucose injection (Figure 8A). Fasting plasma glucose and insulin levels were not modified by UniPR500 treatment (Figure 8A, B) whereas 30 minutes after glucose injection, treated animals showed higher insulin concentrations (Figure 8B), confirming *in vitro* data that highlighted UniPR500 as GSIS enhancer. Taken together these data suggest that UniPR500 was able to improve glucose tolerance in healthy mice increasing GSIS without affecting basal glucose and insulin levels.

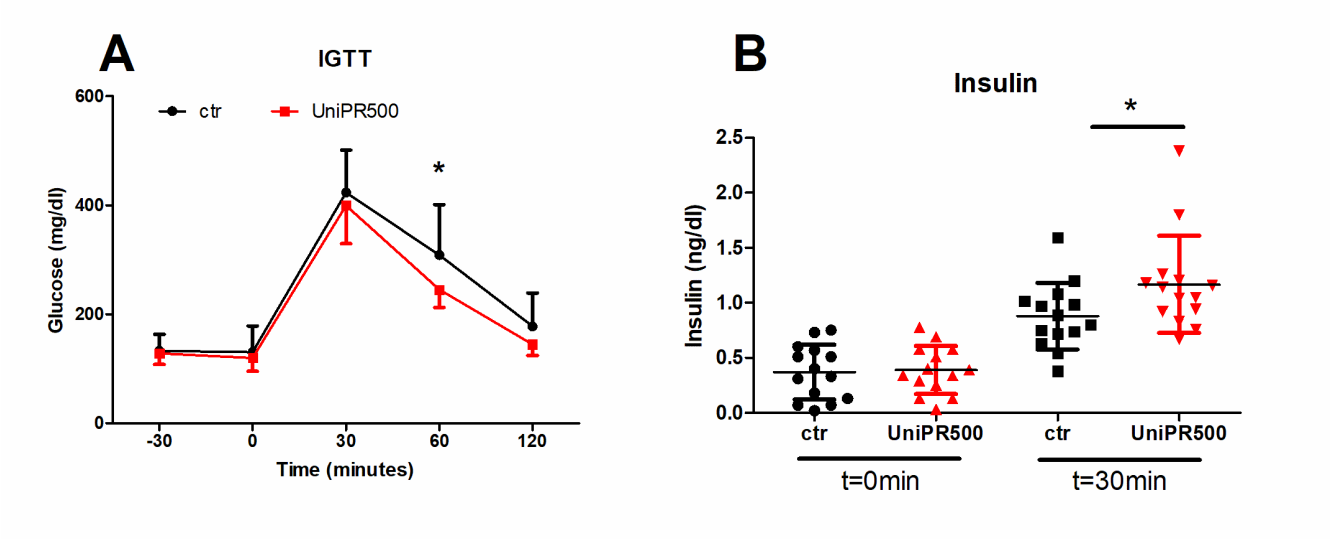

Figure 8. UniPR500 improves glucose tolerance test in mice

*Glucose tolerance test (**A**) and plasma insulin levels (**B**) of male C57BL/6J mice before and after intraperitoneal injection of glucose (2g/kg). UniPR500 30mg/kg was administered 30 minutes before glucose injection. Data are the means ± st. dev.. Two-way ANOVA followed by Bonferroni's post-test. *, p<0.05.*

Afterwards we investigated if UniPR500 was able to control hyperglycaemia in two different non-genetic mouse models of impaired glucose tolerance: 1, the high-fat diet-fed mouse (HFD [13,14,26]) developing an early but persisting slight increase of fasting plasma glucose levels and a slow progressive increase of body weight and

insulin levels mimicking the early phase of type 2 diabetes or a condition of insulin resistance; 2, HFD mouse + low doses of streptozotocin injections, aiming at impairing insulin secretion mimicking a type 1 diabetes [27,28].

The first model was obtained by using C57BL/6J mice fed for 10 weeks with High-Fat Diet (HFD, 60% calories from fat, Mucedola, Milan, Italy) and orally administered with 30 mg/kg UniPR500, or methocel alone, during the last 2 weeks of the HFD treatment (e.g. 9th and the 10th week). Eight weeks of HFD-diet successfully yielded hypercholesterolemic, hyperglycemic, hyperinsulinemic and overweight animals (Figure 9).

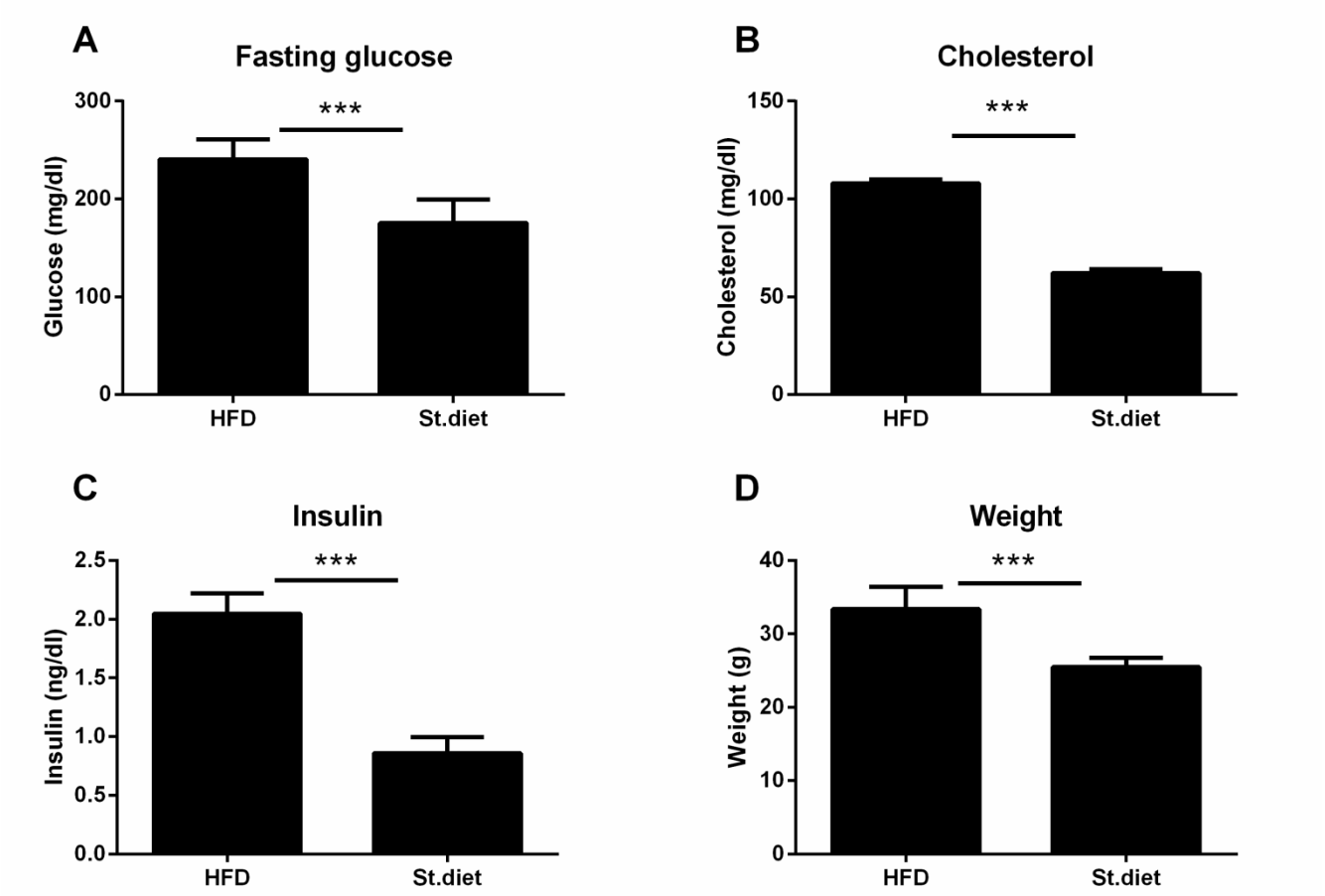

*Figure 9. C57BL/6J mice were fed ad libitum for 8 weeks with standard diet (ctr) or High-Fat Diet (HFD) in order to induce insulin resistance. Glucose (**A**), cholesterol (**B**), insulin fasting (**C**) levels and body weights (**D**) were measured at the end of 8th week. Data are the means ± st.dev. T.test was performed ***, p<0.001.*

Starting from the 9th week and until the end of the 10th week mice were divided in two group: treated group and control group that were daily orally administered with 30mg/kg UniPR500 and methocel, respectively.

At the end of the treatment none of the previous parameters was reverted by UniPR500 treatment. In fact, HFD-mice treated with compound under investigation showed fasting cholesterol, glucose and weight levels comparable to HFD-mice (figure 10).

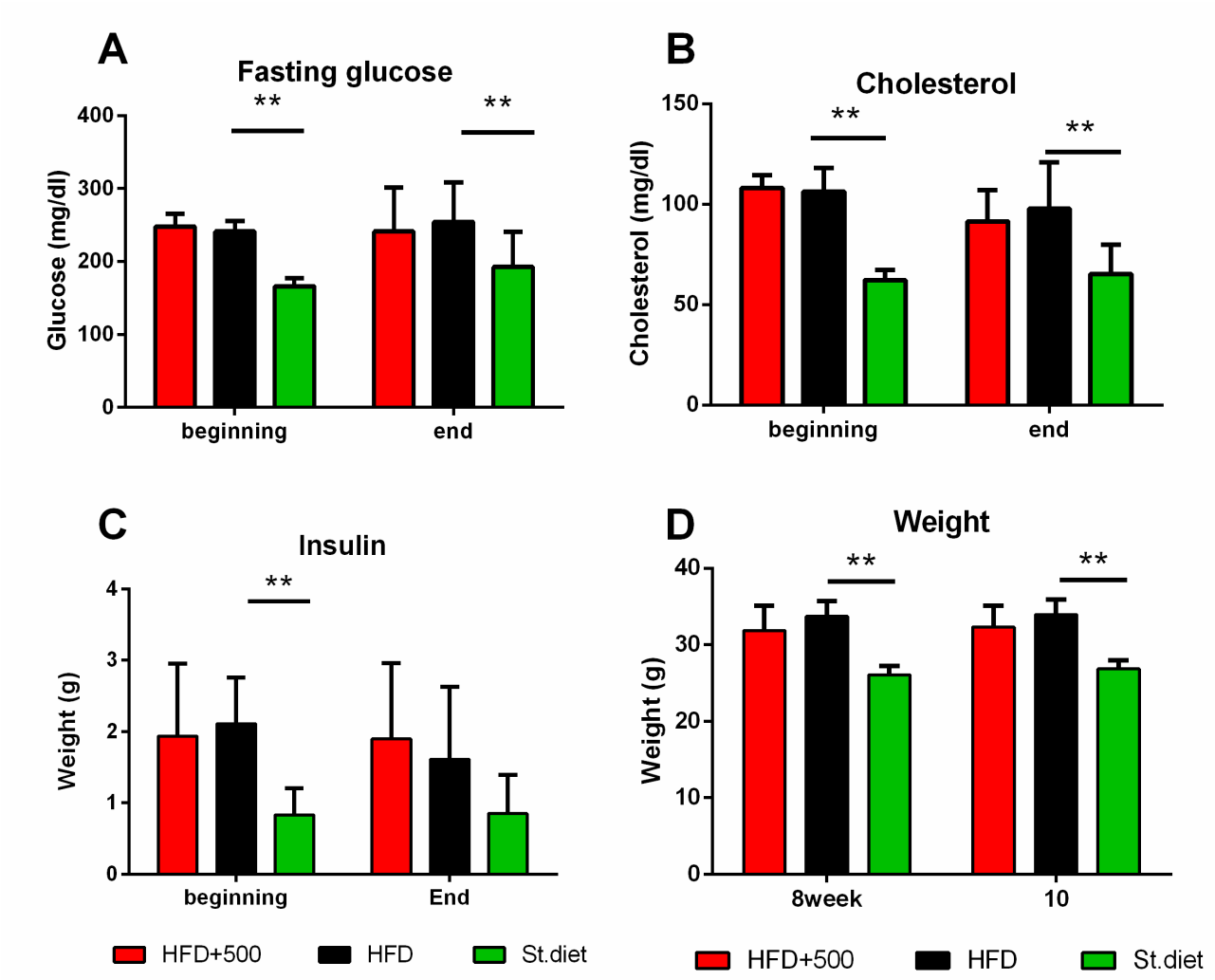

*Figure 10. C57BL/6J standard mice (ctr) or insulin-resistant mice, fed with High-Fat Diet, were orally administered with 30 mg/kg UniPR500 (HFD+500) or methocel (HFD) for 14 days, from the 9th to the 10th week of HFD treatment. Data are the means ± SD. Two-way ANOVA followed by Bonferroni's post-test compared HFD with other treatments. *, p<0.05, **, p<0.01, ***, p<0.001. HFD=High Fat Diet mice, HFD+500=High Fat Diet mice + 14 days UniPR500 30m/kg/die os, ctr=standard diet mice.*

The last administration of UniPR500 or vehicle took place 30 minutes before performing glucose tolerance test. As expected, in these conditions HFD-mice showed poor glucose tolerance when compared to mice fed with a

standard diet (figure 11A) despite the higher plasma insulin levels (Figure 11B) confirming that mice treated with HFD developed insulin-resistance. Nevertheless, HFD mice treated with UniPR500 showed a significant less severe glucose intolerance when compared to HFD-mice, particularly 60 and 120 minutes after glucose injection (figure 11A), also confirmed by AUC (figure 11C). Regarding plasma insulin HFD mice treated with UniPR500 appeared to have higher insulin plasma levels than HFD mice treated with vehicle, but the large scattering of data hamper further proper statistical consideration (figure 11B, D).

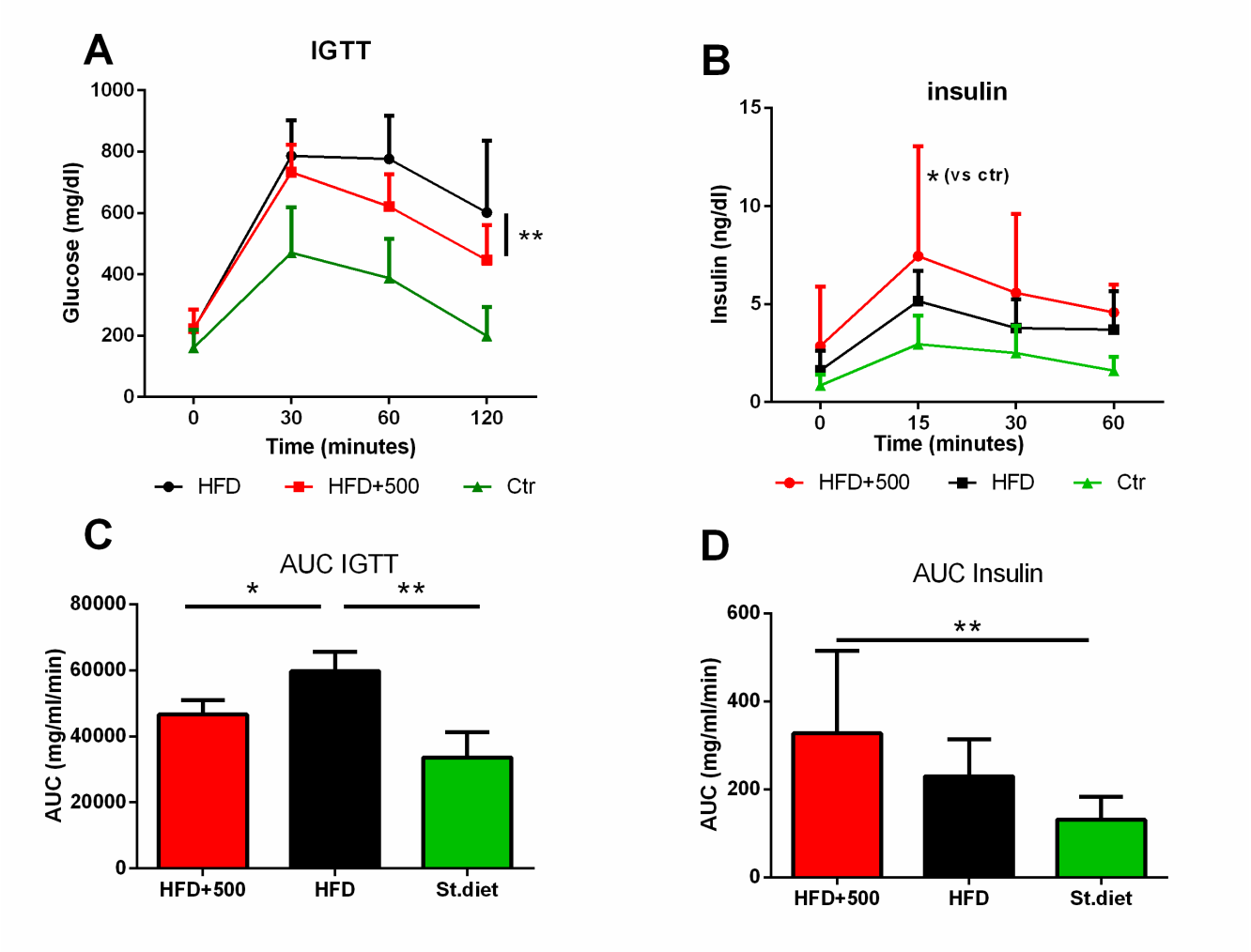

Figure 11. C57BL/6J mice, fed with High-Fat Diet, were orally administered with 30mg/kg UniPR500 (HFD+500) or methocel (HFD) for 14 days, from the 9th to the 10th week of HFD treatment. C57BL/6J mice fed with standard diet for ten weeks (ctr) were orally treated with methocel from the 9th to the 10th week.

*Glucose tolerance test (**A**) and plasma insulin concentration (**B**) after intraperitoneal administration of glucose (2g/kg) 30 minutes after the last oral administration of 30mg/kg UniPR500 or methocel. Panels **C** and **D** represent AUC of glucose plasma levels and AUC of insulin plasma levels, respectively.*

*Data are the means ± st. dev.. Two-way ANOVA followed by Bonferroni's post-test (**A**, **B**) comparing HFD+500*

*with other treatments. One-way ANOVA followed by Dunnett's post-test for panels **C** and **D**, comparing HFD+500 with other treatments. *, p<0.05, **, p<0.01.*

UniPR500 does not improve glucose tolerance test in streptozotocin-treated mice

We finally asked if UniPR500 could improve glucose tolerance in a more severe model of diabetes induced by 5 consecutive administrations (days 1-5 of the experimental procedure) of a low dose of streptozotocin (40mg/kg i.p. 1 x day, 5 consecutive days) followed by HFD diet for 6 weeks (days 8 – 50 of the experimental procedure). This model is not univocally described by Authors as a model of type 1 diabetes [14,28,29], but our histological findings showed an extensive pancreatic damage compromising β-cell function. According to the histopatological features, no significant differences emerged between the streptozotocin-HFD mice treated with UniPR500 and streptozotocin-HFD untreated mice; both groups showed endocrine pancreatic injuries, islet cells vacuolation and lymphocytes infiltration, indicative of Insulin-Dependent Diabetes Mellitus, mild or severe degenerative changes (steatosis) in the liver, slight focal interstitial inflammation in kidneys (supplementary material).

As expected, mice showed high fasting glucose and cholesterol plasma concentrations together with significantly higher body weights when compared with animals fed with standard diet and not receiving streptozotocin injections (data not shown). At 37TH day of the experimental procedure, streptozotocin + HFD mice were randomly divided in 2 groups and daily treated with 30mg/kg UniPR500 or methocel respectively. Compound administration did not modify fasting glucose or cholesterol levels after 14 days of treatment neither changed weight when compared with mice treated only with vehicle (data not shown).

At the 50th day the last administration of UniPR500 30mg/kg or Methocel was done 30 minutes before performing IGTT on streptozotocin + HFD mice (mice fed with standard diet are used as internal control). Streptozotocin + HFD mice showed a very poor glucose tolerance highlighted by an AUC nearly 4-fold higher than control animals. The subchronic treatment with 30mg/kg UniPR500 failed to improve glucose tolerance in these mice and the pancreas histological analysis of these animals could help to explain this data (figure 12). In fact in these mice pancreas resulted severely damage suggesting β-cell function impairment. As expected by a GSIS enhancer, since UniPR500 improves glucose tolerance through the increase of insulin release from healthy β-cells, it can not act if these cells are damaged and no longer working, a condition that reflect a type 1 diabetes rather than a type 2 diabetes or I/R.

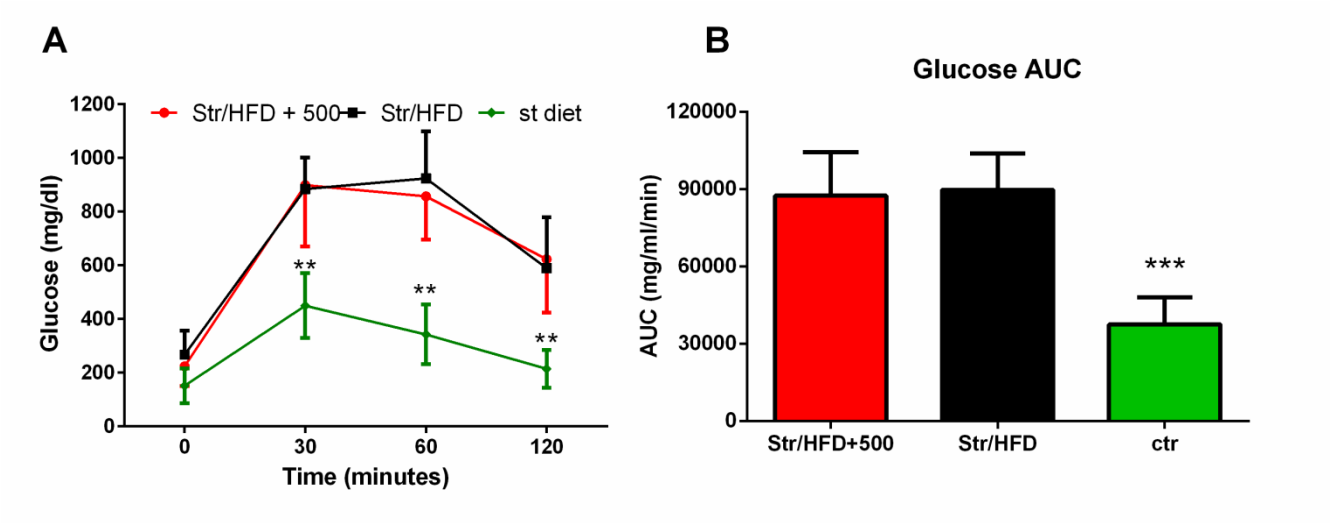

Figure 12. UniPR500 does not modify glucose tolerance of streptozotocin/HFD mice

C57BL/6J mice were injected with streptozotocin 40mg/kg i.p. and fed ad libitum for 42 days with High-Fat Diet. UniPR500 30mg/kg or Methocel were orally administered for 14 days from day 37 to day 50. ***A****, Glucose tolerance test after intraperitoneal administration of glucose (2g/kg) 30 minutes after the last oral administration of 30mg/kg UniPR500 or methocel at the end of treatment* ***B****, AUC of panel* ***A****. Data are the means ± st. dev.. Two-way ANOVA followed by Bonferroni's post-test (****A****) comparing all the treatments, **, p<0.01 for standard diet vs other treatments. One-way ANOVA followed by Dunnett's test for panels* ***B****.***, for standard diet vs other treatments.*

DISCUSSION

Eph/ephrin system is an emerging target for cancer therapy and different pharmacological approaches were developed or are under development by using proteins, peptides, antibodies or small molecules including PPI-inhibitors and kinase inhibitors [7] . In this context, we have recently started a drug discovery program aimed at finding PPI inhibitors targeting Eph/ephrin interaction identifying lithocholic acid (LCA) as hit compound during a screening campaign. Through an iterative lead optimization phase based on molecular simulations [9] synthesis and pharmacological analysis [19], we developed new derivatives endowed with better potency, efficacy, chemico-physical properties and bioavailability [10]. UniPR500 represents the most advanced LCA derivative developed so far targeting the Eph/ephrin interface, also in light of its high selectivity versus the Eph receptors. This compound failed to interact with several targets involved in the control of glucose homeostasis

i.e. PPAR-γ; GLP-1; DDP-4; PTP1B enzyme KATP ion channel and TGR5 (GPBAR1), the critical G-protein coupled receptor activated by LCA and close analogues [18]

Even if Eph/ephrin targeting was primarily addressed at the discovery of new drugs useful for cancer therapy and many proof-of concepts support its efficacy [29–33], its usefulness is going to be studied also in other fields including ALS [34], nerve regeneration and diabetes [35]. As for the latter case Jain et al showed that kinase inhibitors of the EphA receptors increased GSIS both in *in vitro* and in *in vivo* studies [6]. In fact the inhibition of EphA5 forward signaling was shown to increase GSIS , as well the activation of ephrin-A reverse signaling [2]. In this context it is not surprising that pharmacological blockade of EphA receptors by means of a kinase inhibitor resulted in increased GSIS [6].

Here we tested the *in vitro* and *in vivo* efficacy of UniPR500, a new PPI inhibitor targeting Eph-ephrin interaction. It is worth to note that kinase inhibitors are able to block the forward signaling only whereas the PPI-inhibitor UniPR500 used in this study can block both the forward and the reverse signaling. The behavior of a UniPR500 as GSIS enhancer is not obvious since it may increase GSIS by blocking forward signaling controlled by EphA5 but it may also reduce GSIS by inhibiting the reverse signaling controlled by ephrin-A5.

In the previous study [6] a roughly 2-fold increase of insulin secretion upon treatment with Eph kinase inhibitors on MIN6 or mice pancreatic islet cells was described. In our study we used the innovative technique of dynamic perifusion to study real time insulin secretion from human EndoC-βH1 cells [36]. We showed that GSIS peaked between 10 to 20 minutes after glucose stimulation and completely finished after 30 minutes. In our model UniPR500 induced an up to 5-fold increased insulin release which prompted us to evaluate further this compound in *in vivo* models of diabetes.

We next studied the effect of UniPR500 on glucose tolerance in healthy mice and, according to our data on the dynamic perifusion model, we found a significant increase in insulin secretion and glucose tolerance. Overall, this further confirms the efficacy of Eph signaling inhibition as a valid strategy in GSIS potentiation. However UniPR500 appeared less effective than kinase inhibitors [6] as GSIS enhancer in mice and consequently the improvement of glucose tolerance turned out to be minor. The apparently lower efficacy of UniPR500 may be explained by the use of different doses or by considering the different mechanism of action of this molecule in comparison to kinase inhibitors. As mentioned before, kinase inhibitors block only Eph forward signaling whilst a PPI-inhibitor like UniPR500 can reduce insulin release by inhibiting the reverse signaling. In the future may be interesting to evaluate in the same model the effect of the recombinant EphA5-Fc in comparison to Eph kinase inhibitors and Eph/ephrin antagonists. Indeed, EphA5-Fc may be able to block EphA5 forward signaling as

kinase inhibitor, but also able to activate ephrin-A5 reverse signaling at the same time. In this view EphA5-Fc may be even more effective than kinase inhibitors as GSIS enhancer and glucose tolerance improver.

In addition, we studied for the first time the effect of Eph/ephrin targeting on glucose tolerance in a non-genetic mouse model of insulin resistance, where UniPR500 treatment allowed a faster recovery from hyperglycemia. Since no data are available with kinase inhibitors, future studies aimed at evaluating their efficacy could be very interesting.

Finally, as expected by a GSIS enhancer, UniPR500 failed to improve glucose tolerance when IGTT was performed in a condition where mice pancreatic function was severely compromised. New approaches are under investigations to control autoimmune diabetes by modulating the immune response through phosphoinositide 3-kinase-y inhibitors PTPN22 [37], carbamazepine [38], CCL4 administration [39] or Beta cells replacement [40].

CONCLUSIONS

Although further studies are needed to clarify if an alteration of Eph/ephrin system affects pancreas of T2D patients, these data demonstrate that Eph targeting agents are new and promising insulin enhancers, potentially devoid of hypoglicaemic side effect thanks to their action on GSIS rather than on basal insulin release. Moreover, the relative low expression profile of Eph/ephrin system in adult tissues may ensure for this new class of drugs a satisfactory safety profile in clinical use. Nevertheless given the prominent role played by this system during embryogenesis [41], further studies on possible teratogenic effects of these compounds are needed. Interestingly, the literature data highlighted the involvement of Eph/ephrin signaling in vascular and nerve injuries, as well as in the neuropathic pain transmission [42,43], all diabetes comorbidities. Therefore, further investigations are essential to determine the complete pharmacological/toxicological profile of action of this new class of Eph/ephrin antagonists after chronic administration.

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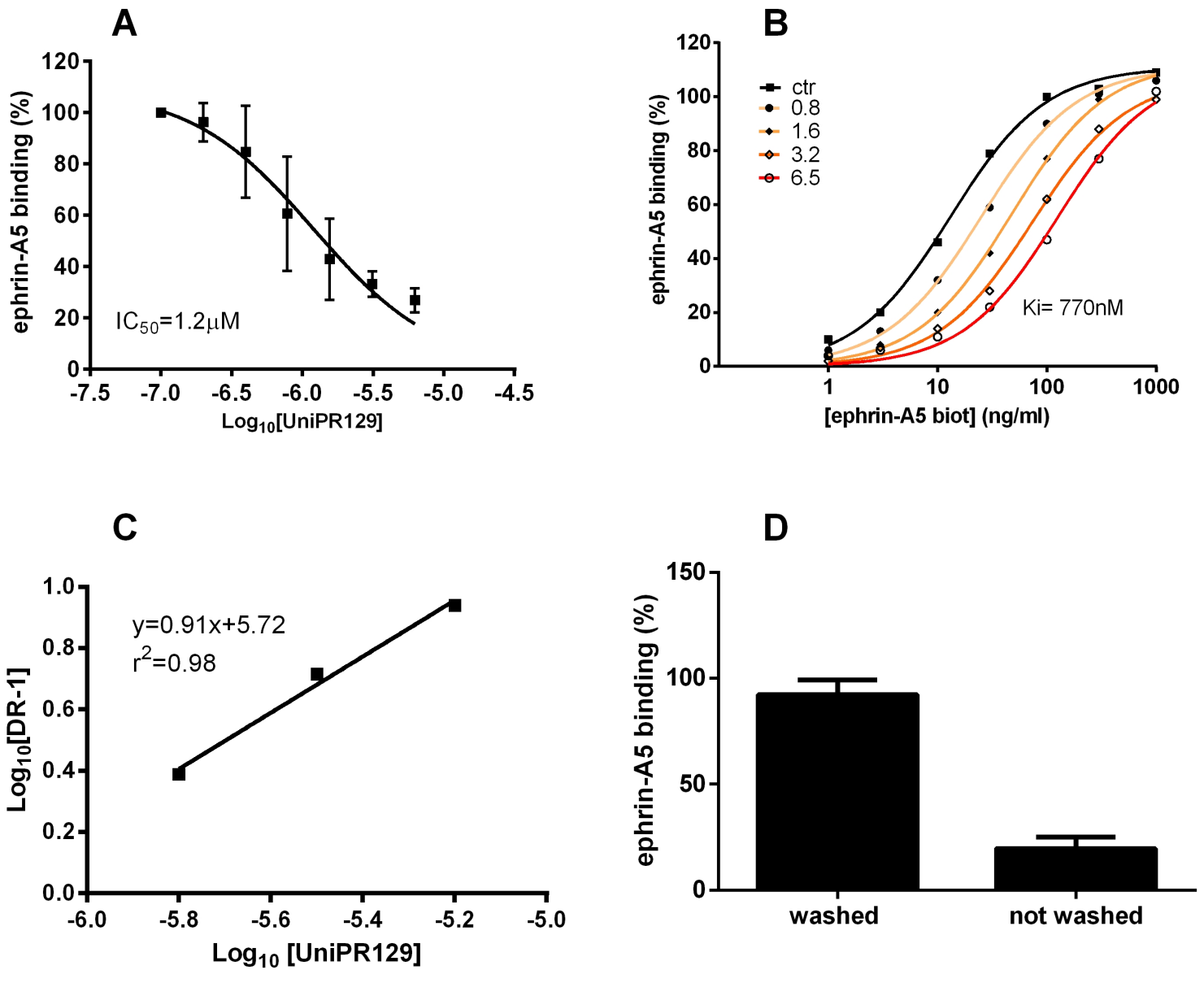
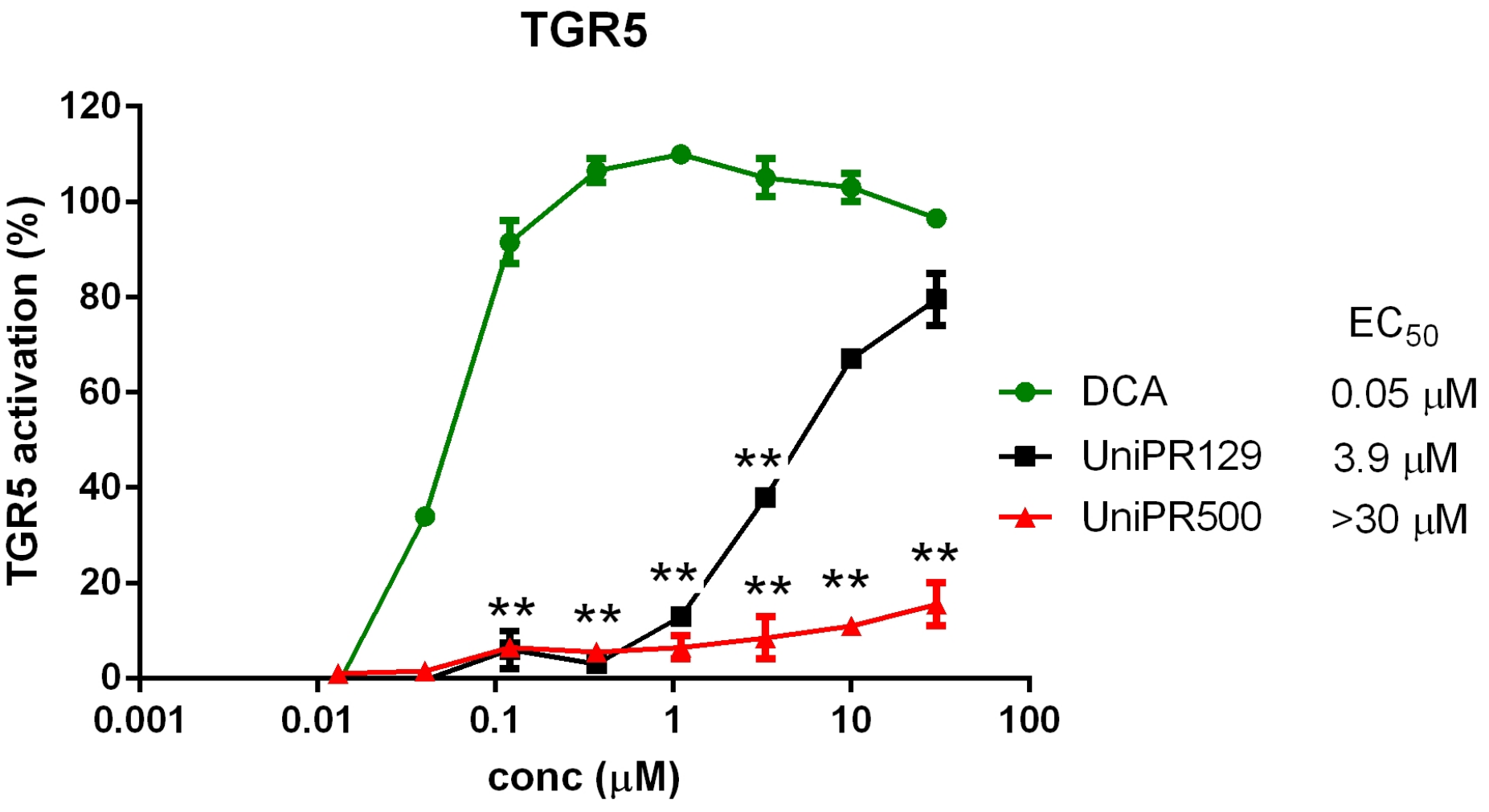
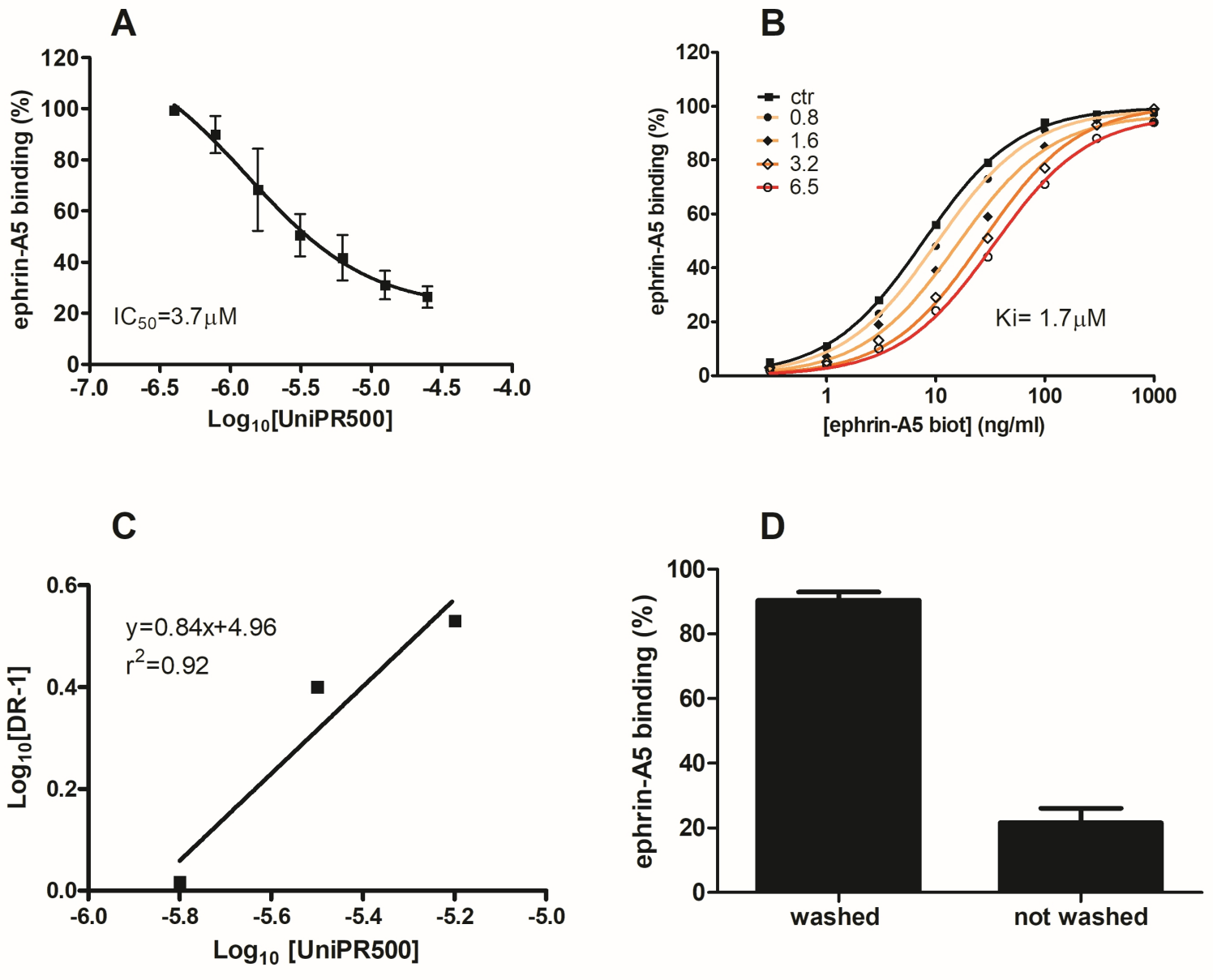
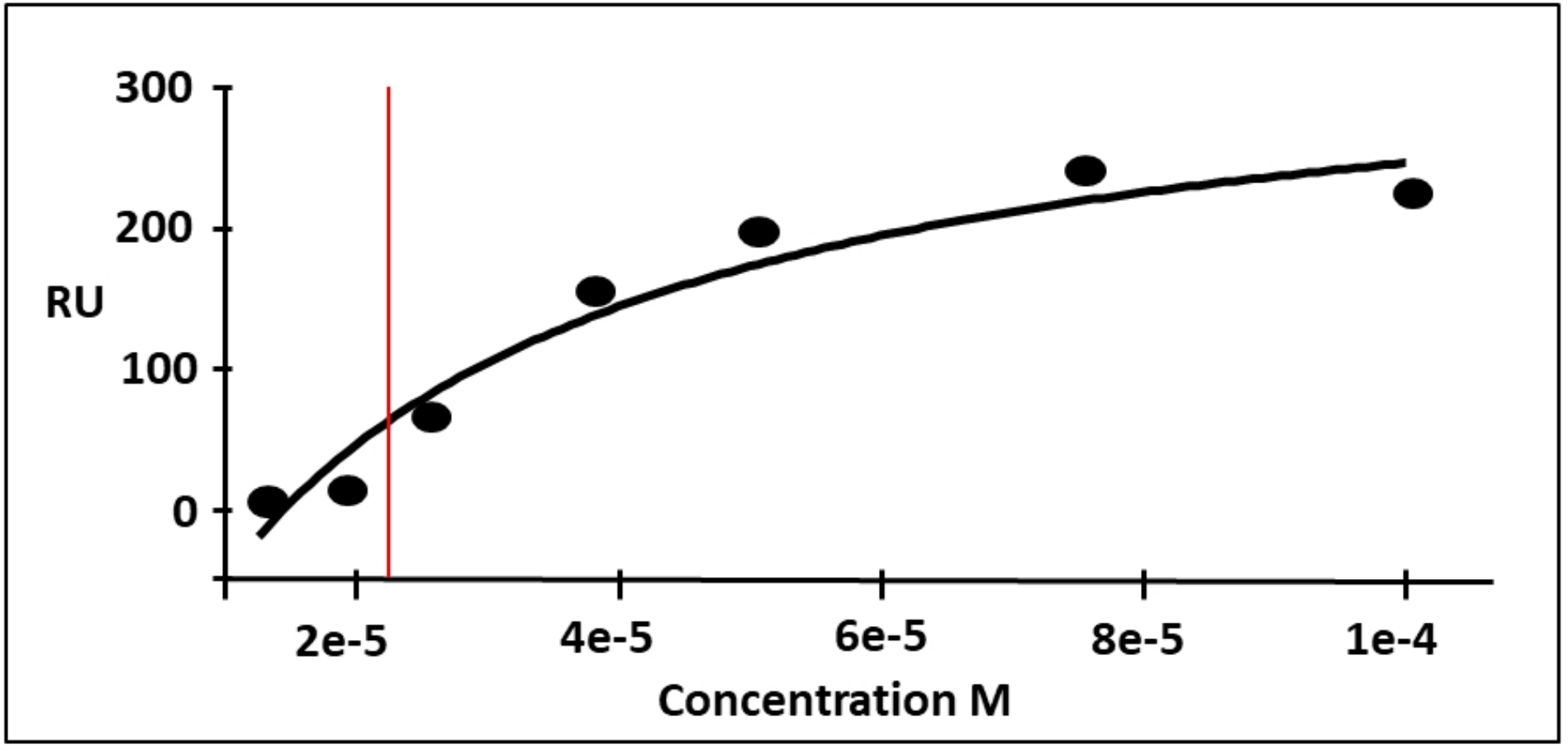
CONFLICT OF INTEREST

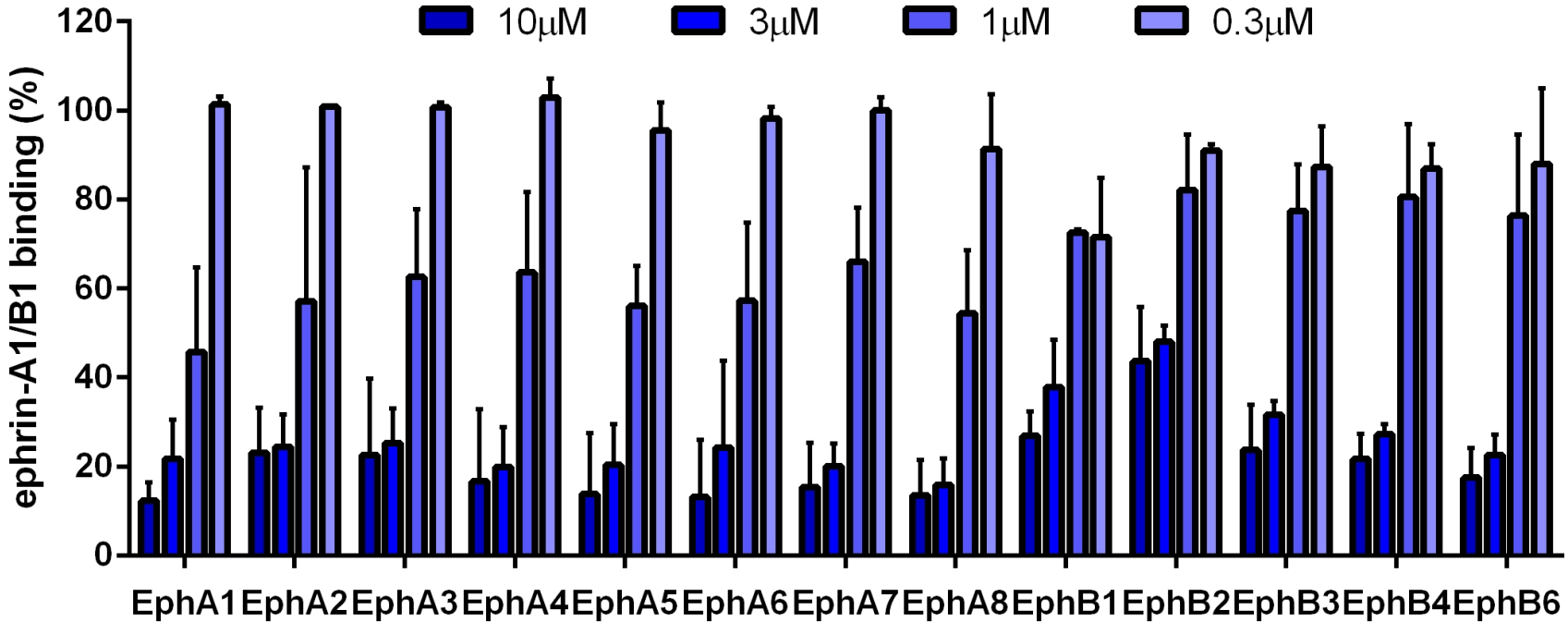
The compound was patented by AL, MI and MT


A
ephrin-A5 binding (%)
120
100
80
60
40
20
0
IC50=1.2μM
-7.5
-7.0
-6.5
-6.0
-5.5
-5.0
-4.5
Log10[UniPR129]
B
ephrin-A5 binding (%)
ctr
0.8
1.6
3.2
6.5
Ki= 770nM
1
10
100
1000
[ephrin-A5 biot] (ng/ml)
C
Log10[DR-1]
y=0.91x+5.72
r2=0.98
1.0
0.8
0.6
0.4
0.2
0.0
-6.0
-5.8
-5.6
-5.4
-5.2
-5.0
Log10 [UniPR129]
D
ephrin-A5 binding (%)
150
100
50
0
washed
not washed

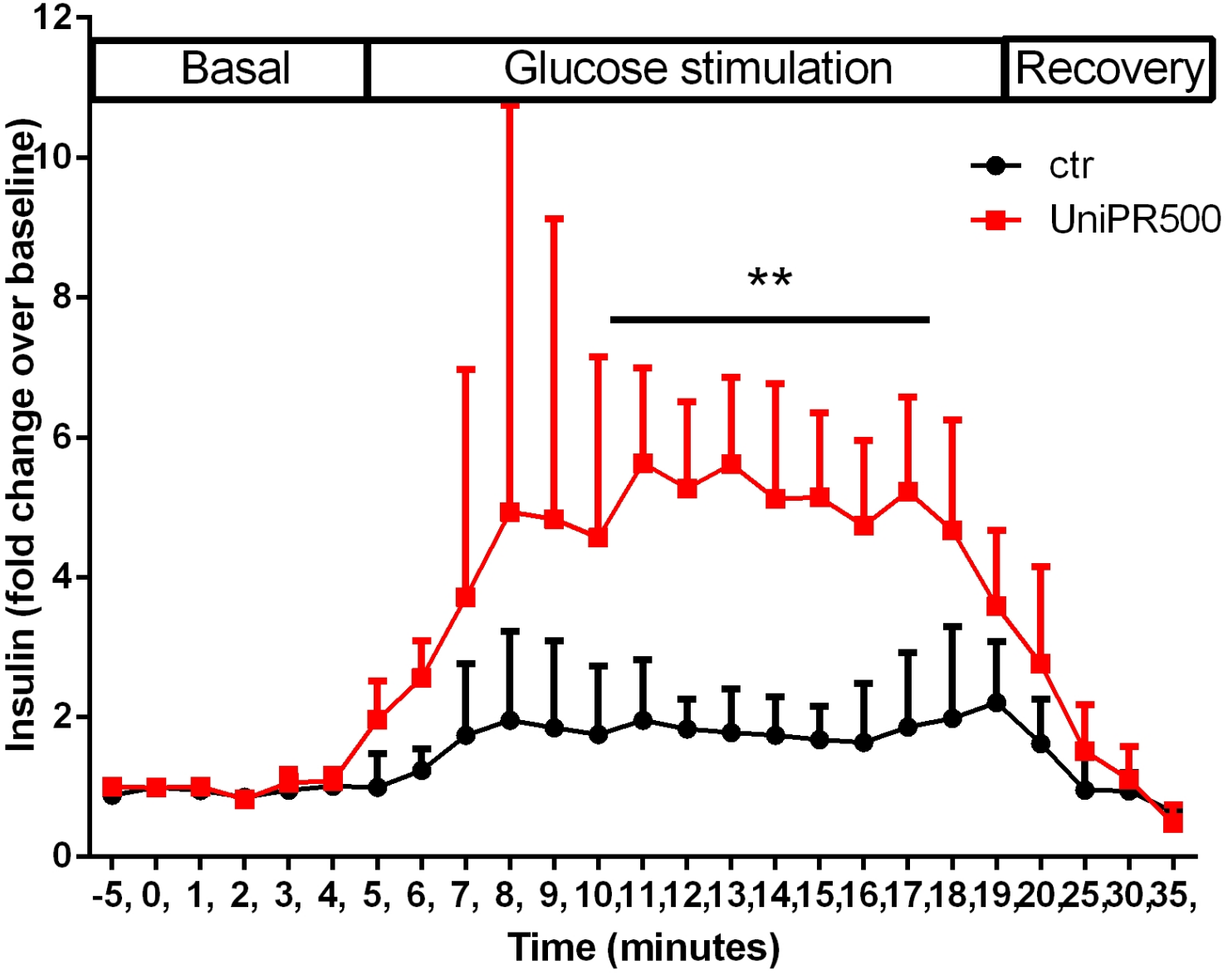

TGR5
TGR5 activation (%)
120
100
80
60
40
20
0
0.001
0.01
0.1
1
10
100
conc (μM)
**
**
**
**
**
**
EC50
DCA
0.05 μM
UniPR129
3.9 μM
UniPR500
>30 μM

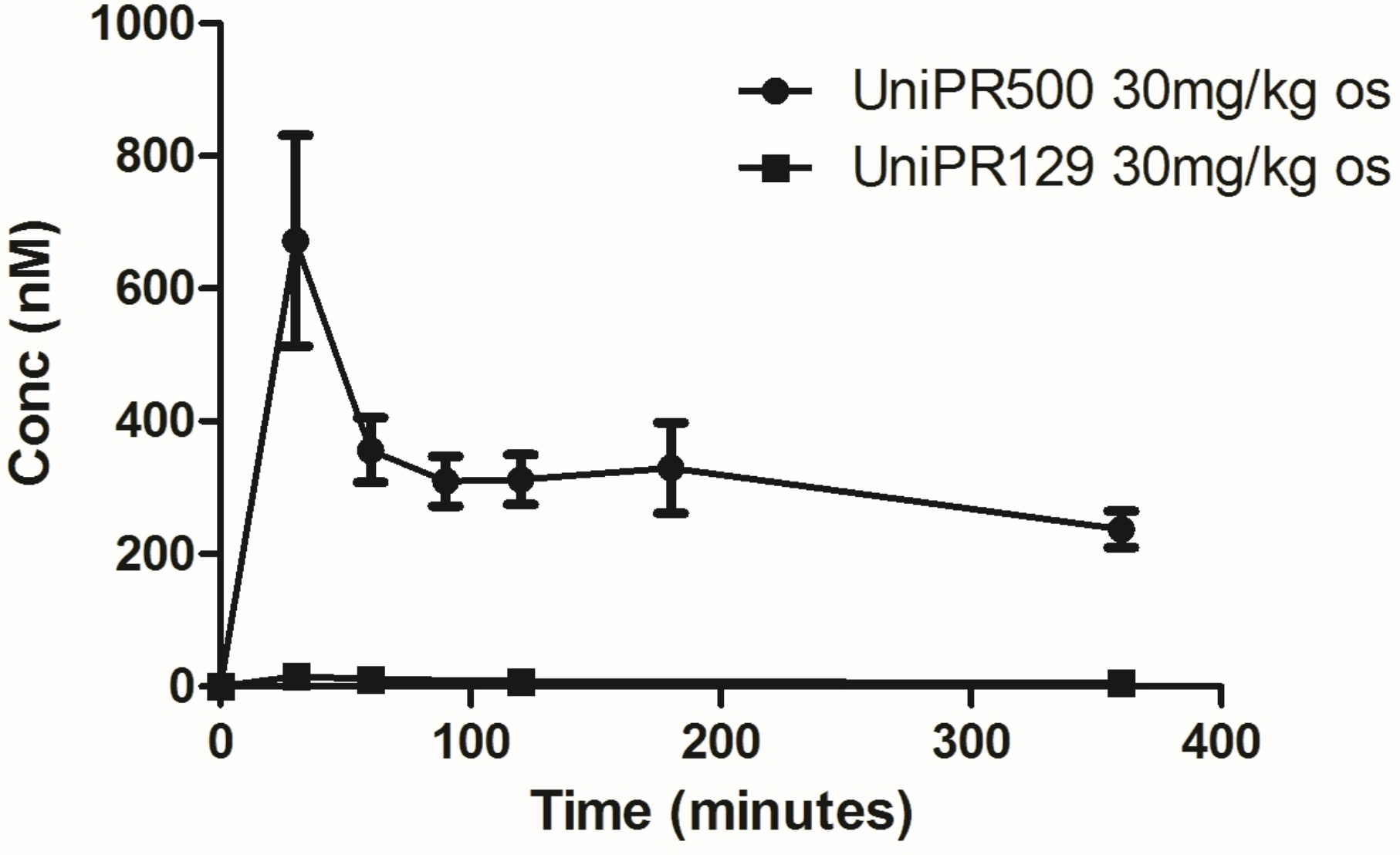

A
ephrin-A5 binding (%)
120
100
80
60
40
20
0
IC50=3.7μM
-7.0
-6.5
-6.0
-5.5
-5.0
-4.5
-4.0
Log10[UniPR500]
B
ephrin-A5 binding (%)
ctr
0.8
1.6
3.2
6.5
Ki= 1.7μM
1
10
100
1000
[ephrin-A5 biot] (ng/ml)
C
Log10[DR-1]
y=0.84x+4.96
r2=0.92
0.6
0.4
0.2
0.0
-6.0
-5.8
-5.6
-5.4
-5.2
-5.0
Log10 [UniPR500]
D
ephrin-A5 binding (%)
100
80
60
40
20
0
washed
not washed

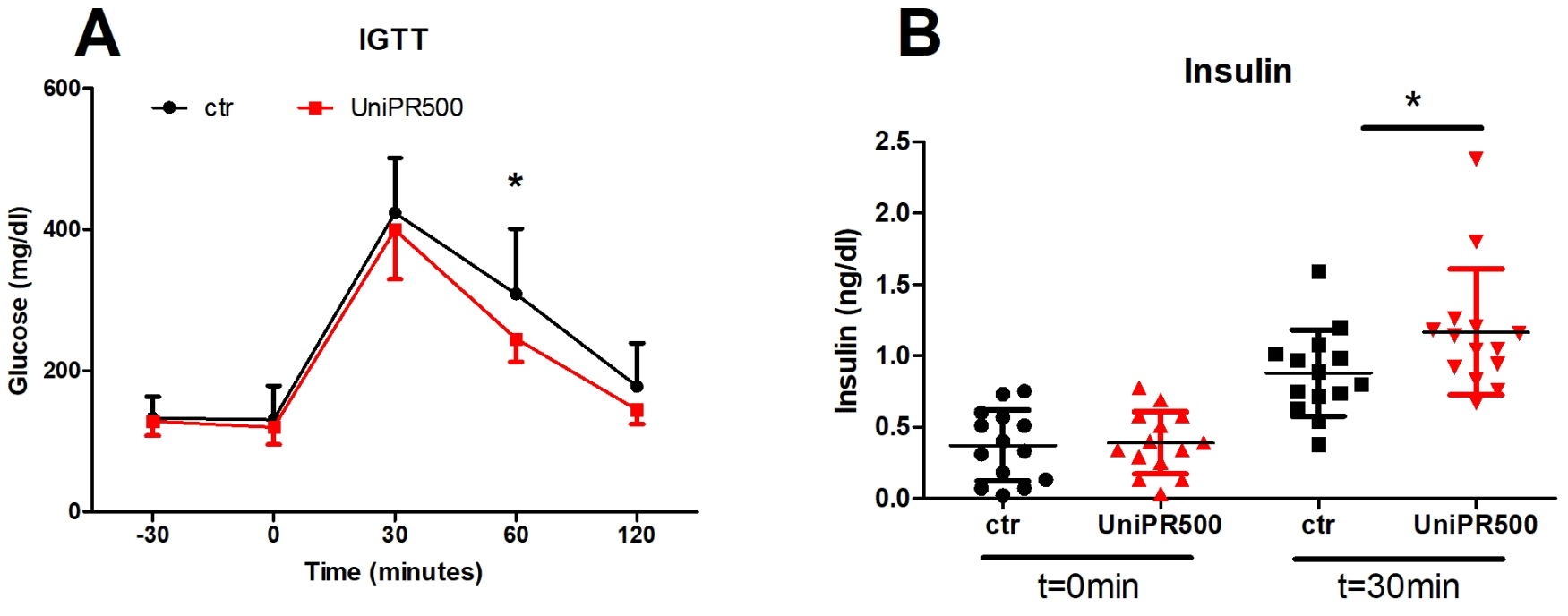

300
200
RU
100
0
2e-5
4e-5
6e-5
8e-5
1e-4
Concentration M

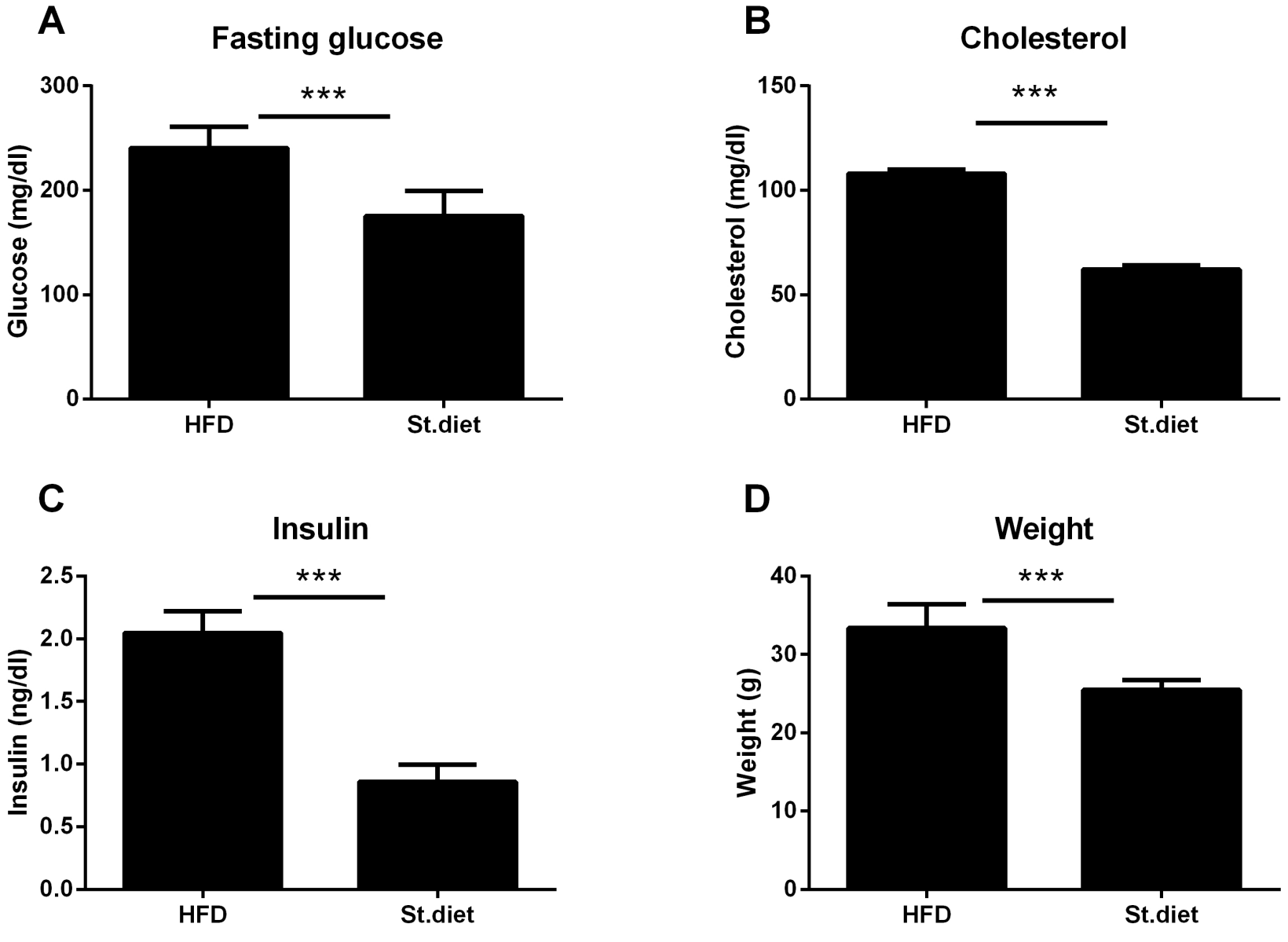


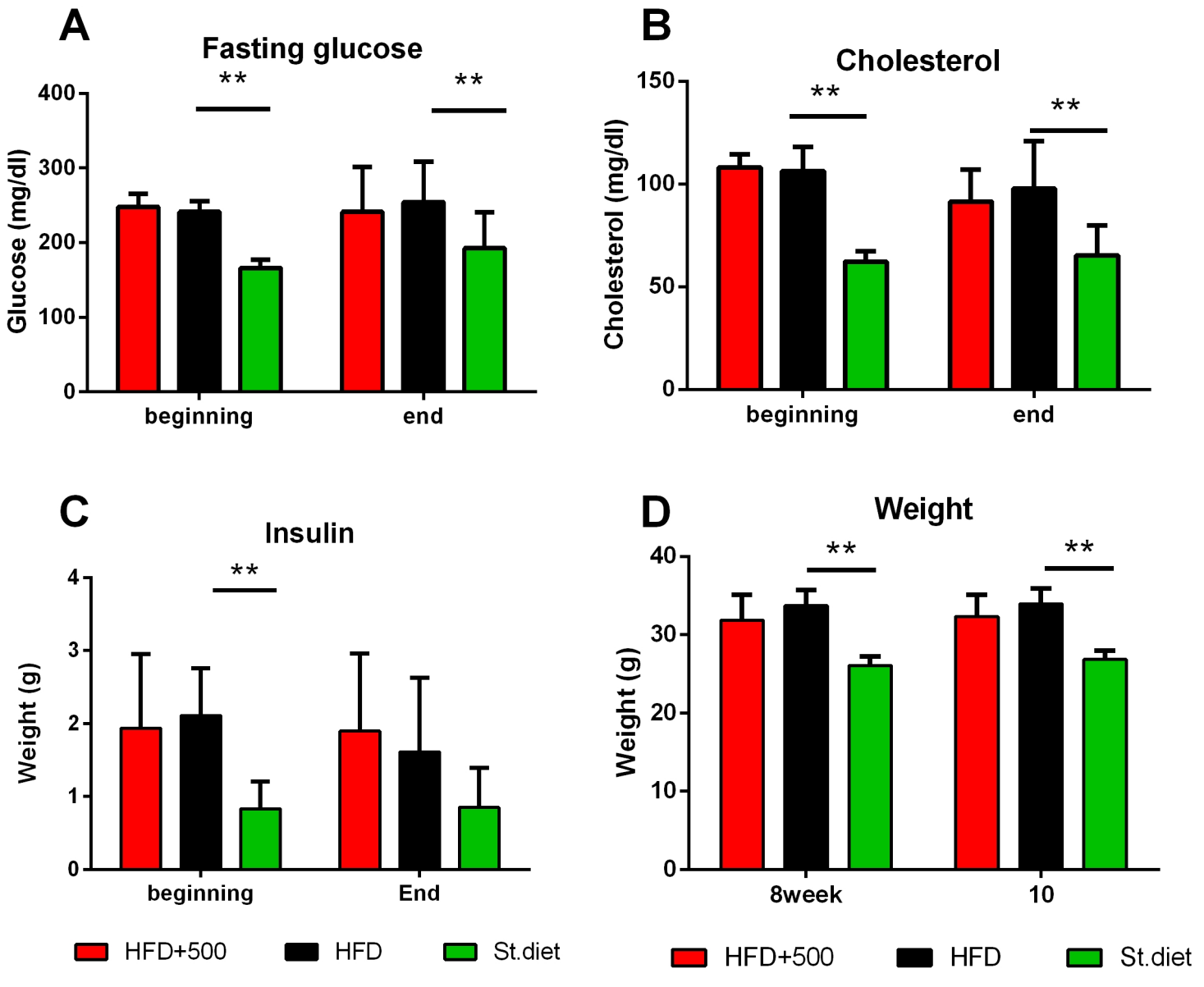
	EphA1	EphA2	EphA3	EphA4	EphA5	EphA6	EphA7	EphA8	EphB1	EphB2	EphB3	EphB4	EphB6
IC_{50}(μM)	1.23	1.14	1.52	1.48	1.29	1.41	1.52	1.18	2.56	5.09	2.47	2.41	2.05

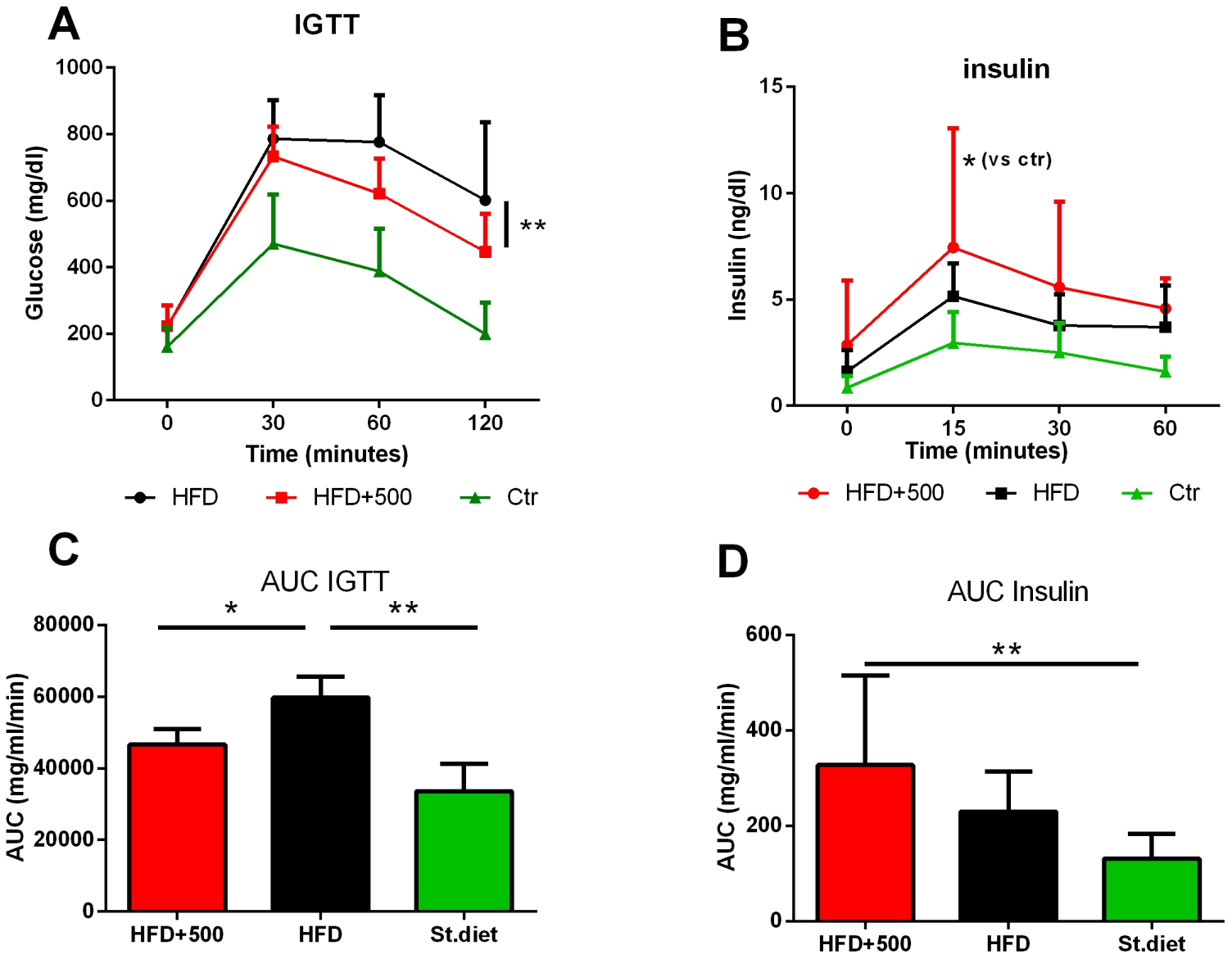

Basal
Glucose stimulation
Recovery
ctr
UniPR500
**
Insulin (fold change over baseline)
0
2
4
6
8
10
12
-5, 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35,
Time (minutes)

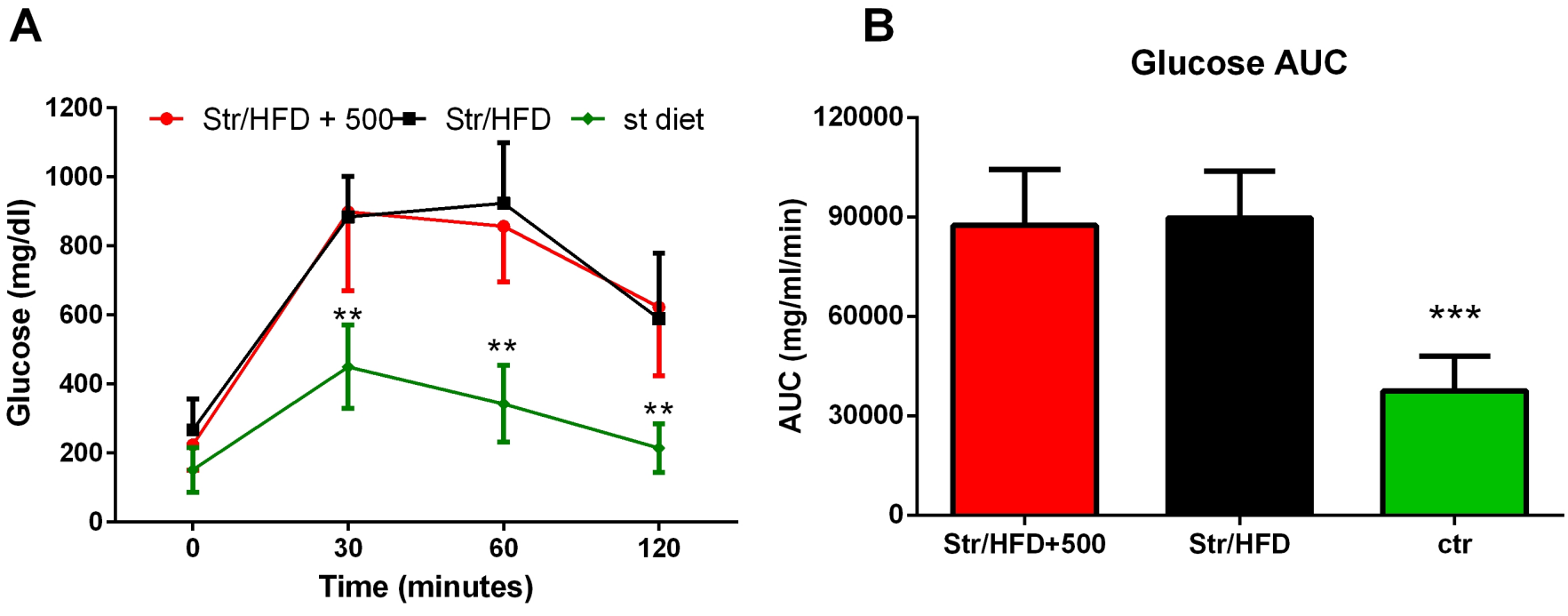

1000
800
600
400
200
0
Conc (nM)
0
100
200
300
400
Time (minutes)
UniPR500 30mg/kg os
UniPR129 30mg/kg os


A
IGTT
ctr
UniPR500
Glucose (mg/dl)
600
400
200
0
-30
0
30
60
120
Time (minutes)
*
B
Insulin
Insulin (ng/dl)
2.5
2.0
1.5
1.0
0.5
0.0
ctr
UniPR500
ctr
UniPR500
t=0min
t=30min
*

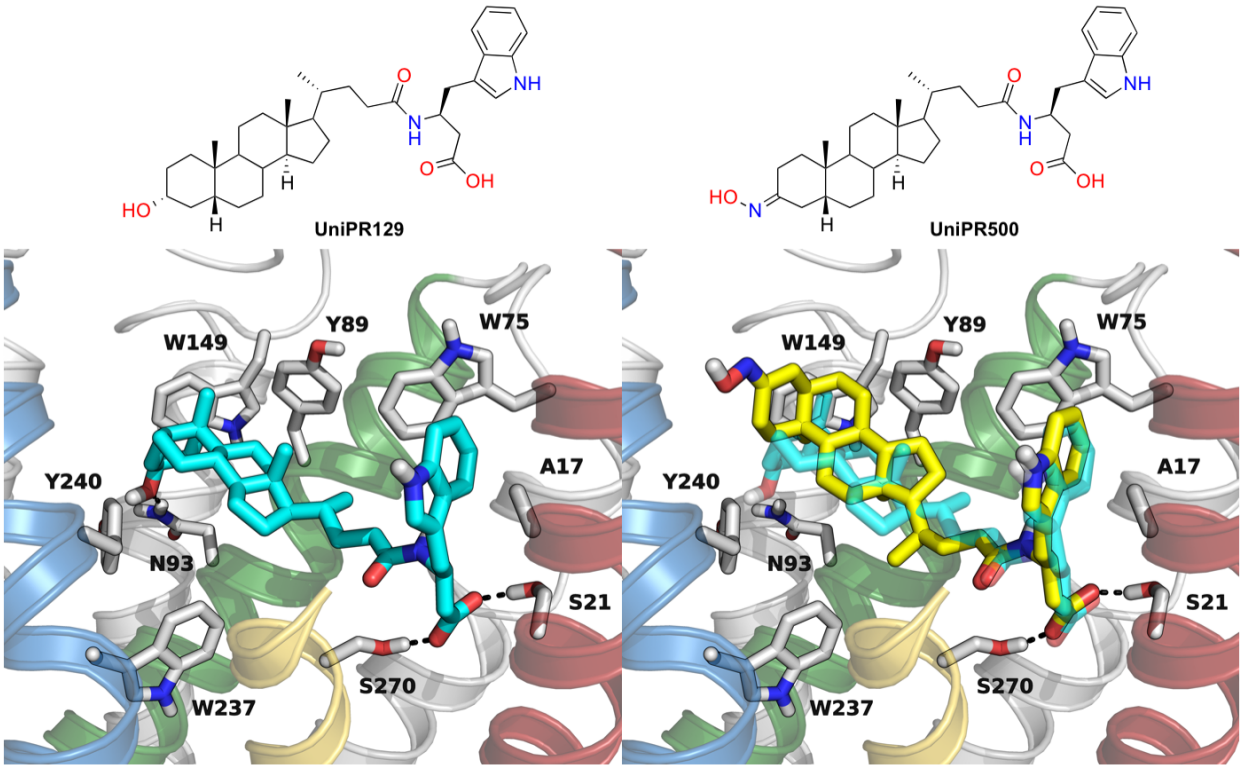

A
Fasting glucose
Glucose (mg/dl)
300
200
100
0
***
HFD
St.diet
B
Cholesterol
Cholesterol (mg/dl)
150
100
50
0
***
HFD
St.diet
C
Insulin
Insulin (ng/dl)
2.5
2.0
1.5
1.0
0.5
0.0
***
HFD
St.diet
D
Weight
Weight (g)
40
30
20
10
0
***
HFD
St.diet


A
Fasting glucose
**
**
Glucose (mg/dl)
400
300
200
100
0
beginning
end
B
Cholesterol
**
**
Cholesterol (mg/dl)
150
100
50
0
beginning
end
C
Insulin
**
Weight (g)
4
3
2
1
0
beginning
End
D
Weight
**
**
Weight (g)
40
30
20
10
0
8week
10
HFD+500
HFD
St.diet
HFD+500
HFD
St.diet


A
IGTT
Glucose (mg/dl)
1000
800
600
400
200
0
0
30
60
120
Time (minutes)
**
HFD
HFD+500
Ctr
B
insulin
Insulin (ng/dl)
15
10
5
0
* (vs ctr)
0
15
30
60
Time (minutes)
HFD+500
HFD
Ctr
C
AUC IGTT
AUC (mg/ml/min)
80000
60000
40000
20000
0
*
**
HFD+500
HFD
St.diet
D
AUC Insulin
AUC (mg/ml/min)
600
400
200
0
**
HFD+500
HFD
St.diet

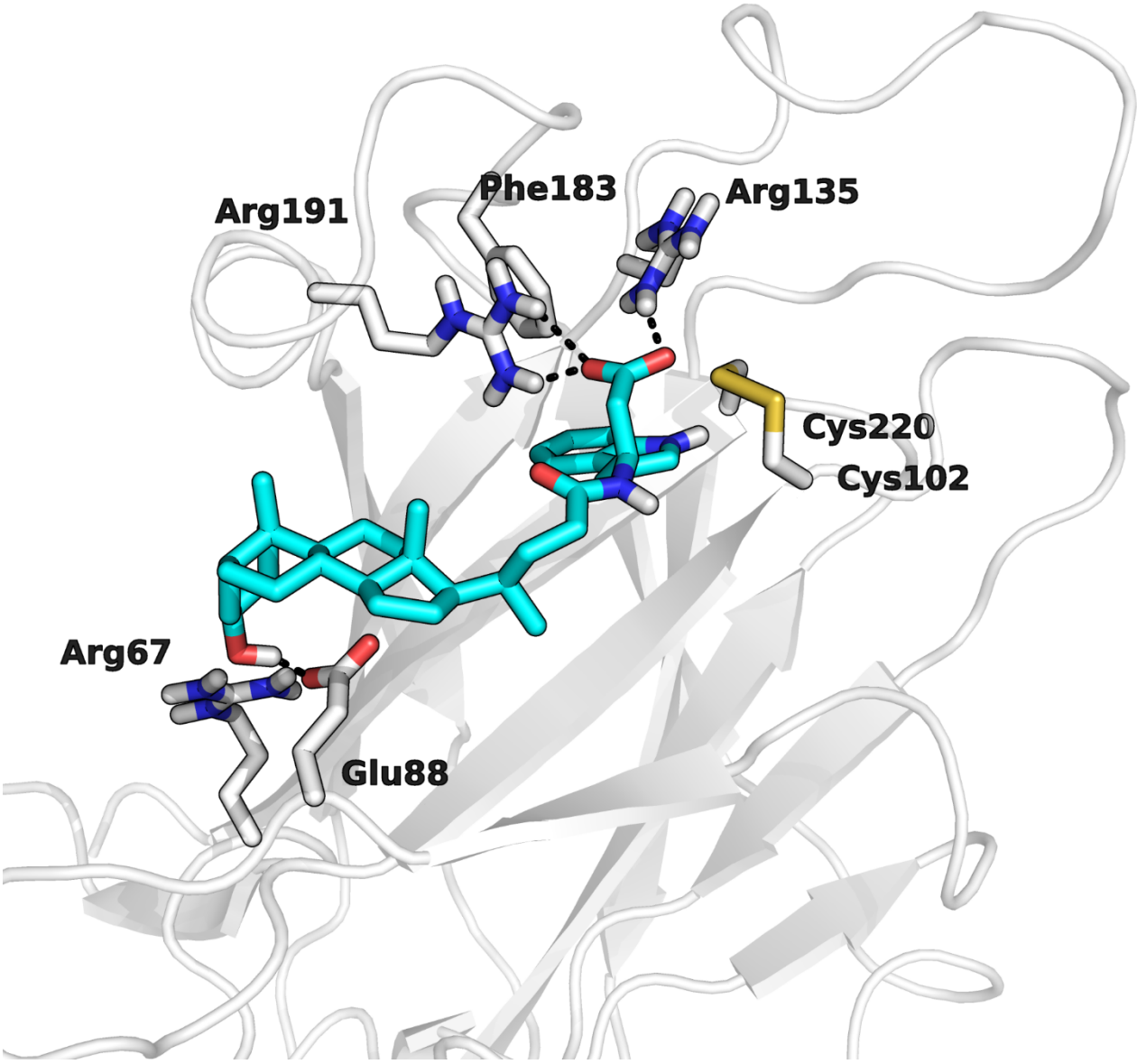

A
Str/HFD + 500
Str/HFD
st diet
Glucose (mg/dl)
1200
1000
800
600
400
200
0
**
**
**
0
30
60
120
Time (minutes)
B
Glucose AUC
AUC (mg/ml/min)
120000
90000
60000
30000
0
***
Str/HFD+500
Str/HFD
ctr

Supplementary material 1

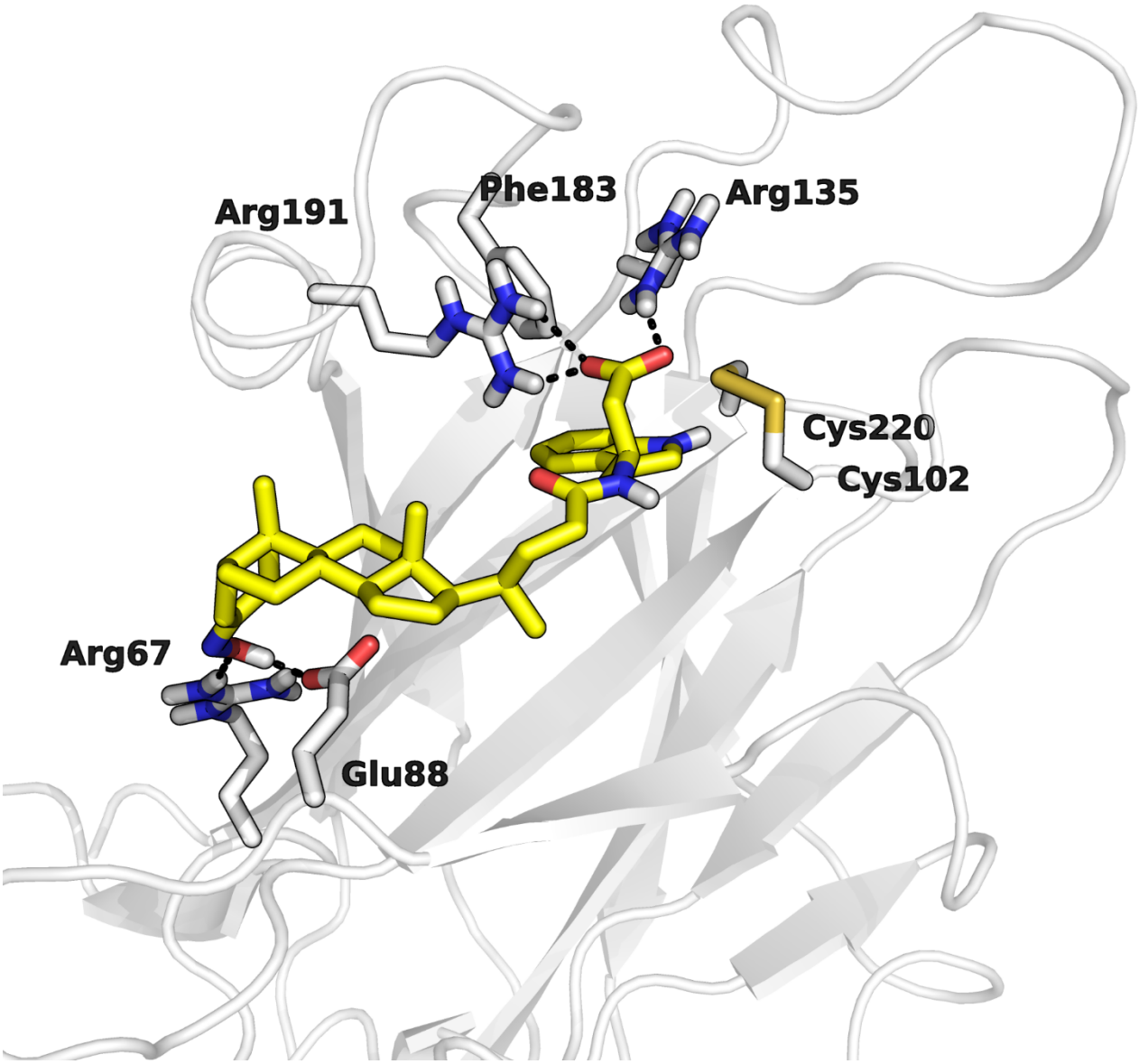


Binding mode of UniPR129 (left, cyan carbon atoms) and of UniPR500 (right, yellow carbon atoms) to TGR5 receptor (white carbon atoms).

Supplementary material 2



Binding pose of UniPR129 (cyan carbon atoms) on EphA5 binding site (white carbon atoms). The overall structure of EphA5 is represented with white ribbons. EphA5 structure was taken from PDB (4ET7.pdb)



Binding pose of UniPR500 (yellow carbon atoms) on EphA5 binding site (white carbon atoms). The overall structure of EphA5 is represented with white ribbons. EphA5 structure was taken from PDB (4ET7.pdb)

Supplementary material 3

Anatomo-pathological analysis were performed on streptozotocin-HFD mice treated and untreated with UniPR500 30mg/kg os for 14 days as described in the main text. Liver, kidneys, pancreas were collected from each mice and samples were immediately fixed in 10% neutral buffered formalin. After paraffin embedding, 4-5 µm thick sections were obtained with microtome (Leica), stained with Hematoxylin and Eosin (H&E) and Periodic acid-Schiff (PAS). Histological slides were examined with Nikon Eclipse E800 microscope (Nikon Corporation, Japan) using Nikon PLAN APO lenses. Sections were photographed at 4x, 10x, 20x and 40x (Nikon PLAN APO lenses) with Camera DIGITAL SIGHT DS-Fi1 (Nikon Corporation, made in Japan); pictures were acquired with DS Camera Control Unit DS-L2 (Nikon Corporation, Japan) and stored in USB device. Histological lesions were graded and classified based on the extension/distribution (focal, multifocal and diffuse) and severity (scant, mild, moderate, severe).

Results:

No gross lesions were detected in treated or untreated mice during necroscopy.

Mice 1-2-3-4-14 (streptozotocin-HFD mice treated with UniPR500 for 14 days)

- Pancreas: In diabetic mice treated for 15 days with UNIPR500, inflammatory or regressive injuries were not observed on the exocrine pancreas; the islets were rare, both small or large, frequently adjacent to small vascular or ductular structures: numerous pancreatic islets cells presented a cytoplasmatic vacuolation (3/5) or perinsuler lymphocytic infiltration (insulitis) (2/5)
- Liver: The liver parenchyma showed multifocal micro and macrovesicular steatosis**,** that affected one or more hepatic lobes, in all the examinated mice (5/5), with scant interstitial or periportal lymphocytic infiltrates.
- Kidney: In all the diabetic treated mice no regressive injuries were observed on the tubular epithelial cells; scant perivascular lymphocytic infiltrates were present in 3 mice (3/5). With the PAS stain, slight thickening of Bowman's capsule were highlighted in all examinated mice (5/5)

Mice 6-8-9-10-12 (streptozotocin-HFD mice untreated with UniPR500)

- Pancreas; pancreatic islets were small or large, frequently adiacent to vascular or ductular structures. In 2 untreated mice (2/5) islet cells showed morphological alterations with a large nucleus or cytoplasm vacuolation; in one mouse (1/5) perinsular lymphocytic infiltration were observed; another mouse showed focal necrosis of acinar cells with mild neutrophilic infiltration.
- Liver; focal or multifocal micro/macrovesicular steatosis were observed in all streptozotocin-HFD untreated mice**.**
- Kidneys: In all the mice no regressive injures affecting the tubular epithelial cells; 3/5 mice present scant or mild perivascular infiltrates of lymphocytes. With PAS stain, slight thickening of Bowman's capsule were highlighted in all mice.

Accordingly to the histopathological features, no significant differences have been showed between the streptozotocin-HFD mice treated with UNIPR500 and streptozotocin-HFD untreated mice; both groups showed endocrine pancreatic injuries, islet cells vacuolation and lymphocytes infiltration, indicative of IDDM, mild or severe degenerative changes (steatosis) in the liver, slight focal interstitial inflammation in kidneys.

Pancreas

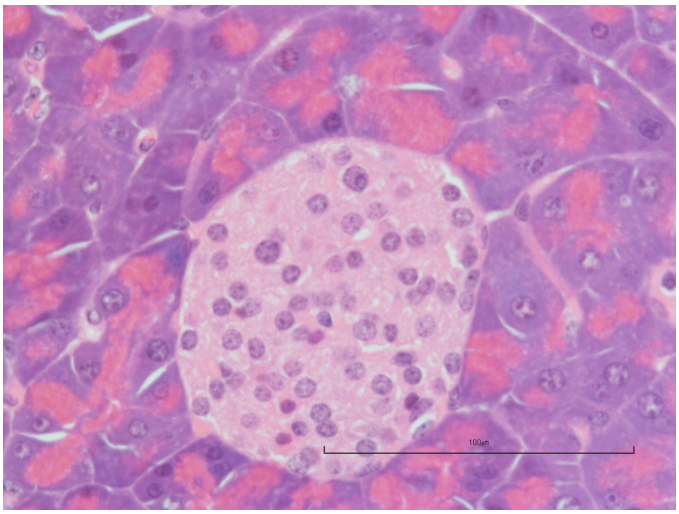


Fig.1-Pancreas of control mouse with normal pancreatic islet (H-E, 40x)

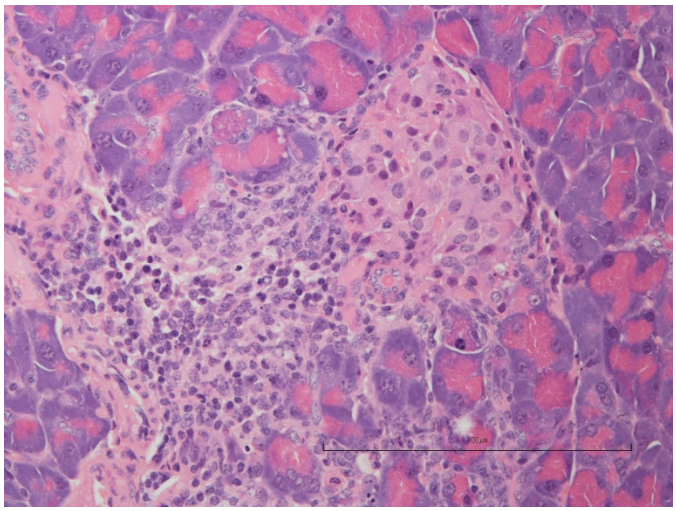


Fig 2 Pancreas- Streptozocin-HFD untreated mouse: peri-insuler severe lymphocytic infiltration (insulitis) (H-E, 40x)

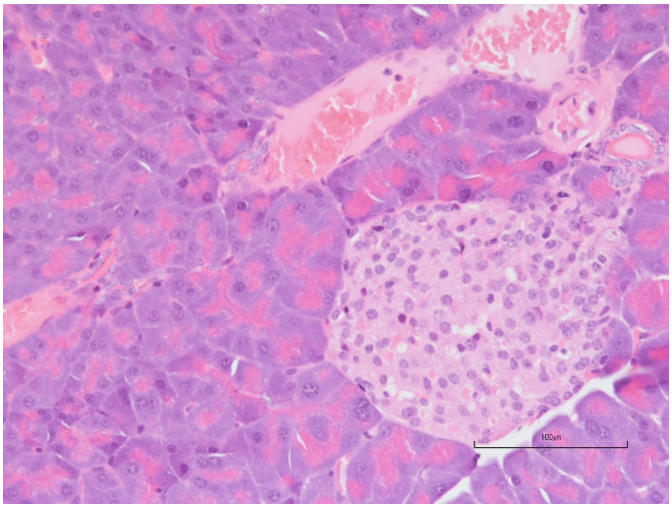


Fig. 3- Pancreas- Streptozocin-HFD mouse treated with UniPR500: mild citoplasmatic vacuolation of pancreatic islets (H-E, 20x)

Kidney

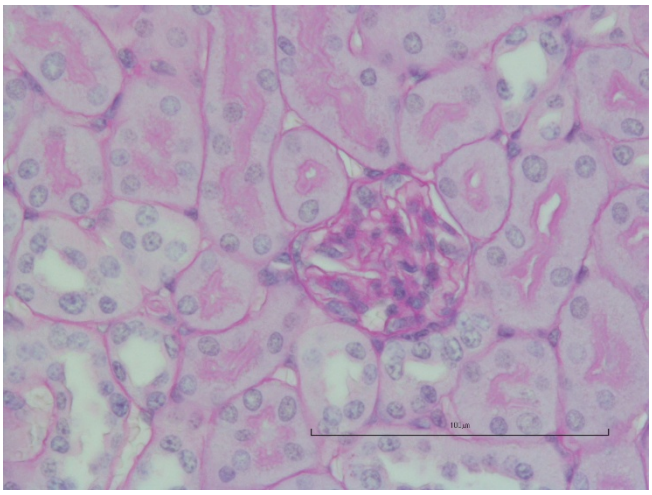


Fig.4: Kidney of control mouse showing the normal histological structure of renal parenchyma, (PAS stain, 40x)

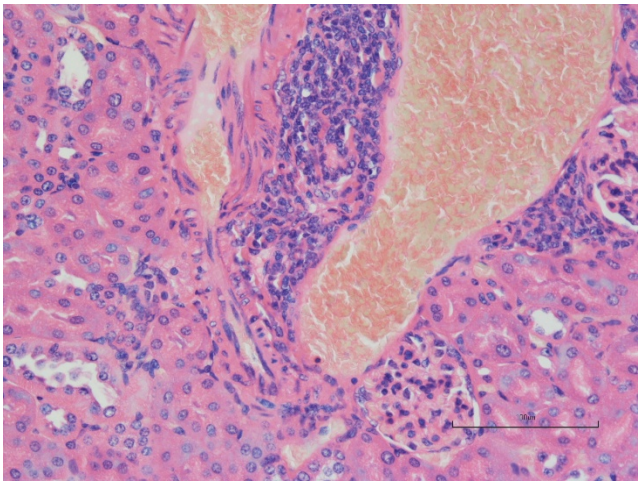


Fig 5-Kidneys-Perivascular infiltrates of lymphocytes in Streptozocin-HFD untreated mice (H-E stain-20x)

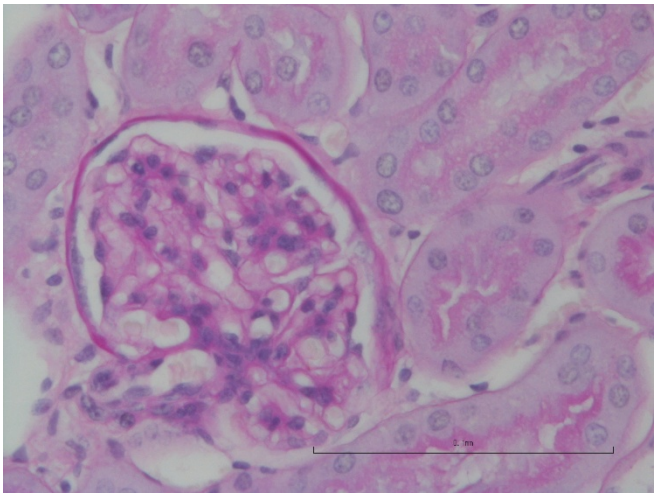


Fig 6 -Kidney- Slight thickening of Bowman's capsule were highlighted in Streptozocin-HFD treated mice with UniPR500 (PAS stain -40x)

Liver

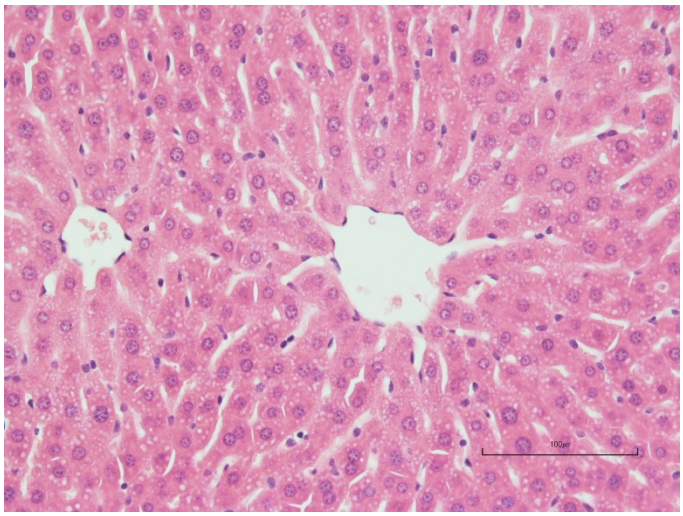


Fig 7- Liver of control mouse with normal hepatocytes (H-E, 20x)

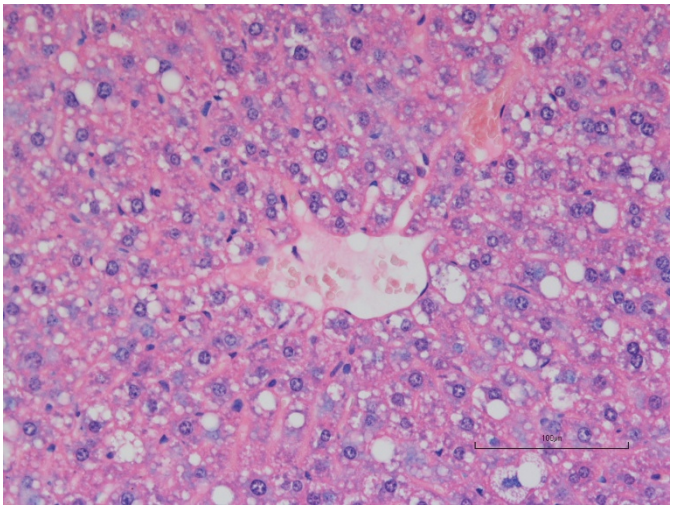


Fig. 8 Liver- Streptozocin-HFD untreated mice showed severe steatosis (H-E, 20X)

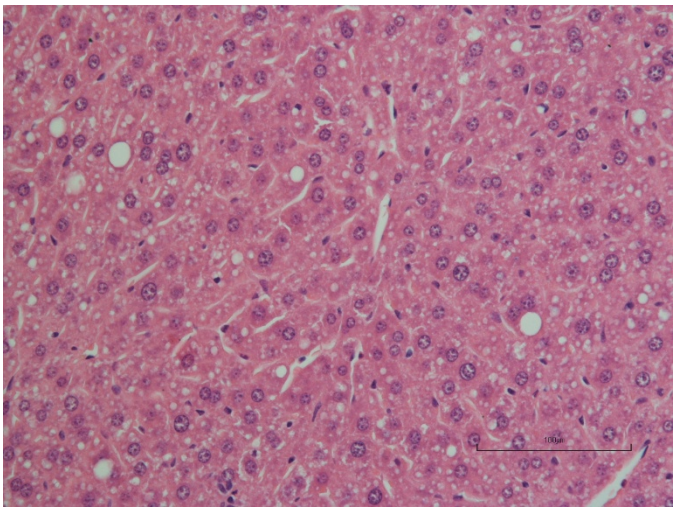


Fig 9 - Liver- Streptozocin-HFD treated mice with UniPR500 showed mild steatosis (H-E, 20X)

Table 1. Selectivity panel for the Eph-ephrin antagonist UniPR500. The compound was tested at single dose (10 μM) in triplicate. The biological activity of a reference standard for each target is also reported.

Target	**test**	**%inhibition or activation (±SD)**	**Standard IC_{50} or EC_{50}**
GLP1, agonism 2181	Cellular functional assay GPCR/cAMP/HTRF	0.4±0.2	GLP-1 (7-37), 0.052nM
GLP1, antagonism 2182	Cellular functional assay GPCR/cAMP/HTRF	13±12	exendin-3(9-39), 5.7nM
PPAR-γ, agonism 2771	Nuclear functional assay Coactivator recruitment/alphascreen	0.0±0.0	Rosiglitazone, 190nM
PPAR-γ, antagonism 2772	Nuclear functional assay Coactivator recruitment/alphascreen	-2.4±6.8	GW9662, 91nM
PTP1B, inhibition 2593	Enzyme assay, fluorogenic substrate	-4.5±0.7	(NH4)6Mo7O24, 12nM
DPP IV, 2942	Enzyme assay, fluorogenic substrate	-3.3±3.5	K579, 3nM
K_{ATP} channel, 0165	Radioligand assay	7.0±9.8	Glibenclamide, 0.25nM

Table 2. Physicochemical properties, ASBT binding and *in vitro* stability of UniPR500

Cpd	log$D_{oct,7.4}$	solubility[a](μM)	% ASBT binding [b]	% remaining compound in plasma[c]	% remaining compound in liver[d]
UniPR129	4.90 ± 0.15 [e]	31.8 ± 4.2 [e]	22.7 ± 2.4	98.3 ± 9.5 [e]	56 ± 5 [e]
UniPR500	4.23 ± 0.11 [e]	51.9 ± 4.4 [e]	92.6 ± 1.5	93.7 ± 11.3 [e]	70 ± 7 [e]

[a] From DMSO stock solution. [b] Percent bound at 10 μM [c] Percent remaining after 24h of incubation, 37 °C [d] Percent remaining after 1 h incubation in the presence of a NADPH-regenerating system in liver S9 fraction [e] data from (Incerti et al., 2017). Data are the means of at least three independent experiments ± SEM.

M. Tognolini, A. Lodola and M. Incerti patented the described compound (Italian patent 0001426534, no extensions).