A new synthetic dual agonist of GPR120/GPR40 induces GLP-1 secretion and improves glucose homeostasis in mice

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ABSTRACT

G-protein coupled receptors 40 and 120 (GPR40 and GPR120) are increasingly emerging as potential therapeutic targets for the treatment of altered glucose homeostasis, and their agonists are under evaluation for their glucagon-like peptide-1 (GLP-1)-mediated therapeutic effects on insulin production and sensitivity. Here, we characterized a new dual GPR40 and GPR120 agonist (DFL23916) and demonstrated that it can induce GLP-1 secretion and improve glucose homeostasis.

Resulting from a rational drug design approach aimed at identifying new dual GPR120/40 agonists able to delay receptor internalization, DFL23916 had a good activity and a very high selectivity towards human GPR120 (long and short isoforms) and GPR40, as well as towards their mouse orthologous, by which it induced both Gαq/11-initiated signal transduction pathways with subsequent Ca^{2+} intracellular spikes and G protein-independent signaling *via* β-arrestin with the same activity. Compared to the endogenous ligand alpha-linolenic acid (ALA), a selective GPR120 agonist (TUG-891) and a well-known dual GPR40 and GPR120 agonist (GW9508), DFL23916 was the most effective in inducing GLP-1 secretion in human and murine enteroendocrine cells, and this could be due to the delayed internalization of the receptor (up to 3 h) that we observed after treatment with DFL23916. With a good pharmacokinetic/ADME profile, DFL23916 significantly increased GLP-1 portal vein levels in healthy mice, demonstrating that it can efficiently induce GLP-1 secretion *in vivo*. Contrary to the selective GPR120 agonist (TUG-891), DFL23916 significantly improved also glucose homeostasis in mice undergoing an oral glucose tolerance test (OGTT).

1. Introduction

Glucose homeostasis requires a perfect balance between the rate of glucose entering the circulation and the rate of glucose removal from the circulation, which is physiologically governed by the fine interplay of insulin, glucagon, amylin, and incretin hormones [1]. Altered glucose homeostasis and insulin resistance are key factors in the pathophysiology of metabolic syndrome (MeS) and are associated with an increased risk of developing type 2 diabetes (T2D) [2]. Tight glycemic control can be achieved in MeS and T2D patients with conventional anti-hyperglycemic medications and has been shown to reduce MeS progression and the onset of diabetes complications [3]. However, this therapeutic approach also leads to an increased risk of hypoglycemia,

which is associated with acute cognitive impairment, increased risk of falls, rebound hyperglycemia with consequent loss of glycemic control, acute coronary syndrome and increased mortality [3]. Thus, reaching a balanced glycemic control whilst avoiding hypoglycemia remains an urgent clinical need and an open challenge for health professionals.

In this context, one possible therapeutic solution could be aimed at enhancing insulin secretion through glucose-dependent mechanisms, being potentially able to restore the defective physiological pathway minimizing the risk of hypoglycemia [1]. Among insulin-stimulating factors, G-protein coupled receptors 40 and 120 (GPR40 and GPR120) strongly emerged as attractive potential pharmacological targets due to their key role in regulating metabolism and glucose homeostasis. Several studies have demonstrated the association of GPR120 and GPR40

inhibition with the occurrence and development of metabolic disorders, such as obesity and T2D [4], while their agonism has been associated with protective effects [5]. The binding of GPR40 and GPR120 by ω-3 fatty acids (such as α-Linolenic Acid (ALA)) results in fact in various physiological activities, which ultimately improve metabolic homeostasis and alleviate insulin resistance-related consequences [5]. GPR40, also known as free fatty acid receptor 1 (FFA1), is predominantly expressed in pancreatic β-cells and gut enteroendocrine cells where its activation ultimately results in insulin secretion stimulation [6]. GPR120, also known as free fatty acid receptor 4 (FFA4), is mainly expressed in adipocytes, in which it promotes cell differentiation and maturation, macrophages, in which it inhibits inflammatory cytokine release through β-arrestin signaling [7], and gut enteroendocrine cells [8,9]. In these latter cells, free ω-3 fatty acids bind to both GPR40 and GPR120 and induce intracellular calcium spikes, which result in the secretion of incretin hormones, such as glucagon-like peptide-1 (GLP-1) [7]. GLP-1 plays crucial roles in the regulation of glycemia, stimulating glucose-dependent insulin secretion following food ingestion, inducing pro-insulin gene expression and activating β-cell proliferation pathways [10]. Agonists of GLP-1 and inhibitors of its inactivating enzyme dipeptidyl peptidase 4 (DPP4) have been approved for therapeutic use and are now routinely used in patients due to their anti-hyperglycemic effects [11].

In T2D patients, the incretin effect is almost completely deficient mainly because of reduced postprandial GLP-1 secretion [12]. The selective activation of GPR40 or GPR120 has proven effective in inducing GLP-1 secretion in several preclinical models of diabetes, suggesting a potential therapeutic efficacy of this strategy in metabolic disorders [13, 14]. However, it is becoming increasingly clearer that the simultaneous activation of both GPR40 and GPR120 with a dual agonist — rather than the activation of only one of them with specific agonists — may be more therapeutically useful [15]. In this context, even though a few molecules able to bind and activate only GPR120 (TUG-891 [16]) or both GPR40 and GPR120 (GW9508 [17] and another compound [15]) have been already developed, none of them has been fully characterized for its binding selectivity, activity *in vitro* in different species and for its effects on glycemic control *in vivo*.

In this study, we analyzed the capacity of a new dual GPR40 and GPR120 agonist (DFL23916) (Fig. 1) to effectively activate the receptors and induce GLP-1 secretion *in vitro* (in both murine and human enteroendocrine cell lines) and *in vivo*. Then, we investigated whether orally administered DFL23916 can regulate glucose homeostasis in an oral glucose tolerance test (OGTT) mouse model.

2. Materials and methods

2.1. Chemicals and reagents

The compound DFL23916 was synthetized at Dompé Farmaceutici SpA as described in Patent number WO 2018029150 A1. TUG-891 (cat. No. 4601) and GW9508 (cat. No. 2649) were purchased by Tocris Bio-Techne SRL (cat. 4601), while α-linolenic acid (ALA) (cat. No. L2376) and Sitagliptin as phosphate monohydrate salt (cat. No. Y0001812) were purchased by Sigma Aldrich.

When necessary, all stock solutions were serially diluted in DMSO and then further diluted in assay buffer containing 0.01% BSA.
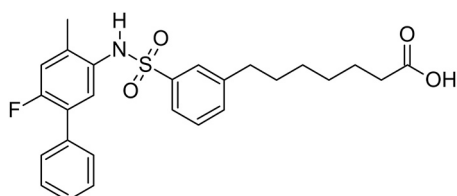

Fig. 1. Chemical structure of compound DFL23916.

2.2. In vitro studies

2.2.1. Cell lines and culture medium

The Compound Profiling experiments were done using chinese hamster ovary (CHO)-K1 cells stably transfected with GPR40 or GPR43, mitochondrial-targeted Photina® (Ca^{2+}-activated photoprotein) CHO (CHO-mito Photina) cells stably transfected with GPR120S (Short Isoform) or GPR120 (Long Isoform) and CHO-chAMPion stably transfected with GPR119 or TGR5 or GLP-1R. The complete culture media for the assays were: DMEM F-12 (1:1) MIXTURE (LONZA), 1 mM Sodium Pyruvate (LONZA), 20 × 7.5% Sodium Bicarbonate (LONZA), 1 μM Hepes (LONZA), 1X Penicillin/Streptomycin (LONZA), 10% of Fetal Bovine Serum (Euroclone).

CHO GPR120-S (short isoform) / β-arrestin cell line were maintained in F-12 Nutrient Mixture (HAM) (MEM/EBSS, Euroclone, cat. EC B2071L) supplemented with 2 mM Ultraglutamine 1 (BioWhittaker cat. BE17–605E/U1), 10% FBS (Fetal Bovine Serum, Euroclone cat. ECS0180L), 1% Penicillin / Streptomycin (100x solution Euroclone cat. B3001D) and 0.4 mg/mL G418 (InvivoGen cat. antgn-5). For Arrestin-2 recruitment and calcium mobilization on mouse GPR120 and internalization assay were used human embryonic kidney 293 (HEK293) cells.

The GLP-1 secretion experiments *in vitro* were done using the murine STC-1 cells, grown in DMEM high glucose (GIBCO) with 5% Fetal Bovine Serum (GIBCO) and 2 mM glutamine (GIBCO) and the human NCI-H716, grown in RPMI (Life Technology, A10491) supplemented with 10% Fetal Bovine Serum (GIBCO) and 2 mM glutamine (GIBCO). For cell adhesion and endocrine differentiation, NCI-H716 were grown in dishes coated with Matrigel (Corning Matrigel Basement Membrane Matrix, 356234) in high-glucose DMEM, 10% FBS, 2 mM glutamine (GIBCO), as previously described (18).

2.2.2. Calcium-flux measurement

Fluorescent Imaging Plate Reader (FLIPR) is a method used for detecting GPCR activation by measuring changes in intracellular Ca^{2+} concentration using calcium-sensitive dyes. A FLIPR-based functional assay for GPR120 was adapted to fluorescence imaging at imaging-based plate reader FDSS6000 as the read-out system. α-linolenic acid (ALA, Sigma Aldrich cat. L2376) was used as reference activator. The compounds were tested in a dose-dependent format (8 concentrations starting from 100 μM, in triplicate and with 0.5 log distance between concentrations).

CHO-mito-Photina GPR120 cells (1 × 10^4) were seeded in 384 assay plates (Greiner 781091) in complete medium (25 μL/well). Assay plates were incubated for 24 h (incubator at 37 °C/5% CO2/90% humidity). Prior to the robotic run, the culture medium was removed by manually flicking the plate. Then 10 μL Tyrode's buffer (130 mM NaCl, 5 mM KCl, 2 mM CaCl$_2$, 1 mM MgCl$_2$, 5 mM NaHCO$_3$, 20 mM HEPES, pH 7.4, sterile filtered) were added to all wells and the assay plates loaded into the Cytomat of the robotic system. The GPR120 activity in response to compound addition was monitored using a fluorescent Ca^{2+} sensitive dye (Fluo8 No Wash Calcium Assay kit (ABD Bioquest, cat. 36316). Cells were loaded by adding 10 μL/well of 2x Fluo-8 No Wash sensitive dye in Tyrode buffer and incubated for 2 h at room temperature under a yellow light sodium lamp. Ten μL/well of test compounds and controls (3x concentrated in Tyrode buffer) were transferred onto the cells with first pipetting head of the FDSS6000 and the emitted fluorescence was monitored over a period of 7 min. Ten μL/well of 4x concentrated reference agonist at ~EC50 (5 μM ALA final) were transferred from an intermediate plate II onto the cells using the second pipetting head of the FDSS6000 and the emitted fluorescence was monitored over a period of additional 3 min.

For GPR40 and GPR43 assay, FLIPRTETRA real time kinetic cellular assay screening system and a calcium fluorescent dye have been used as readout to measure the calcium increase due to receptor activation in CHO-K1 cells. The compounds were tested in a dose-dependent format (8 concentrations starting from 1 mM, in duplicate and with 0.5 log

Table 1

Real-time PCR primers.

Primers	Amplicon context sequence	Efficiency (%)	Assay Design
hGAPDH	GTATGACAACGAATTTGGCTACAGCAACAGGGTGGTGGACCTCATGGCCCACA TGGCCTCCAAGGAGTAAGACCCCTGGACCACCAGCCCCAGCAAGAGCACAAGA GGAAGAGAGAGACCCTCACTGCTGGGGAGTCCCTGCCACAC	97	Exonic
hFFAR1	TGGAGTGTGGTGCTTAATCCGCTGGTGACCGGTTACTTGGGAAGGGGTCCTGGCC TGAAGACAGTGTGTGCGGCAAGAACGCAAGGGGGCAAGTCCCAGAA	100	Exonic
hFFAR2	TGCCTTTCACCAGCTTGGTATCCCTTCCTGACTGAATTGTCCTACTCAAAGGAGC ATAAGTCAGAGATGCACGAAGAAGTAGTTAGGTATAGAAGCACCTGCCGG	96	Exonic
hGLP1R	AGCTCCCCGGAGGAGCAGCTCCTGTTCCTCTACATCATCTACACGGTGGGCTACGCACT CTCCTTCTCTGCTCTGGTTATCGCCTCTGCGATCCTCCTCGGCTTCAGACACCTGCACTGCACC AGGAACTACATCCACCTGAACCTGTTTGCATCCTTCATCCTGCGAGCA	98	Intron-spanning
hGpbar1	CAGTGTCGACCTGGACTTGAACTAAAGGAAGGGCCTCTGCTGACTCCTACCA GAGCATCCGTCCAGCTCAGCCATCCAGCCTGTCTCTACCGGG	97	Exonic
hGPR119	GCCATGGCTCCTGCATGTTCCATCTTTCGAATCTGCTGGCTGTGCATGGAGGCAATCTTGA GCATGTCGCAGTAGAAGAAGACAAAGAGGAGCATGGCTGGGAAG	100	Exonic
hO3far1	GGCTATTCGGCGGTCGCCGCTCTGCCTCTCTGCGTCTTCTTCCGAGTCGTCCCGC AACGGCTCCCCGGCGCCGACCAGGAAATTTCGATTTGCACACTGATTTGGCCCA CCATTCCTGGAGAGATCTCGTGGGATGTCTCTTTTGTTACTTTGAACTTCTTGGTGC CAGGACTGGTC	99	Intron-spanning
mGAPDH	TGGGAGTTGCTGTTGAAGTCGCAGGAGACAACCTGGTCCTCAGTGTAGCCCAAGATGCC CTTCAGTGGGCCCTCAGATGCCTGCTTCACCACCTTCTTGATGTCA	101	Exonic
mFFAR1	CGTGGGACACTGCGCCTCGGATGGCTAACAAGTTCAATGGAAAGCCCAGCGCAAAG GCAGATACATAGAGAGCGAAGGAGAGCTGTGGGGGCAGG	99	Exonic
mFFAR2	TTTTACCTGCCAAACCAGATTTTCTTTTTAATCCTTCCTTTCAGGTCATTGTCAATGTC AGACAGACGGGT ACCAAGACATCTCCTAGACCCAGTGACTGGTGACACAGAGGATAAGCATGTGACCCAGAT CCCGGCATAACAGT	101	Exonic
mGLP1R	CTATGCCTGCTGGCCGGATGGGCCCCCAGGTTCCTTCGTGAATGTCAGCTGCCCCTGGTACCTGCCCTG GGCCAGTAGTGTGCTACAAGGCCATGTGTACCGGTTCTGCACAGCTGAGGGTCTCTGGCTACATAAGGACAACTC	100	Intron-spanning
mGpbar1	CTGGAGGACAGCTGCCCAAAGGTGTCTACGAGTGCTTCGAGGAAGAGCCAAGAGGGACAATCCAG GCCCCAGCACTGCCTACCACACCAGTAGCCAATGCAGCATTGACCTGGACTTGAATT AGGGAAGCAGC	101	Exonic
mGPR119	TGTGATTGCATGTTCTTGAGAGAAGTCCTGCAGCGTCTTAGCCATCGAGCTCCGGATGGCTGATAGTGAC GATGTGATAGGCGCTTTCTCTGGTCCTCTCTGGACC	101	Intron-spanning
mO3far1	CACATTGGATTGGCCCAACCGCATAGGAGAAATCTCATGGGATGTGTTTTTTGTGACTTTGAACT TCCTGGTGCCGGGACTGGTCATTGTGATCAGTTACTCCAAAATTTTACAGATCACGAAAGCATCGCGGAAGA GGCTTACGCTGAGCTTGGCATACTCTGAGAGCCACCAGATCCGAGTGTCCCAAC	102	Intron-spanning

dilution steps). Ten thousand cells/well were seeded in 384 MATRIX black/clear bottom (MTP) in complete medium (25 µL/well). Twenty-four hours after seeding the culture medium was removed by manually flicking the plate over the sink. Cells were loaded with 20 µL/well of 0.5X Fluo-8 No Wash sensitive dye in Tyrode's buffer and then incubated for 1 h at room temperature under a yellow light sodium lamp. Ten µL/well of test compounds and controls 3X concentrated in Tyrode's buffer were injected onto the cells with the FLIPRTETRA. The kinetic response was monitored over a period of roughly 4 min.

TGR5, GPR119 and GLP-1R were expressed in CHO-chAMPion cells. This cell line expresses a Ca^{2+} sensitive photoprotein and a cAMP gated channel (CNG). Activation of the target by a specific agonist triggers an increase of intracellular cAMP levels, which induces channel opening allowing the influx of external Ca^{2+}, which then stimulates the emission of light consequent to the activation of the Ca^{2+} sensitive photoprotein. The compounds were tested in a dose-dependent format (8 concentrations starting from 1 mM, in duplicate and with 0.5 log dilution steps). Ten thousand cells/well were seeded in 384 MTP in complete medium (25 µL/well). Twenty-four hours after seeding the culture medium was removed by manually flicking the plate over the sink.

Cells were loaded with 25 µL/well of coelenterazine (10 µM final concentration) in Ca^{2+}-free Tyrode's buffer (130 mM NaCl, 5 mM KCl, 1 mM MgCl$_2$, 5 mM NaHCO$_3$, 20 mM HEPES, pH 7.4, sterile filtered) and 3-Isobutyl-1-methylxanthine (IBMX, Sigma Aldrich cat. I7018) (500 µM final concentration) and then incubated for 3–3 h:30′ at room temperature under a yellow light sodium lamp. Cell plate volume was flattened by aspiration with the CyBi®-Vario pipettor to reach 20 µL in all the wells. Ten µL/well of test compounds and controls 3X concentrated in Ca^{2+}-free Tyrode's buffer were injected off-line with the CyBi®-Vario pipettor. After 10 min the injection of 10 µL of a 10 mM Ca^{2+} Tyrode's buffer (2.8 mM Ca^{2+} final concentration) was performed

at the FLIPRTETRA and the emitted flash luminescence was then recorded.

2.2.3. Arrestin-2 assay towards human GPR120

Compound was tested in a dose response experiment (8 concentrations in triplicate and with 0.5 log distance between concentrations) against the GPR120-S / β-arrestin stably expressed in a CHO β-arrestin cell line.

GPR120-S / β-arrestin (7500 cells/well) were seeded in 384 MTP in complete medium (25 µL/well); 24 h after seeding the medium is removed and replaced with 20 µL/well of assay buffer. Five µL/well of test compounds and controls at 5X-concentration in assay buffer, containing 2.5% Dimethyl sulfoxide (DMSO, Sigma Aldrich cat. 34869) (plus or minus 0.01% Bovine Serum Albumin (BSA, Sigma Aldrich cat. A3059), were added to the assay plate by the CyBi®-Well. Cells were incubated for 3–4 h at room temperature. Plate volume was flattened to 10 µL/well by aspiration with the CyBi®-Well. Ten µL/well of detection mix were added to the assay plate by the CyBi®-Well. Plates were then incubated for 1 h at room temperature. Recruitment of β-Arrestin was measured by luminescence imaging by FLIPRTETRA.

2.2.4. Arrestin-2 recruitment and Calcium mobilization on mouse GPR120

Arrestin-2 recruitment and Calcium mobilization Assay on GPR120 was performed at Eurofins DiscoverX Corporation as a contract service. A summary of the protocol and the reference for the assay is listed on the Eurofins DiscoverX website (https://www.discoverx.com/targets/targets-by-family?fam=Freefattyacid). In brief, HEK-293 cells were used to test 10 increasing concentrations in duplicate for DFL23916, ALA and GW9508, and the same experiment has been performed two times. Compound was tested in a dose response experiment (8–10 concentrations in duplicate and with 0.5 log distance between concentrations) to

evaluate Arrestin-2 recruitment and Calcium mobilization on mouse GPR120.

2.2.5. Internalization assay on GPR120

Internalization Assay on GPR120 was performed at Eurofins DiscoverX Corporation as a contract service. A summary of the protocol and the reference for the assay are listed on the Eurofins DiscoverX website (https://www.discoverx.com/targets/targets-by-family?fam=Freefatt yacid). Compound was tested in a dose response experiment (10 concentrations in duplicate and with 0.5 log distance between concentrations) to evaluate internalization assay on GPR120.

2.2.6. Selectivity and in-vitro ADME-Tox

PPAR gamma, CCK1 and CCK2 assays and all *in vitro* ADME-Tox assays were carried out at Eurofins Panlabs Inc. as a contract service. A summary of each assay protocol and the reference for each assay are listed on the Eurofins website: https://www.eurofinsdiscoveryservices.com/services/.

2.2.7. Real-time PCR in STC-1 and NCI-H716 cells

Three different pellets of STC-1, undifferentiated and differentiated NCI-H716 cells were collected and mRNAs were extracted following the guideline procedure reported in Trizol sheet (https://tools.thermofisher.com/content/sfs/manuals/trizol_reagent.pdf). mRNAs were retro-transcribed using iSCRIPT Supermix protocol (http://www.bio-rad.com/webroot/web/pdf/lsr/literature/10020178.pdf). SsoAdvanced Universal SYBR Green Supermix (Biorad) was used. Real-time PCRs were performed using PrimerPCR assays (Biorad) custom made in CFX96 real-time Thermal Cycler (Biorad). Primers to evaluate the expression of GPR120, GPR40, GPR43, GLP1R, TGR5 and GPR119 were spotted on 96 wells plate. For each primer, the amplicon context sequence, the efficiency and the assay design are specified in Table 1. Primers are designed on a region shared by all different isoforms of the same gene, so all isoforms are amplified although there is not possible to understand which isoform is more expressed. The plate comprises two housekeeping genes (GAPDH and ACTβ-data not shown), which were used to normalize gene expression levels, and several controls for genomic DNA presence, for the transcription efficiency, for RNA impurity and fragmentation. The results of three different biological samples performed in duplicate were calculated as $2^{(Dct\ normalizer\ -\ Dct\ sample)}$ to obtain the expression of the genes.

2.2.8. STC-1 and NCI-H716 cells stimulation

STC-1 cells (2×10^5) were seeded in 12 wells and 24 h later were treated with selected compounds. NCI-H716 cells (1×10^6) were seeded in matrigel coated 12 wells and treated 48 h hours later. The day of the experiment, wells were washed once with 1 mL of PBS 1X (GIBCO) and then cells were stimulated with 0.5 mL of DMEM serum-free without phenol-red (GIBCO, cat. 21063) containing the appropriate concentration of selected compounds for the indicated timepoints. All the tested molecules were dissolved in DMSO 100% at a stock concentration of 10 mM. From this stock, working solutions were prepared in DMEM serum-free. The indicated DMSO concentration used was the reference control in all assays, while the endogenous agonist ALA was used as positive control. After stimulations, surnatants were collected, centrifuged at 4000 rpm for 4 min at 4 °C and stored at −80 °C for the subsequent GLP1 and LDH detection.

For the target validation, agonist treatments were combined with antagonist ones, both the compounds were added to the cells in the same moment, in STC-1 at the concentration of 30 μM for each compound for 30′ at 37 °C/5% CO_2, in differentiated NCI-H716 100 μM for 30′ at 37 °C/5% CO_2. Each experimental point was repeated in three biological replicates and in experimental duplicate each time.

2.2.9. Secreted GLP-1 quantification in STC-1 and differentiated NCI-H716 cells

Total GLP-1 was detected by ELISA (Merk Millipore, EGLP-36 K) according to the manufacturer instructions using 50 μL of each surnatant obtained after cell stimulation with selected molecules. This kit was used to quantify active GLP-1 7–36 and inactive 9–36 fragments and has no significant cross-reactivity with GLP2, GIP, Glucagon, Oxyntomodulin. It has a range of detection which spread from 4.1 pM to 1000 pM. The limit of detection is 1.5 pM and the intra-assay variation between 1% and 2% CV. The inter-assay variation is around 12% CV.

2.3. In vivo studies

All the experiments were conducted in conformity with the Italian Guidelines for Care and Use of Laboratory Animals (D.L.116/92) and with the European Directive (2010/63/UE). The study was approved by the national ethics committee for research animal welfare of the Italian Ministry of Health (authorization no. 318/2019-PR). Most of the *in vivo* and *ex vivo* experimental procedures followed the international guidelines for preclinical studies in neuromuscular diseases, available on the TREAT-NMD website (https://treat-nmd.org/research-overview/preclinical-research/experimental-protocols-for-dmd-animal-models/).

2.3.1. Animals

The pharmacokinetic and pharmacodynamic studies were performed on eight-week-old male C57BL/6 mice (25–30 g of weight) purchased from Charles River Laboratories (Milan, Italy). Once receipt from the supplier, the animals were subjected to health examinations and acceptance.

The animals were housed in a single exclusive room with air-conditioned to provide a minimum of 15 air changes/hour and in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (publication no. 85–23, revised 1996). The environmental controls were set to maintain temperature within the range 22 °C and relative humidity within the range 50–60% with an approximate 12-hours light and 12-hours dark cycle that is controlled automatically. The animals were housed, in groups of five, in cages suitable for the species, also during dosing and feeding periods. The animals were acclimatized to local housing conditions for approximately 10 days. Food (Mucedola Standard GLP diet) and water were available *ad libitum* throughout the study.

All animals were weighed on the day of each treatment. Clinical signs were monitored at regular intervals throughout the study in order to assess any reaction to treatment. Each animal was uniquely identified with a colored spray on the back before the experiment. The experiment was carried on in agreement with the Italian Law (D. L.vo 116/92).

2.3.2. Pharmacokinetics studies in the mouse

DFL23916 was orally administered at 15 mg/kg to male C57BL/6 mice (n = 6) to determinate its oral exposure. On day of treatment, an exact amount of the compound was dissolved in 10% v/v DMSO and 90% v/v of Phosphate Buffer 0.2 M pH 8 to obtain a final concentration of 5 mg/mL. After oral treatment, blood (approximately 60–80 μL) was sampled from retromandibular plexus at the following timepoints: 15, 30, 60, 120, 240, 480 min. Blood samples were collected in heparinized eppendorfs (Heparin Vister 5000 U.I/mL) gently mixed and placed immediately on ice with salt; then eppendorfs were centrifuged (3500xg, at 4 °C for 15 min) and the resulted plasma collected and transferred to uniquely labeled eppendorfs and frozen at −20 °C till the HPLC-MS/MS analysis. At the end of the study animals were euthanized by cervical dislocation under deep isoflurane anesthesia.

2.3.3. In vivo GLP-1 secretion in the mouse

For the *in vivo* GLP-1 secretion test, male C57BL/6 mice were starved for 24 h, weighted and anesthetized with isoflurane (5% for induction and 2% for maintenance of general anesthesia). A midline abdominal

Table 2
AC50 potency values of DFL23916 in various human functional assays. Data are represented as mean of $\pm$ SEM.

Assay	DFL23916
hGPR120 Short Ca^{2+} $AC_{50} \pm SE$	16.6 $\pm$ 0.9 μM
hGPR40 Ca^{2+} $AC_{50} \pm SE$	13.7 $\pm$ 0.7 μM
hGPR120 β-Arrestin-2 $AC_{50} \pm SE$	10.2 $\pm$ 0.5 μM
hGPR40 β-Arrestin-2 $AC_{50} \pm SE$	11.5 $\pm$ 0.8 μM
hGLP1R, hGPR43, hGPR119, hTGR5 (agonist effect) AC_{50}	>1 mM
hPPARγ, CCK1 and CCK2 (agonist effect) AC_{50}	>30 μM

Table 3
AC50 potency values of DFL23916 in various mouse functional assays. Data are represented as mean of $\pm$ SEM.

	mGPR120 Ca^{2+} $AC_{50} \pm SE$	mGPR120 β-Arrestin-2 $AC_{50} \pm SE$	mGPR40 Calcium flux $AC_{50} \pm SE$	mGPR40 β-Arrestin-2 $AC_{50} \pm SE$
DFL23916	13.2 $\pm$ 1.1 μM	7.8 $\pm$ 0.6 μM	Not determined	9.4 $\pm$ 0.8 μM

incision was made, the abdominal wall was opened at the linea alba, and the portal vein was exposed by displacing the intestinal duct. To assess GLP-1 secretion, blood samples (120 μL) were collected from the portal vein, under a stereomicroscope, at time 0 and 15 min after vehicle (5 μL/g body weight), α-linolenic acid (30 mg/kg) or DFL23916 (30 mg/kg) direct administration into the proximal colon by a 32 G needle syringe. The vehicle used to dissolve test compounds was 15% v/v PEG400 + 85% v/v Phosphate Buffer Solution 0.2 M pH 7.5. Pre heparinized 29 G syringes were used to collect blood by puncture of the portal vein. Blood samples were then collected in tubes with EDTA (BD Microtainer), plasma was separated by centrifugation at 4.500 rpm for 5 min and stored at − 20 °C for later GLP-1 detection.

Plasma GLP-1 concentration was measured by enzyme immunoassay using a GLP-1 ELISA kit (Millipore, cat. EZGLP1T-36 K) as indicated by manufacturer's instructions. Briefly, GLP-1 total quantification was measured spectrophotometrically by the increased absorbency at 450 nm, corrected from the absorbency at 590 nm. GLP-1 pM concentration was derived by interpolation from a reference curve generated in the same assay and calculated by Infinity 200 Software.

2.3.4. Oral glucose tolerance test in the mouse
For the oral glucose tolerance test (OGTT), mice were orally administrated with vehicle (10 μL/g of body weight), Sitagliptin (10 mg/kg), TUG-891 (30 mg/kg) or DFL23916 (30 or 100 mg/kg). The administration volume of each compound was the same as for the vehicle: 20% v/v DMSO + 80% v/v Phosphate Buffer Solution 0.1 M pH 8. Mice were starved overnight, weighted and orally administrated as described above. Forty-five minutes following the oral administration of the compounds, a bolus of 20% glucose (2 mg/g body weight) was orally given to the mice and glycaemia was measured at time − 45′, 0′, 15′, 30′, 60′, 90′ and 120′ by tail vein bleeding with a portable glucometer (Johnson & Johnson).

2.3.5. Blinding and randomization
For *in vivo* experiments, drug treatments were conducted in a blinded fashion. In all *in vivo* tests, randomization of animals and treatments were performed by different experimenters. Animals were allocated to experimental groups based on their baseline responses to behavioral test. In order to reduce the effect of extraneous variables on behavioral assessment, concurrent vehicle-treated groups were present throughout all experiments. All treatments were performed in the morning, between

08:00 and 9:00 a.m; behavioral testing was conducted between 10:00 a. m. and 15:00 p.m. No animal was excluded from statistical analysis.

2.4. Statistics

All experimental data were expressed as mean $\pm$ standard error of the mean (SEM). Multiple statistical comparisons between groups were performed by one-way analysis of variance (ANOVA), with Bonferroni *post hoc* analysis and Holm-Sidak's multiple comparisons when the null hypothesis was rejected (p < 0.05). This allowed the evaluation of intra- and inter-group variability, as well as inter-group statistical comparison, while controlling the experiment-wise error rate for false positive (type I error). All data collected follow with good approximation a normal distribution, being included in the 95% confidence interval of the mean; this generally allows for the clear identification of outliers, if any, and for the application of the statistical analyses described above. No outliers were found during the present study. Missing data in the results were then related only to overt technical issues during the experimental procedures, which led to the exclusion of those specific samples from the analysis.

3. Results

3.1. Physicochemical characterization of DFL23916

DFL23916 displayed a pKa1 of 4.98 $\pm$ 0.01 and pKa2 of 8.85 $\pm$ 0.03, a moderate aqueous solubility (0.7 mg/mL at pH 7.4) and LogP of 5.06 $\pm$ 0.05 and LogD$_{7.4}$ of 2.63 $\pm$ 0.01. Starting from the Structure–Activity Relationships (SAR), we designed a compound with a suitable lipophilicity to balance biological activity and ADME-PK properties. This allows to overcome the main limitations of the currently known GPR120 and GPR40 ligands, characterized by relatively high lipophilicity, which is associated with poor pharmacokinetic properties, toxicology and metabolic instability.

3.2. In vitro characterization of DFL23916

3.2.1. DFL23916 is a dual agonist of human GPR120 and GPR40
To examine the ability of our compound DFL23916 to effectively activate GPR120 and GPR40, we developed a functional cell-based assay. Since both receptors have been shown to couple to Gαq/11-initiated signal transduction pathways and, as such, to induce Ca^{2+} intracellular spikes, we first evaluated the activity of our compound in a Ca^{2+} mobilization assays using Chinese hamster ovary (CHO) cells stably transfected with human *GPR120* and *GPR40* genes. DFL23916
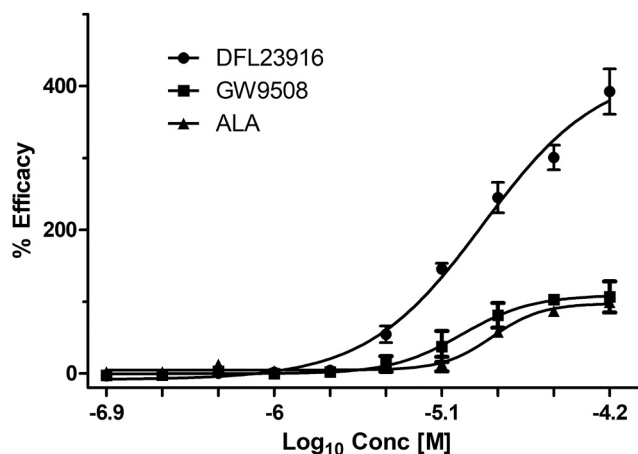

Fig. 2. Concentration-response curves for DFL23916, ALA and GW9508 on mouse GPR120 in Calcium mobilization assay performed in HEK-293 cells. Data are represented as mean of $\pm$ SEM (ten concentrations in duplicate).
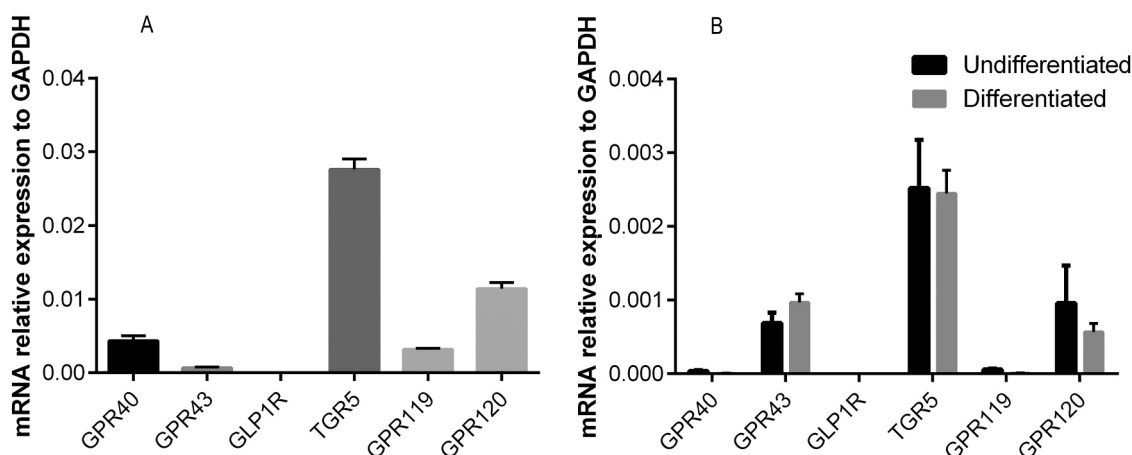
Fig. 3. mRNA expression relative GAPDH in STC1 cells (A) and in undifferentiated and differentiated NCIH716 cells (B). Data are represented as mean of three independent experiments ± SEM.

showed an AC50 of 16.6 ± 0.9 μM on human GPR120 short isoform and AC50 of 13.7 ± 0.7 μM on human GPR40 (Table 2), thus demonstrating its capacity to bind and activate both receptors. We tested the compound also towards the long isoform of human GPR120 finding a similar potency (data not shown). Since GPR120 and GPR40 have also been reported to couple to G protein-independent β-arrestin 2-mediated pathways [5], we examined the activity of DFL23916 using also a PathHunter® β-Arrestin assay developed by DiscoverX and found an activity of 10.2 ± 0.5 μM and 11.5 ± 0.8 μM towards GPR120 and GPR40, respectively (Table 2). We also compared the activity of DFL23916 with that of other GPR120 and GPR40 agonists: the endogenous fatty acid agonist α-linolenic acid (ALA), the GPR120 and GPR40 agonist GW9508 [17], and the GPR120 selective agonist TUG-891 [16], finding AC50 values of 5.38 ± 0.7 μM (hGPR120) and 5.28 ± 0.9 μM (hGPR40) for ALA, of 2.38 ± 0.4 μM (hGPR120) and 0.175 ± 0.08 μM (hGPR40) for GW9508 and of 1.16 ± 0.1 μM (hGPR120) and 10.0 ± 1.1 μM (hGPR40) for TUG-891.

These data clearly demonstrated that DFL23916 is a dual agonist able to signal the heterotrimeric G proteins Gq/11 and to activate functionally distinct G protein-independent signaling *via* β-arrestins.

To assess the selectivity of DFL23916 for GPR120 and GPR40, we tested the compound for its capacity to activate also other G protein-coupled receptors (GPCRs) involved in GLP-1 release in human

enteroendocrine cells. DFL23916 showed no activity towards several receptors as hGLP1R, hGPR43, hGPR119, hTGR5, with an AC50 > 1 mM. Moreover, we tested our compound towards PPAR-γ (a key factor for the regulation of fatty acid storage and glucose metabolism) and cholecystokinin receptors (CCK1 and CCK2), and no DFL23916 activity was detected towards these receptors, thus demonstrating a very high specificity for GPR120 and GPR40 (Table 2).

3.2.2. DFL23916 is a dual agonist of mouse GPR120 and GPR40

Cross species selectivity is a known issue for the development and preclinical evaluation of GPR120 and GPR40 agonists, especially for synthetic chemical ones [13,19]. To assess the cross-species selectivity of DFL23916, we examined its affinity for mouse orthologs of GPR120 (mGPR120) and GPR40 (mGPR40). Using the Ca^{2+} mobilization assay on mouse GPR120, DFL23916 exhibited an AC50 of 13.2 ± 1.1 μM showing an efficacy of 396% compared to the endogenous ligand ALA (AC50 of 14.8 ± 1.7 μM) and to the GPR120 and GPR40 dual agonist GW9508 [17] (AC50 of 10.2 ± 0.9 μM) (Table 3 and Fig. 2). The recorded efficacy of DFL23916 might be due to a super-agonist phenomenon.

We then assessed the activity on mouse GPR120 and GPR40 by evaluating β-arrestin 2 recruitment and found an AC50 of 7.8 ± 0.6 μM and 9.4 ± 0.8 μM, respectively (Table 3).
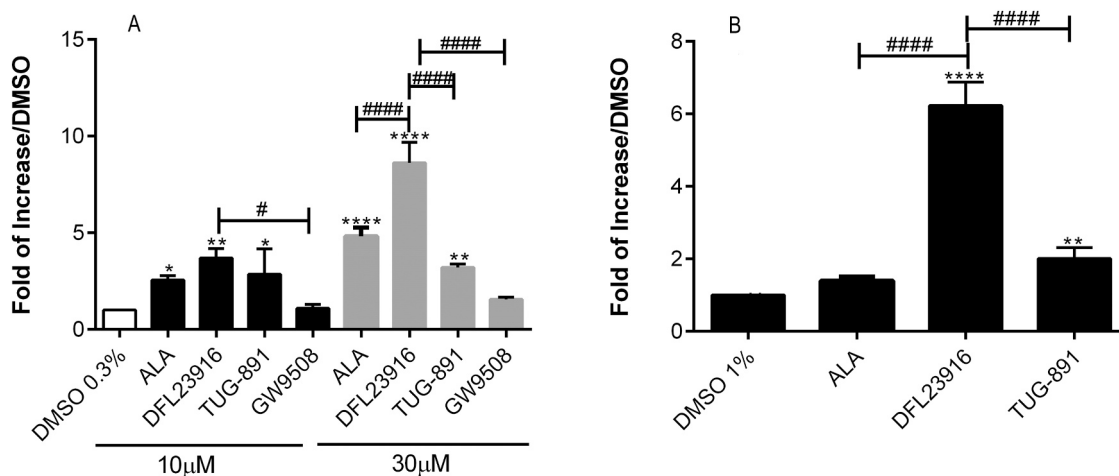

Fig. 4. GLP-1 secretion in STC1 cells (A) and in differentiated NCI-H716 (B). Data are represented as mean of three independent experiments ± SEM. STC1 cells were treated for 30 min with indicated concentrations and compounds, while NCI-H716 cells were treated for 30 min with 100 μM of indicated compounds. The statistical significance is referred to the relative DMSO (*) or DFL23916 (#) and was calculated by one-way ANOVA followed by Bonferroni *post hoc* analysis (#### $p < 0.0001$; # $p < 0.05$; **** $p < 0.0001$; ** $p < 0.005$; * $p < 0.05$).
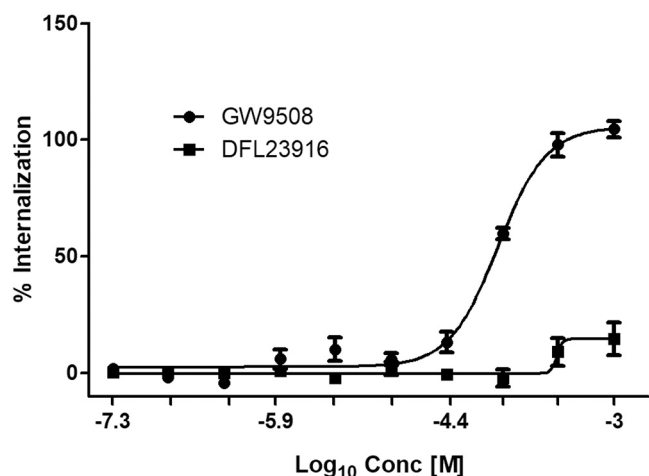
Fig. 5. Concentration-response curves for DFL23916 and GW9508 on human GPR120 internalization assay. Data are represented as mean of ± SEM (ten concentrations in duplicate).

3.2.3. DFL23916 stimulates GLP-1 secretion in human NCI-H716 and in mouse STC-1 enteroendocrine cell lines

Having assessed the potency and affinity of DFL23916 for both human and murine GPR120 and GPR40, we then investigated its capacity to stimulate GLP-1 secretion through the activation of GPR120 and GPR40 in both human and mouse enteroendocrine cells. To this end, we used NCI-H716 cells, which are frequently used as a model to study human enteroendocrine L-cell physiology [20], and murine intestinal secreting tumor cells (STC-1), which possess many features of native intestinal enteroendocrine cells [21]. NCI-H716 and STC-1 cells were characterized for the expression of the GPCRs that are crucially involved in glucose-metabolism (GPR120, GPR40, GPR43, GPR119, TGR5 and GLP1R), and the expression of human GPR120 and GPR40 and mouse orthologs was detected in human and mouse cells, respectively (Fig. 3).

The experiment for GLP-1 secretion analysis was set up in terms of incubation time (30 min in both cell types) and compounds' concentration (30 µM for STC-1 cells (Fig. 4A) and 100 µM for NCI-H716 cells

as reported in the literature [22] (Fig. 4B)). After the experimental set up, we compared the GLP-1 secretion induced by: our compound DFL23916, the endogenous ligand ALA, the selective GPR120 agonist TUG-891 [16] and the well-described GPR120 and GPR40 agonist GW9508 [6]. In NCI-H716 cells, GLP-1 secretion induced by DFL23916 was significantly higher than that induced by ALA ($p < 0.0001$) and TUG-891 ($p < 0.0001$), and also in STC-1 cells, DFL23916 induced a significantly greater GLP-1 excretion compared to that induced by ALA ($p < 0.0001$), GW9508 ($p < 0.0001$) and TUG-891 ($p < 0.0001$) (Fig. 4).

These results demonstrated that DFL23916 was the most efficient in inducing GLP-1 secretion in both human NCI-H716 and mouse STC-1 enteroendocrine cell lines. Surprisingly, in our hands, GLP-1 secretion induced by GW9508 was scarce and did not significantly differ compared to control; thus, this compound was no longer used for GLP-1 induction comparison studies in subsequent *in vivo* experiments.

3.2.4. DFL23916 delays GPR120 internalization

The internalization of GPR120 after agonist administration represents a key challenge for the use of GPR120 agonists treatment, as it impedes a sustained GLP-1 excretion [16,23]. We thus investigated whether the higher efficiency of DFL23916 in inducing GLP-1 secretion *in vitro* could be due to a different kinetic of post-binding GPR120 endocytosis compared to the other tested compounds. We used the Enzyme Fragment Complementation (EFC) technology with β-galactosidase developed by DiscoverX and tested DFL23916 in dose response curve up to 100 µM. Interestingly, DFL23916 significantly delayed the internalization of human GPR120 up to 3 h after exposure, showing an IC50 > 100 µM, compared to the dual agonist GW9508, which showed an IC50 of 9.5 ± 1.3 µM (Fig. 5). The same results were obtained also for mouse GPR120 (data not shown).

These findings are noteworthy especially considering the available data regarding the selective GPR120 agonist TUG-891 and the endogenous ligand ALA, for which it has been demonstrated that GPR120 endocytosis increases in a quasi-linear fashion, reaching the complete internalization within 40 min after exposure [16].

3.2.5. Mechanism of action of DFL23916

To confirm the dual receptor binding capacity of our high selective
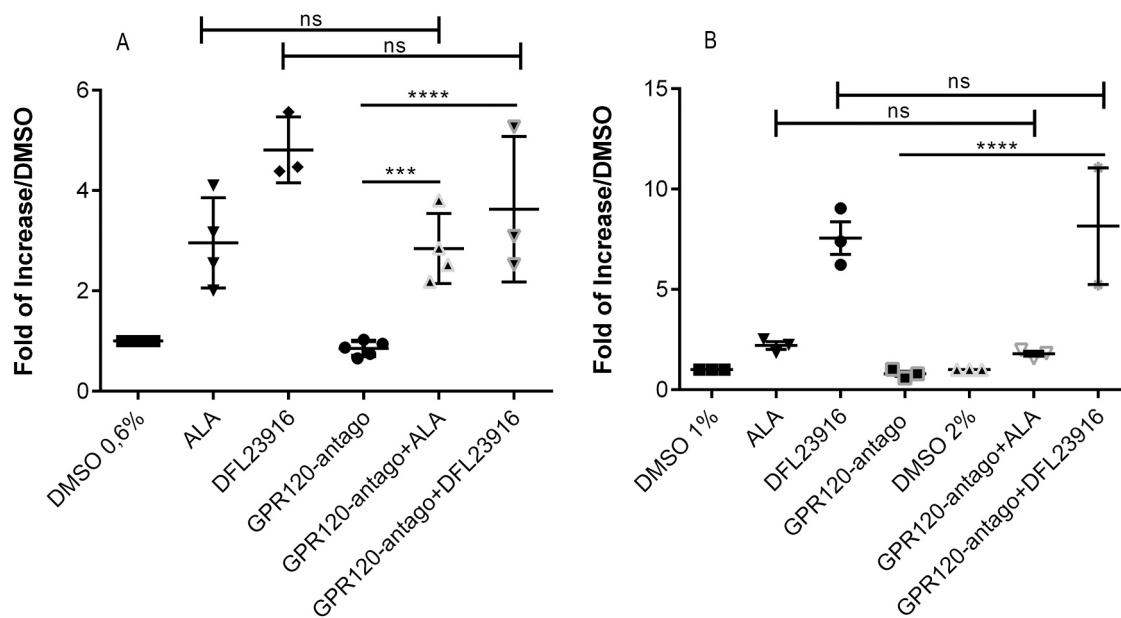

Fig. 6. DFL23916 induces GLP-1 secretion by activating both GPR120 and GPR40. GLP-1 secretion after single and combo treatments with DFL23916 or ALA and a specific GPR120 inhibitor in STC-1 cells (A) and in differentiated NCI-H716 cells (B). Data are represented as mean of an average of three independent experiments ± SEM (****p < 0.0001; ***p < 0.001).

Table 4

In vitro ADME-TOX properties of DFL23916. Data are represented as mean of ± SEM.

Assay	Results		
Metabolic stability in liver microsomes Test conc: 1 μM (n = 2)	Mouse: Clearance: 16.0 ± 3.2 μL/min/mg; $t_{1/2} = 88.5 \pm 17.8$ min Human: Clearance: 10.1 ± 2.4 μL/min/mg; $t_{1/2} = 141.1 \pm 33.7$ min		
Intrinsic Clearance in intestinal S9 with cofactors (NADPH, glutathione, UDPGA and PAPS). Test conc: 10 μM (n = 2)	Mouse: Clearance <5.8 μL/min/mg; t1/2 > 120 min Human: Clearance <5.8 μL/min/mg; t1/2 > 120 min		
Permeability on MDCKII-MDR1 Test conc: 10 μM (n = 3)			**Papp (10^{-6} cm/sec)**
	A to B		1.5 ± 0.4
	B to A		0.7 ± 0.05
Solubility and stability in SGF, FeSIF and FaSIF (n = 3)		**Solubility (μM)**	**Stability at 200 μM (%)**
	SGF (pH 1.6)	5.8 ± 2.3	78.9 ± 6.5
	FeSIF (pH 5)	198.9 ± 11.5	89.6 ± 9.7
	FaSIF (pH 6.5)	75.4 ± 17.2	100.7 ± 2.0
Cytotoxicity (%) (Cell number, Intracellular free calcium, nuclear size, membrane permeability, mitochondrial membrane potential, n = 3)	No effect was observed at 10 μM		
AMES Test (strains: TA98, TA100, TA1535, TA1537 ± S9 fraction, n = 3)	No effect was observed up to 100 μM		
hERG Test (automated patch-clamp, n = 2)	IC50 > 100 μM		

Table 5

Pharmacokinetic parameters for DFL23916 following oral dosing at 15 mg/kg in mice. Data are represented as mean of ± SEM (n = 6).

Parameter	Result
T1/2 (h)	2.2 ± 0.2
Tmax (h)	0.42 ± 0.15
Cmax (ng/mL)	198 ± 13
AUC0-Tlast (ng/mL·h)	480 ± 148
AUC 0–Tinf (ng/mL·h)	571 ± 79
MRT0-Tinf (h)	3.4 ± 0.3

molecule, we then performed mechanism of action studies. Since no GPR120 neutralizing antibody was commercially available at the time the experiments were performed, we took advantage of a selective GPR120 antagonist, Xanthene 39, which was previously used for competition studies with other GPR120 agonists [22], and administrated it simultaneously with DFL23916 or ALA (Fig. 6). Measuring GLP-1 after 30′ of incubation, we observed that GPR120 antagonist Xanthene 39 given in combination with DFL23916 and ALA only slightly reduced GLP-1 secretion in both STC-1 and differentiated NCI-H716 cells (Fig. 6). Notably, although the variance among some values of GLP-1 secretion obtained after the combination of DFL23916 with the GPR120 antagonist is quite large, the values are significantly above those of negative control (GPR120 antagonist alone, p < 0.0001 in both STC-1 and NCI-H716 cells) and similar to those obtained in the same condition with ALA (no statistical difference was observed between ALA+GPR120 antagonist and DFL23916 +GPR120 antagonist), which is known to activate both receptors (GPR120 and GPR40). Thus, even if a trend of reduction can be found when DFL23916 is given with a GPR120 antagonist, this trend is also detectable with ALA and surely due to the absence of the GPR120-mediated GLP-1 production. This little,

not significant reduction in GLP-1 secretion observed after the addition of the GPR120 antagonist demonstrated that it most probably prevented the activation of the receptor by DFL23916 – maybe thanks to an overlap on the same portion of GPR120 binding site – and that our compound has indeed a dual receptor binding capacity and can induce the secretion of incretin GLP-1 activating both GPR120 and GPR40 [18].

3.3. In vivo characterization of DFL23916

3.3.1. ADME-PK Characterization of DFL23916

Once assessed the *in vitro* pharmacology of DFL23916, we further characterized the compound to evaluate the possibility of testing it *in vivo* (Table 4). DFL23916 displayed a good metabolic stability in liver microsomes with a half-life of 88.5 ± 17.8 min in mouse and 141.1 ± 33.7 min in human. Since GPR120 and GPR40 are abundantly expressed in the intestine, we designed a compound with high stability in the gastro and intestinal fluids, high stability towards intestinal microsomes, and lastly, low gastro-intestinal absorption rate to ensure low epithelial permeability. As a matter of fact, DFL23916 displayed high stability (in the range of 79–101%) in simulated gastric fluid (SGF), fed-simulated intestinal fluid (FeSIF) and fasted-simulated intestinal fluid (FaSIF), Table 3. Furthermore, we found an optimal metabolic stability in mouse and human intestinal S9 cells plus cofactors, with a half-life greater than 120 min, and we observed a low cell permeability (Papp $1.5 \pm 0.4\ 10^{-6}$ cm/sec) in Madin-Darby canine kidney epithelial type II (MDCKII)-MDR1 cells. Finally, the compound did not show any sign of cytotoxicity, genotoxicity and cardiotoxicity (Table 4), demonstrating the *in vitro* safe pharmacology of DFL23916 and opening the way for *in vivo* studies.

To determine its oral exposure, DFL23916 was administered to male C57BL/6 mice (n = 6) at 15 mg/kg. DFL23916 was absorbed rapidly (Tmax at 25 min) and showed a mean residence time (MRT) of 3.4 h, being still present up to 8 h at a concentration of about 20 ng/mL (Table 5). Furthermore, the oral half-life of DFL23916 was at 2.2 h (Table 5).

3.3.2. Effect of DFL23916 on GLP-1 secretion in C57BL/6 mice

To prove the ability of DFL23916 to induce GLP-1 secretion also *in vivo*, we set up a protocol to detect GLP-1 plasma levels in the portal vein following DFL23916 direct administration into the proximal colon of C57BL/6 mice. Plasma GLP-1 concentration was measured at 0 and 15 min following the colonic administration of: *i.* vehicle, used as control of injection, *ii.* ALA used as positive control and administered at 30 mg/kg, or *iii.* DFL23916 at 30 mg/kg (the dose was selected based on PK results in mice) in three different groups of mice (Fig. 6). GW9508 was not included in the experimental design because it did not induce GLP-1 secretion *in vitro*, and we did not include TUG-891 because we were ethically committed to reduce the number of animals for this experiment. This was meant to be only a proof-of-concept experiment, which would be followed by the investigation of the potential therapeutic effects in the OGTT.

No differences in basal plasma GLP-1 levels (T0′) were found in mice belonging to the different experimental groups. Notably, DFL23916 significantly increased GLP-1 levels at 15 min post administration compared to T0′ (p = 0.0014), and this increment was comparable to that observed after the administration of ALA (p = 0.023) (Fig. 7A). To demonstrate the effect of ALA and DFL23916 on GLP-1 secretion compared to the vehicle, we then calculated and compared the fold of increase for each group at 15 min after administration of the compounds and found that ALA and DFL23916 induced a statistically significant increase of GLP-1 secretion compared to vehicle (p = 0.0039 and 0.0033, respectively) (Fig. 7B). These data demonstrate the ability of DFL23916 to induce a significant increase of GLP-1 plasma levels *in vivo* 15 min after the colonic administration and provide the proof-of-concept of drug's capacity to induce the enteroendocrine secretion of GLP-1 *in vivo*.
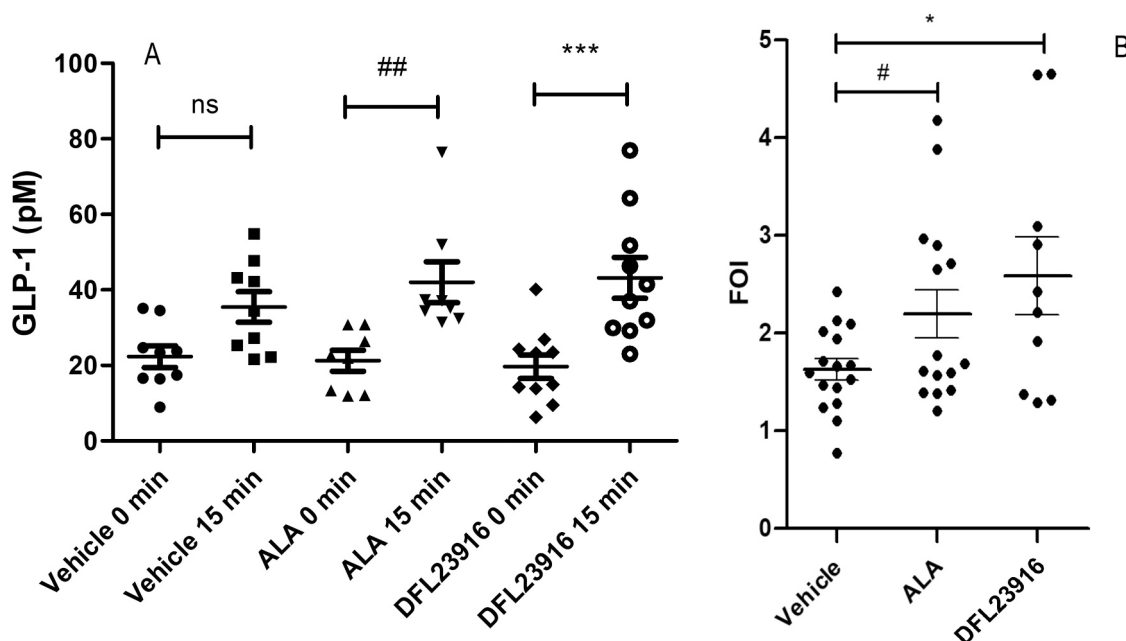
Fig. 7. Effect of DFL23916 on GLP-1 plasma levels. (A) Values distribution is reported in the dot plot, indicating the mean ± S.E.M. for each group: Vehicle (15% v/v PEG400 + 85% v/v Phosphate Buffer Solution 0.2 M pH 7.5, n = 9), ALA 30 mg/kg (n = 8) and DFL23916 30 mg/kg (n = 10), at time 0 and 15 min following colonic administration. Statistically significant differences were found by one-way ANOVA followed by Bonferroni *post hoc* analysis (DFL23916 0 min *vs* DFL23916 15 min, *** $p = 0.0014$; ALA 0 min *vs* ALA 15 min, ## $p = 0.023$). (B) GLP-1 secretion fold of increase: effect of DFL23916 and ALA on GLP-1 plasma levels by fold of increase. Values distribution is reported in the dot plot, indicating the mean value ± S.E.M. of fold of increase for each group. # $p = 0.039$, ALA 15 min *vs* vehicle 15 min; * $p = 0.033$, DFL23916 15 min *vs* vehicle 15 min.

3.3.3. Effects of DFL23916 on glucose tolerance in C57BL/6 mice

To evaluate the effect of DFL23916 on glycemic control and glucose tolerance *in vivo*, we performed an oral glucose tolerance test (OGTT) following oral administration of the compound. As positive control for this experiment, we used Sitagliptin, a potent orally active inhibitor of the GLP-1 inactivating enzyme dipeptidyl peptidase IV (DPP-IV), because it is approved and available in the market for the treatment of T2D and has been demonstrated to decrease the glycemic curve in OGTT in mice [24]. The anti-hyperglycemic effect of DFL23916 was compared to that of the selective GPR120 agonist TUG-891 and was evaluated at

30 mg/kg (the dose that was effective in GLP-1 *in vivo* secretion experiment) and at 100 mg/kg, to assess the half-log dose response. Compounds were orally administered 45 min prior to glucose challenge and in this time frame we did not observe any significant effect on mouse glycemia, demonstrating that, in absence of glucose, DFL23916 has no glucose-lowering effect. After glucose bolus, mouse glycemia was then evaluated at different time points comparing the effects of DFL23916 with those of Sitagliptin and TUG-891, as well as the vehicle (Fig. 8A). As expected, Sitagliptin at 10 mg/kg significantly ameliorated glucose tolerance by 24.8% (p = 0.0001) compared to the vehicle, while the
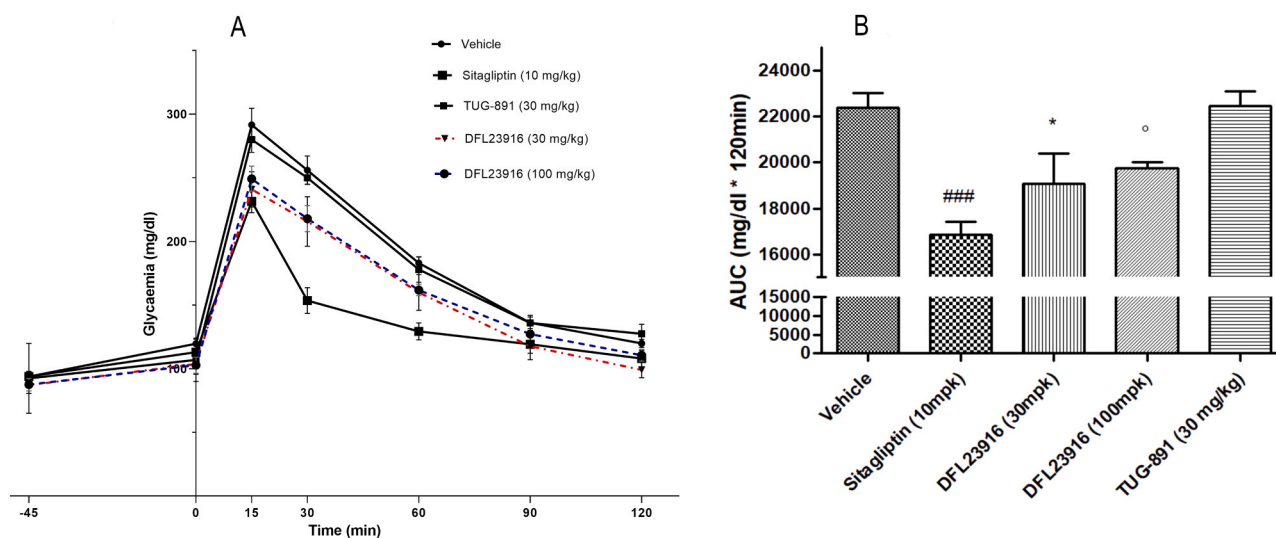

Fig. 8. Effect of DFL23916 on glucose tolerance *in vivo*. Glycemic response curves (A) and the area under the curve (AUC) (B) indicate the effect of vehicle (20% v/v DMSO + 80% v/v Phosphate Buffer Solution 0.1 M pH 8; n = 8), Sitagliptin 10 mg/kg (n = 6), TUG-891 30 mg/kg (n = 8) and DFL23916 at 30 mg/kg (n = 8) and 100 mg/kg (n = 8) on the glucose response in C57BL/6 mice during the OGTT. Each value is expressed as mean ± S.E.M. Statistically significant differences were found by one-way ANOVA followed by Holm-Sidak's multiple comparisons test (Sitagliptin 10 mg/kg *vs* vehicle, ### $p = 0.0001$; DFL23916 30 mg/kg *vs* vehicle, * $p = 0.011$; DFL23916 100 mg/kg *vs* vehicle, ° $p = 0.036$).

effect on the glycemia excursion was not significant after administration of TUG-891 at 30 mg/kg. Notably, oral administration of DFL23916 significantly reduced the glycemia excursion after glucose challenge compared to the vehicle at the dose of 100 mg/kg (11.8%, p = 0.036), and the reduction was even greater (15%, p = 0.011) at the lower dose of 30 mg/kg (Fig. 8B).

These results showed that a single oral administration of DFL23916, both at the dose of 30 mg/kg and 100 mg/kg, induced the amelioration of glucose tolerance in healthy C57BL/6 mice compared to vehicle-treated mice.

4. Discussion

Metformin, sulfonylureas and insulin are key drugs for the treatment of MeS and T2D, however their anti-hyperglycemic action exposes patients to increased risk for hypoglycemia [25] and does not induce important incretin effects. New therapeutic strategies able to induce glucose homeostasis in a more physiological, glucose-dependent manner, are thus necessary to obtain a balanced glycemic control in patients with altered glucose homeostasis. In this context, great interest has risen around GPR40 and GPR120 as key regulators of insulin secretion, β-cell survival and insulin sensitivity, and thus as potential therapeutic targets for the treatment of MeS and T2D [1]. In this study, we characterized a new dual GPR40 and GPR120 agonist, coded DFL23916, and investigated its effects in inducing GLP-1 secretion *in vitro* and *in vivo* and in improving glucose homeostasis *in vivo*.

The dual activation of both GPR120 and GPR40 is increasingly considered as a potentially effective therapeutic strategy to achieve long-term glucose homeostasis, and this is due to the combined activity of the beneficial effects exerted by each receptor [15]. It has been suggested that GPR120/GPR40 dual activation can lead to a synergistic effect on GLP-1 release, insulin secretion and β-cell maintenance [16], promoting, for example, both glucose-stimulated insulin secretion by β cells through GPR40 and the survival of these cells through GPR120. Taking advantage of the deep knowledge of structure activity relationships, a rational drug design approach was undertaken to identify a new dual GPR120/40 agonist class with the ability to delay receptor internalization, in which DFL23916 represents the selected Lead Compound with PK/ADME profile suitable for our indication.

DFL23916 showed a good activity towards hGPR120 (long and short isoforms) and hGPR40, as well as towards their mouse orthologous. Notably, the activity of our compound towards both receptors is low micromolar-ranged, which is satisfactory however, as it is comparable with that of free fatty acids, the endogenous ligands of GPR120 and GPR40, and with that of other well-known synthetic agonists of them (GW9508 and TUG-891). Activating GPR120 and GPR40, DFL23916 can induce both Gαq/11-initiated signal transduction pathways with subsequent Ca^{2+} intracellular spikes and G protein-independent signaling *via* β-arrestin with the same activity, indicating that DFL23916 has a biased agonism through two independent signaling pathways and can thus potentially lead to different pharmacological and therapeutic effects. Interestingly, DFL23916 showed a super-agonist behavior on mGPR120, which could be due to a higher affinity of the compound for the mouse orthologous compared to human GPR120. Future ligand binding experiments will further investigate this phenomenon.

After the activation, the rapid internalization of GPR120 has been previously described in case of treatment with GPR120 agonists (TUG-891 and ALA induced the complete GPR120 internalization within 40 min [16]) and represents a serious challenge for drug development in this context [23]. Interestingly, the activation of GPR120 by our compound delayed the receptor internalization up to 3 h. On one hand, such late receptor endocytosis implies the engaging possibility of prolonged and repeated treatment with DFL23916 without losing the activity and the therapeutic effects, and, on the other hand, together with the super-agonist behavior observed on mGPR120, it can explain the increased efficiency of DFL23916 in stimulating GLP-1 secretion after 30

and 60 min in enteroendocrine cells, despite having a slightly lower AC50 compared to the other tested compounds ALA, TUG-891 and GW9508 (Fig. 4). Notably, GW9508 was no longer included in subsequent *in vivo* studies because in our hands it did not induce GLP-1 secretion *in vitro*.

Mechanism of action studies further confirmed that the higher GLP-1 secretion that we observed *in vitro* – compared to the other tested compounds – was the result of the dual GPR120/GPR40 activation mediated by DFL23916, while dose-response studies demonstrated that the greater GLP-1 production obtained with DFL23916 was not the result of an off-target stimulation of other receptors expressed in STC-1 and NCI-H716 cells (as GPR43, GPR119 and TGR5), as a complete absence of DFL23916 activity was detected towards these receptors (2-log selectivity). Notably, the high selectivity of our compound towards GPR120 and GPR40 is even more striking considering the relatively high concentrations at which it has to be used.

Keeping in mind that to successfully activate GPR40 and GPR120 *in vivo*, a compound has to reach distal small intestine and proximal colon, where the receptors are mainly expressed [26,27], and thus requires high stability in gastric fluids and high stability as well as moderate solubility in intestinal fluids, we evaluated the pharmacokinetic of DFL23916. Indeed, our results showed that DFL23916 fuls these characteristics displaying a good balance between solubility and lipophilicity, which does not allow its passage through the epithelial barrier, and also good solubility and stability in simulated gastric and intestinal fluids. These data and the suitable ADME profile together with the pharmacokinetic study in mice showed that only 0.2% of the administered DFL23916 dose was systemically absorbed. This, in association with the functional *in vivo* studies described below, further supports the topical delivery of the active compound to the colon and rectum where GPR40 and GPR120 are expressed; however, further biodistribution studies are ongoing to assess the distribution of the compound in the animal body.

Several *in vivo* studies have reported that the activation of GPR40 or GPR120 through selective or dual agonist administration has glucose-lowering effects in preclinical models of diabetes, obese mice and OGTT models [7, 15, 28]. In order to investigate the effects of our compound *in vivo*, we first set up a procedure for GLP-1 detection in portal vein blood and observed that local administration of DFL23916 induced a 2.6-fold increase of GLP-1 secretion in healthy mice comparable with that induced by ALA, thus providing the proof-of-concept of DFL23916 capacity to efficiently induce GLP-1 secretion *in vivo*. Then, we tested our compound on glucose homeostasis in mice through an OGTT, comparing its anti-hyperglycemic effect with that of Sitagliptin, a commercially available potent orally active dipeptidyl peptidase IV (DPP-4) inhibitor [24], and of TUG-891, a selective agonist of GPR120 that, contrary to the dual agonist GW9508, we observed being able to induced GLP-1 secretion *in vitro*. While TUG-891 did not significantly alter the glycemic excursion in OGTT mice, orally administered DFL23916 significantly ameliorated the glycemic response following the glucose load, thus proving its efficacy in improving glucose homeostasis *in vivo*. These data demonstrated that our compound is more efficient than the selective GPR120 agonist in modulating the glycemic response *in vivo*, and this is probably due to the delayed internalization of GPR120 that we observed after DFL23916 administration compared to TUG-891, which may allow for a longer and continuous receptor stimulation and GLP-1 secretion.

These results are especially remarkable considering the dual activity of DFL23916 in terms of activated receptors (GPR120 and GPR40) and transduction pathways (Gαq/11-initiated signaling with Ca^{2+} intracellular spikes and G protein-independent signaling *via* β-arrestin). In fact, in the case of chronic/repeated treatment, the beneficial effects of a prolonged dual activation of both GPR120 and GPR40 can lead to a synergistic positive action on both insulin secretion induction (through GPR40) and insulin resistance alleviation (through GPR120), which was not appreciable in this acute-response experiment; on the other hand,

the prolonged activation of both the intracellular Ca^{2+} and β-arrestin pathways can potentially result in a combined anti-hyperglycemic and anti-inflammatory effect, which would be different from the effect obtained with drugs that do not activate both pathways, such as sitagliptin.

Altogether the *in vivo* and *in vitro* data highlight the potential therapeutically advantageous characteristics of DFL23916, strongly encouraging further investigations on the use of this compound for the treatment of disorders characterized by altered glucose homeostasis.

5. Conclusions

The present study proposes a new dual GPR40/GPR120 agonist (DFL23916) as a potential novel drug for the treatment of impaired glucose homeostasis. DFL23916 has good activity and high specificity towards both human and mouse GPR120 and GPR40, displaying in particular a striking super agonist behavior on mGPR120. Showing good pharmacokinetic characteristics and safe ADME profile, the compound delays receptor internalization and increases glucose tolerance *in vivo* by inducing GLP-1 secretion. These properties of DFL23916, and in particular its dual activity combined with the delayed receptor internalization, make it stand out among the other compounds and may allow to overcome key open challenges for pharmacological agonism of GPR120 and GPR40 and for MeS and T2D treatment. Thus, further studies aimed at evaluating the effects of acute and/or chronic administration of DFL23916 in impaired glucose tolerance mouse models, such as diet-induced obesity or genetically modified *ob/ob* mice, may finally identify a good candidate for clinical experimentation.

Funding

Conflict of interest statement

Gianluca Bianchini, Anna Sirico, Rubina Novelli and Andrea Aramini are employees of Dompé Farmaceutici s.p.a., Italy. The company has interest in the development of GPR120/GPR40 agonists for the treatment of altered glucose homeostasis conditions. The other authors declare no conflict of interest.

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