

Evaluation of tetrahydropyridazine-based peripherally restricted dual inhibitors of CB1R and inducible nitric oxide synthase (iNOS) for treating metabolic syndrome disorders

Pinaki Bhattacharjee^a, Szabolcs Dvorácskó^{a,b,1}, Océane Pointeau^{c,d}, Biswajit Kundu^a, Nicholas Rutland^a, Henry Puhl III^e, Jie Liu^f, Grzegorz Godlewski^f, Sergio A. Hassan^g, Tony Jourdan^{c,d}, Resat Cinar^b, Malliga R. Iyer^{a,*}

^a Section on Medicinal Chemistry, National Institute on Alcohol Abuse and Alcoholism (NIAAA), National Institutes of Health (NIH), 5625 Fishers Lane, Rockville, MD 20852, USA

^b Section on Fibrotic Disorders, National Institute on Alcohol Abuse and Alcoholism (NIAAA), National Institutes of Health (NIH), 5625 Fishers Lane, Rockville, MD 20852, USA

^c UFR Sciences Vie Terre Environnement, Université Bourgogne Europe, 21000 Dijon, France

^d INSERM Research Center U1231, Team PADYS, 21000 Dijon, France

^e Laboratory of Biophotonics and Quantum Biology, National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, 5625 Fishers Lane, Rockville, MD 20852, USA

^f Laboratory of Physiologic Studies, National Institute on Alcohol Abuse and Alcoholism (NIAAA), National Institutes of Health (NIH), 5625 Fishers Lane, Rockville, MD 20852, USA

^g Bioinformatics and Computational Biosciences Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA

ARTICLE INFO

Keywords:

CB1R, Cannabinoid-1 receptor
DIO, Diet-induced obesity
ECS, Endocannabinoid system
HFD, High fat diet
iNOS, inducible nitric oxide synthase

ABSTRACT

Background and purpose: The endocannabinoid system is a key regulator of metabolism, sparking interest in cannabinoid type 1 receptor (CB1R) antagonists as potential treatments for obesity and related conditions collectively called metabolic syndrome disorders. However, the neuropsychiatric liabilities associated with centrally acting CB1R antagonists led researchers to focus on developing peripherally restricted compounds that do not cross the blood-brain barrier (BBB).

This study aimed to synthesize and evaluate novel CB1R antagonists based on tetrahydropyridazine core incorporating physicochemical design principles that would allow for negligible BBB penetration. The efficacy of the compounds was assessed in rodent models of diet induced obesity and diabetes.

Experimental approach: In this study, we employed a rational-design approach along with structure-based modeling to develop small-molecule CB1R antagonists that are peripherally acting. Pharmacological profiles of two racemic compounds PB19A and PB95 were evaluated in cannabinoid receptor binding studies, and functional [³⁵S]-GTPγS assays. Further chiral separation of enantiomers allowed for the evaluation of respective eutomers in in vitro ADME studies along with in vivo pharmacokinetic and tissue distribution studies in mice. The results showed that the compounds are orally bioavailable and had negligible brain penetration. The design features also incorporated putative amidine moieties which inhibit the pro-inflammatory enzyme; inducible nitric oxide synthase (iNOS). Both biochemical and in vitro cell-based assays showed the CB1R antagonists having iNOS inhibitor properties. In vivo CB1R functional antagonism was assessed by upper gastrointestinal motility assay. The efficacy of our CB1R antagonists was compared with brain penetrant ibipinabant in a diet-induced obesity mouse model, assessing effects on lipid metabolism biomarkers, food intake, body weight reduction, glucose tolerance and insulin resistance.

Key results: Novel compounds PB19AE2 and PB95E2 were designed and evaluated as peripherally restricted CB1R antagonists. In high fat diet fed mice, these compounds improved metabolic parameters, modestly reduced food intake, and ameliorated hepatic lipid metabolism markers.

* Corresponding author.

E-mail address: malliga.iyer@nih.gov (M.R. Iyer).

¹ Current address: Laboratory of Biomolecular Structure and Pharmacology, Institute of Biochemistry, HUN-REN Biological Research Centre, Temesvári krt. 62, 6726 Szeged, Hungary.

<https://doi.org/10.1016/j.metabol.2025.156291>

Received 26 December 2024; Accepted 2 May 2025

Available online 12 May 2025

0026-0495/© 2025 Published by Elsevier Inc.

Conclusion and implications: Overall, PB19AE2 and PB95E2 are orally bioavailable, peripherally acting CB1 antagonists and their preliminary evaluation show promising potential in utilizing the pyridazine-based compounds for generating potent leads for treating obesity- associated disorders.

1. Introduction

The endocannabinoid system (ECS) is a complex and widespread neuromodulatory signaling network that plays a crucial role in maintaining homeostasis in the human body [1,2]. Discovered in the early 1990s, the ECS has since been recognized as a vital component in regulating various physiological and pathophysiological processes [3,4]. Due to the wide-ranging physiological functions performed by the endocannabinoid system comprising its endogenous ligands, enzymes and receptors, molecules acting on this signaling pathway have been the subject of detailed studies [2,4,5]. Endocannabinoids are naturally occurring endogenous, lipid-based neurotransmitters produced by the body. The two main endocannabinoid ligands identified and produced on demand are *N*-arachidonylethanolamine (AEA) and 2-arachidonoyl-glycerol (2-AG) [2,6]. Notably, the ECS is also the primary target for exogenous cannabinoids, including tetrahydrocannabinol (THC), which is the psychoactive component of cannabis [1]. Recent research on endocannabinoids has fundamentally altered our understanding of human physiology and the management of numerous disorders. Studies have uncovered important details on the endocannabinoid system signaling including the two well-studied G-protein receptor targets the Gi/o protein-coupled cannabinoid receptor type 1 (CB1) and type 2 (CB2) [7,8]. Among these two receptors, CB1R is extensively distributed throughout the central nervous system (CNS) especially in hippocampus, cortex, cerebellum, and basal ganglia and is accountable for the psychotropic effects of the active ingredient of marijuana and synthetic agonists [9]. CB1R is expressed in many peripheral tissues, including the liver, pancreas, skeletal muscle, and adipose tissue, testis, and ileum. Conversely, while CB2R is mostly noted on immune cells, studies now vouch for its presence in the brain [10,11]. The CB1R has received significant attention in the last three decades for its involvement in the regulation of food intake, behavior, and de novo lipogenesis [12]. While many targets and agents are implicated in the pharmacotherapy of obesity [13], antagonism of CB1R is a valid mechanism to treat obesity and related diseases e.g. insulin resistance, dyslipidemia and other diabetic complications. These findings formed the basis for the development of the first-in-class CB1R antagonist/inverse agonist rimonabant, which promoted weight loss, improved dyslipidemia, glycemic control, and reduced hepatic steatosis in overweight people with metabolic syndrome (MetS) [14,15]. Nevertheless, since blocking CB1R in the brain is linked to anxiety and related neuropsychiatric liabilities the development of CNS penetrant, systemically acting CB1R orthosteric antagonists has limited potential as a therapeutically viable approach

[16–18]. Hence compounds that are restricted to the periphery and selectively target CB1R without causing adverse effects in the central nervous system (CNS) are sought as a renewed therapeutic approach [19].

The 3,4-diarylpyrazoline chemotype was reported by Lange and coworkers around twenty years ago as their first attempt to create CB1R antagonists/inverse agonists [20,21]. An extensively studied compound ibipinabant/SLV-319, entered the clinic trials, yet did not progress when rimonabant's travails came to fore. Further Lange et al. showed that expansion of the central five membered core to a six-membered 1,4,5,6-tetrahydropyridazines scaffold allows for the generation of compounds which modulate the CB1R [22]. This approach however enumerated only a very small set of compounds that were evaluated with no effects reported on any rodent models (Fig. 1).

Growing body of data suggest that preclinically, peripherally acting CB1R antagonist may rescue metabolic shortcomings in obese subjects [23–25]. This has prompted the development and testing of several chemical entities with reduced BBB access in rodent models to validate the promise of the therapeutic potential of peripherally acting CB1R antagonists [17,24,26]. Further, promising clinical validation has now been reported for monlunabant (MRI-1891/INV-202) in a Phase 2A clinical trial [27]. Additional advancements in this approach included combining CB1R and enzymatic targets like inducible nitric oxide synthase (iNOS) and adenosine monophosphate kinase (AMPK) to create hybrid antagonists [27–29]. iNOS, an enzyme catalyzing the production of NO is a signaling pathway whose overactivation may promote obesity, diabetes and renal dysfunction [30–32]. A substantial up-regulation of iNOS is considered the main perpetrator of autotoxicity under oxidative stress such as obesity [33,34]. The expression of iNOS has been observed to be enhanced in both muscle and adipose tissues in genetic and dietary models of obesity [35]. As shown previously, unimolecular entities bearing hybrid-CB1R/iNOS inhibition properties is an ideal target-combination to treat metabolic and fibroproliferative disorders [28,29,36–38].

Our ongoing efforts in exploiting the therapeutic potential of peripheral CB1R antagonists, led us to this study, where we employed rational design and structure-based modeling to develop 1,4,5,6-tetrahydropyridazine-based racemic CB1R antagonists PB19A and PB95 avoiding CNS penetration to abrogate the neuropsychiatric side effects. In vitro profiles were evaluated through cannabinoid receptor binding studies and functional assay were conducted using GTPγS assays. Further characterization was carried out by the separation of component enantiomers where potent CB1R antagonism was shown to reside with

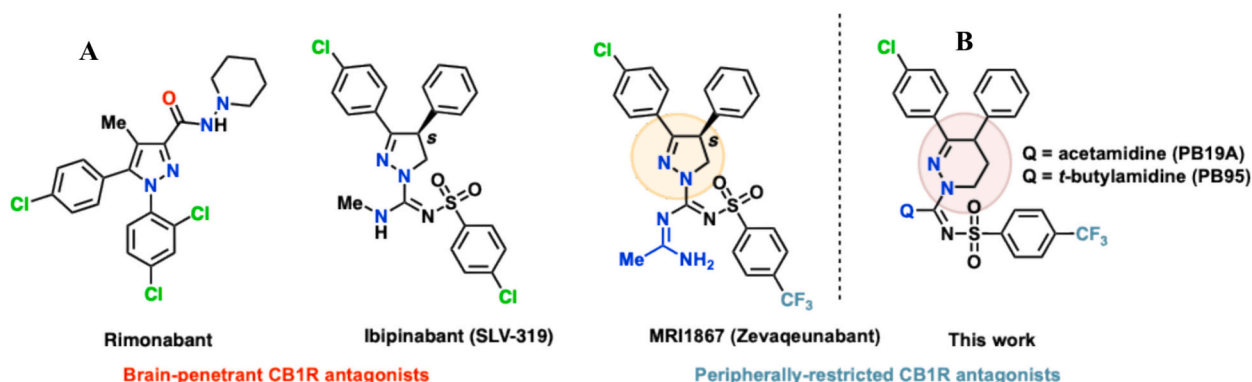
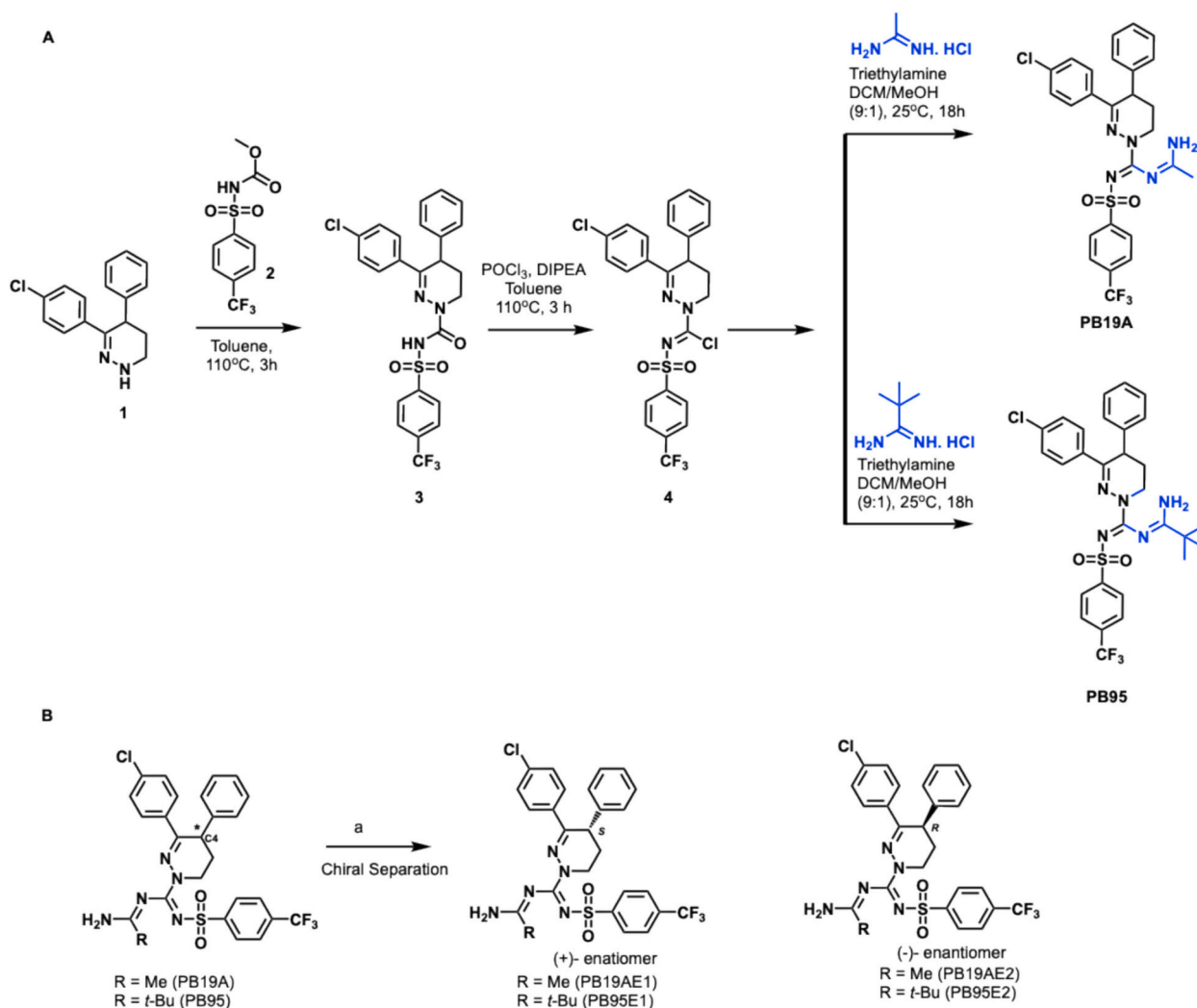


Fig. 1. (A) Centrally acting and peripherally restricted CB1R antagonists (B) Tetrahydropyridazine based CB1R antagonists.



Scheme 1. (A) Synthesis of compounds PB19A and PB95 (B) Enantiomeric separation of lead compounds using Chiral chromatography (a) Conditions: PB19A ColumnTek ENANTIOCEL C2-5,4,6 (ID)X 250 (L) mm and for PB95: Phenomenex Lux 5 uM Amylose-1, 30 (ID) X 250 (L) mm.

(–) enantiomers PB19AE2 and PB95E2. Subsequently the antagonists were tested in *in vitro* ADME assays and mice tissue distribution studies to assess their brain penetrant properties. *In vivo* engagement of CB1R was assessed by the compounds ability to reverse the inhibition of upper gastrointestinal (GI) motility caused by CB1R agonist, arachidonoyl 2-chloroethylamide (ACEA) [29]. The efficacy of chiral CB1R antagonists was evaluated in a diet-induced obesity mouse model, assessing effects on food intake, body weight reduction and other metabolic parameters contributing to diabetes.

2. Materials and methods

2.1. Chemistry

The synthesis of the compounds was carried out as shown in Scheme 1 A/B. Commercially available reagents were procured and used as is. Prohibitively expensive reagents and/or intermediates were synthesized based on reported procedures where indicated. Melting points were determined on a MPA100 Stanford Research System apparatus and are uncorrected. Proton (^1H NMR and ^{13}C NMR) spectra were recorded on a Jeol 400 or Bruker 800 MHz spectrometer in CDCl_3 (unless otherwise noted) with the values given in ppm (TMS as internal standard) and J (Hz) assignments of ^1H resonance couplings were determined by Mes-trenova software. Mass spectra (HRMS) were recorded on a VG 7070E

spectrometer or a JEOL SX102a mass spectrometer. Thin layer chromatography (TLC) analyses were carried out on Analtech/Miles Scientific silica gel GHLF 0.25 mm plates using various gradients of EtOAc:*n*-hexanes or $\text{CHCl}_3/\text{MeOH}$ containing 1 % NH_4OH . TLC plates were visualized under UV light or by staining in an iodine chamber. Flash column chromatography was performed on Teledyne ISCO Combiflash system. Product yields are un-optimized. All compounds tested had ≥ 95 % purity based on a combination of TLC, ^1H NMR, ^{13}C NMR, LC/MS, high-resolution mass spec and X-ray (where indicated). Detection on LC-MS was carried out on Agilent 1200 using either Luna C18 3 mm (3×75 mm) where the mobile phase was 4 % to 100 % acetonitrile (0.05 % TFA) standard gradient or Agilent EC-C18 poroshell 2.7 mM (3×50 mm) where the mobile phase was 50 % to 98 % acetonitrile (0.1 % formic acid). The LC-MS chromatogram showed the expected molecular (MH^+) ion as well as a single peak at UV (254 nm) which was used to compute purity. Chiral rotation data was obtained using Anton Paar polarimeter under shown conditions. X-ray facility at Georgetown University was utilized to obtain crystal structure of select compound(s).

Chiral separation for component enantiomers were carried out at Lotus Separations, Princeton University NJ, USA.

2.2. Radioligand binding and functional assay

2.2.1. CB1R binding assay

The assay was performed as described previously [39]. Detailed methods are available in Supplementary Information.

2.2.2. GTP γ S binding assay

The assay was performed as described previously [36]. Detailed methods are available in Supplementary Information.

2.2.3. CB2R binding assay

The assay was performed as described previously [28,29]. Detailed methods are available in Supplementary Information.

2.2.4. CB1R GRAB_{CB2.0} assay

The assay was performed as previously described [40]. Detailed methods are available in Supplementary Information.

2.3. X-ray data

The crystal structure data for PB19AE1 has been deposited at the joint CCDC/FIZ Karlsruhe deposition service. The data have been assigned the following deposition numbers: CCDC 2412342.

2.4. Molecular modeling and dynamics simulations

The computational modeling and molecular dynamics (MD) simulations were carried out as described previously [27,41–43]. The initial conformation of CB1R was taken from the cryo-EM structure of its complex with zevaquenabant (9B9Z), which is similar (C α -rmsd $\sim$ 1 Å) to those bound to monlunabant (9BA0) and taranabant (PDB ID: 9B9Y) [43]. Given the multiple potential conformers of PB19AE2, we performed quantum chemical geometry optimization at the DFT level using the B3LYP functional and the 6-31G** basis set for four main conformers, both in the gas phase and in two solvents (using the SMD continuum model as implemented in Gaussian 16, revision C.01): water and *n*-octanol, the latter serving as a mimic of a lipid membrane environment (SI Table S1 and SI Fig. S12). The central six-membered ring with Arm 2 in an axial position (pc_2) was found to be the most stable in all three environments, followed by the chair-inverted conformer (pc_1) with the arm in the equatorial position; the latter was $\sim$ 1.5 kcal/mol less stable in the membrane. The reverse conformers, $pc_1(r)$ and $pc_2(r)$, where the central dihedral ψ is rotated 180 degrees, were also considered. These four conformers (upper panels of SI Fig. S13) were initially docked into the CB1R binding pocket with arms 1 and 2 inside the narrow hydrophobic cavity and oriented as in all previous compounds of the series [27,41–43]. Flipping of these arms (lower panels of SI Fig. S13) leads to alternate potential binding modes since our earlier studies have shown that the cavity readily adapts through slight reorientations of side chains and backbone shifts. Such modes were studied previously but not considered here because the recent cryo-EM structures of CB1R bound to similar four-armed compounds give us confidence that they could be disregarded [27,41–43]. MD simulations were performed with NAMD at 37 °C, 1 atm, 120 mM of NaCl and standard protonation states at pH 7.4, using the CHARMM (param36) forcefield. Statistics of the interactions between the ligand and the receptor, and between the ligand and water, were obtained as described previously [43].

2.5. In vitro iNOS inhibition assays

The assay was performed as described previously [28,29,41]. Detailed methods are available in Supplementary Information.

Briefly, inducible nitric oxide synthase (iNOS) activity was measured by the conversion rate of [3 H]-L-arginine to [3 H]-L-citrulline using a NOS Activity Assay Kit (Cayman Chemical). The tested compounds were dissolved in DMSO to prepare 10 mM stock solutions. Compounds were

tested at a final concentration of 10 μ M and incubated at 37 °C for 30 min in assay buffer containing reaction buffer (2 $\times$), 10 mM NADPH, [3 H]-L-arginine, and HPLC-grade water, along with recombinant mouse iNOS enzyme (1 unit/sample, Cayman #60864). The reaction was stopped after 30 min using the stop buffer provided in the kit, and subsequent steps were performed according to the supplier's protocol. The specificity of iNOS activity measured was further confirmed by its inhibition by 1 mM N-nitro-L-arginine (L-NNA). Afterward, samples were mixed with Ultima Gold XR scintillation cocktail, and radioactivity was quantified using a TRI-CARB liquid scintillation analyzer. Enzyme activity was expressed as the percentage conversion of [3 H]-L-arginine to [3 H]-L-citrulline.

2.6. In vitro ADME assays (Metabolic stability, Caco, MDCK-MDR1)

The assays were performed by Bienta/Enamine and detailed methods are available in Supplementary Information.

2.7. Animals

All animal procedures were conducted in accordance with the rules and regulations of the Institutional Animal Care and Use Committee of the National Institutes of Alcohol Abuse and Alcoholism (NIAAA) under the protocols of LPS-GK1 and APAFIS no. 40985, UAR, Epina-sur-Orge, France). Thirteen-week-old male C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed under a 12-h light/dark cycle and fed a standard diet, ad libitum (Teklad NIH-31; Envigo, Huntingdon, UK). To generate diet-induced obesity (DIO) mice, official French regulations for the use and care of laboratory animals were followed throughout the experiments. The experimental protocol was approved by the local ethics committee (CE2A, Dijon, France) for animal experimentation (APAFIS no. 40985). Six-week-old male wild-type C57BL/6J mice (JANVIER LABS, Le Genest St Isle, France) were maintained under a 12:12-h light-dark cycle and fed ad libitum a high-fat diet (cat. no. E15742–34, 60 % of calories from fat [lard], 20 % from protein, and 20 % from carbohydrates; SSNIFF, Soest, Germany) for 18 weeks. C57BL/6J mice were kept on a standard laboratory diet (STD; AO4; UAR, Epina-sur-Orge, France) for same duration.

2.8. Experimental drug treatment

The compounds were administered by intraperitoneal injections or oral gavage as indicated. The vehicle was a 1:1:18 ratio of DMSO: Tween 80: Saline. Formulations were applied at 2, 6, 20 and 60 mg/mL concentrations to achieve doses of 3 and/or 10 mg/kg as indicated.

2.9. Tissue levels of drugs and PK

The extraction of the tissues and mass spectrometry conditions were used as described previously [38,41]. Briefly, 30 μ L of serum or tissues weighing 50–80 mg were homogenized in 0.5 mL of ice-cold methanol/Tris buffer (50 mM, pH 8.0), 1:1, containing 100 ng [13 C $_6$]-MRI-1867 as internal standard. Homogenates were extracted two times with CHCl $_3$: MeOH (2:1, vol/vol), dried under nitrogen flow, and reconstituted with MeOH after precipitating proteins with ice-cold acetone. Tissue levels of PB19AE2 and PB95E2 were determined by LC-MS/MS using an Agilent 6470 triple quadrupole mass spectrometer (Agilent Technologies) coupled to an Agilent 1200 LC system (Agilent Technologies). Liquid chromatographic separation was obtained using 2.5 μ L injections of samples onto a InfinityLab Poroshell 120 EC-C18, (3.0 $\times$ 100 mm, 2.7 Micron) from the Agilent Technologies. The autosampler temperature was set at 4 °C and the column was maintained at 34 °C during the whole analysis. Gradient elution (350 μ L/min) began at 10 % B (MeOH, 0.1 % formic acid) with linear increase towards 50%B for the first 0.5 min, followed by a linear increase towards 85 % B at 3 min; this was

maintained until 14 min and then increasing linearly again to 100 % B at 18 min; this was maintained until 38 min. At minute 39, the gradient changed linear to the initial setting which was maintained for 6 min. The Agilent 6470 triple quadrupole mass spectrometer was set for electrospray ionization (ESI). The electrospray interface was operated in positive ion mode. The source parameters were as follows; capillary voltage, 3500 V; gas temperature, 300 °C; drying gas, 5 L/min; Sheath gas temperature, 250 °C; Sheath gas flow, 10 L/min; and nitrogen was used as the nebulizing gas with a pressure of 45 psig. Collision-induced dissociation (CID) energy was performed by using nitrogen. Levels of each compound were analyzed by multiple reactions monitoring. The molecular ion and fragments for each compound were measured. The molecular ion and fragments for each compound were measured as follows as follows: m/z 562.1 → 271 for PB19AE2, m/z 604.2 → 521.1 for PB95E2. The amounts of compounds in the samples were determined against standard curve using [$^{13}\text{C}_6$]-MRI-1867 [44] as internal standard. Values are expressed as ng/mL or ng/g. The amounts of compounds in the samples were determined against standard curve using [$^{13}\text{C}_6$]-MRI-1867 [44] as internal standard. Values are expressed as ng/mL or ng/g. For PK analysis, compounds were dissolved in a vehicle of DMSO:Tween 80:saline (1:1:18) and administered by oral gavage of 3 or 10 mg/kg doses as indicated for each compound. After the indicated time point (15 min, 30 min, 1, 2, 8 and 24 h) blood samples were collected from the tail. Samples were centrifuged and serum was collected and extracted and measured by LC-MS/MS spectrometry.

2.10. Food intake and body weight reduction studies

For DIO experiments, compounds or vehicle were administered daily by oral gavage. Body weight and individual food intake were monitored daily. After euthanasia, tissues were collected, weighed, and snap frozen in liquid nitrogen. Trunk blood was collected to determine endocrine and biochemical parameters.

2.11. Glucose tolerance test and serum chemistry

The assays were performed following 2 week of daily oral doses of PB19AE2 and PB95E2, as previously described [29,45]. I.P. glucose tolerance tests were performed after 6-h fasting by i.p. administration of glucose at 1.5 mg/kg and subsequent sampling of tail blood at 15-min intervals. Blood glucose levels were measured from tail blood. Leptin, glucagon, GIP, GLP-1, ghrelin, resistin and PAI-1 were assessed from 1 single sample using the Bio-Plex Pro Mouse Diabetes 8-Plex Assay (Biorad Laboratories, Hercules, USA, cat.171-A7001M). Serum TG, cholesterol, HDL, LDL, ALT and AST were assessed using the automated Vista 1500 and its dedicated reagents (Siemens, Erlangen, Germany). The assays were performed following 1 week of daily oral doses of PB19AE2 and PB95E2, as previously described [29,45]. I.P. glucose tolerance tests were performed after overnight fasting by i.p. administration of glucose at 1.5 mg/kg and subsequent sampling of tail blood at 15-min intervals.

2.12. Upper GI motility assay

The assay was performed as described previously [39]. To assess in vivo CB1R antagonism, PB19AE2 and PB95E2 along with rimonabant was administered 30 min after an oral dose of the CB1R agonist arachidonyl-2'-chloroethylamide (ACEA), 5 mg/kg, to male 8- to 10-week-old mice 1 h prior to an oral bolus of 10 % charcoal suspension in 5 % gum arabic. Thirty minutes later, the mice were sacrificed, and the distance traveled by the head of the marker between the pylorus and the cecum was measured and expressed as percentage of total length of the small intestine.

Table 1

In vitro pharmacology data of dual-target novel compounds.^a

Compounds	CB1R K _i (nM)	CB2R K _i (nM)	[^{35}S]- GTPγS binding (nM) IC ₅₀ (nM)	iNOS inhibition (%) (Radio isotopic assay)	iNOS inhibition (%) Mouse RAW 264.7 cells
Rimonabant	3.6 ± 0.7	1580	25 ± 2 [28]	–	ND
S-MRI-1867	1.8 ± 0.3	>1000	40 ± 7 [28]	49 ± 0.4	ND
PB19A	32 ± 4	ND	93 ± 5	ND	64
PB19AE1	>1000	ND	>1000	ND	ND
PB19AE2	13 ± 2.1	47 ± 4.1	26 ± 1.1	27 ± 0.4	ND
PB95	12 ± 1.8	ND	18 ± 1.6	ND	66
PB95E1	>1000	ND	>1000	ND	ND
PB95E2	3.5 ± 0.2	43 ± 3.4	15 ± 0.6	23 ± 4.1	ND

^a Binding affinity for CB1R, was determined from binding displacement isotherms using [^3H]-CP55940 as the labeled ligand and crude mouse brain plasma membranes. K_i values were calculated using the Cheng-Prusoff equation utilizing GraphPad Prism 9 (GraphPad Software, Boston, MA, USA). GTPγS-functional assay was performed using [^{35}S]-GTPγS and CB1R-expressing crude mouse brain membranes in the presence of the agonist CP55,940 (1 μM). iNOS experiments were carried out using both biochemical and cell-based assays where indicated. Data represent mean ± SEM from 3 independent experiments performed in triplicates.

2.13. Drugs

Stock solutions of rimonabant, PB19AE2 and PB95E2 were made in DMSO, and aliquots were kept in –20 °C freezer. For animal studies, drugs were further dissolved in DMSO:Tween 80:saline (10:3:87) immediately before administration. This solvent composition was particularly critical for keeping PB95AE2 in clear solution.

2.14. Statistical analysis

Values are mean ± SEM of $n = 4$ samples. Results were analyzed using two-way ANOVA followed by Dunnett post-hoc test. For DIO mice experiments, statistical analysis was performed with GraphPad Prism (version 10.4.1 for Windows, San Diego, CA) by an analysis of variance (ANOVA) followed by Dunnett post-hoc test for comparisons vs vehicle treated DIO mice. Statistical significance was set at $p < 0.05$. All summary results are presented as mean ± standard error of the mean. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to vehicle-treated DIO littermates.

3. Results and discussion

3.1. Pharmacological characterization of PB19AE2 and PB95E2 as CB1R peripherally restricted antagonists

In the current study, two novel compounds (PB19A and PB95) were rationally designed based on strategic modifications of 1,4,5,6-tetrahydropyridazines to reduce brain penetration [22,28,29]. Our prior work with S-MRI-1867 (zevaquenabant) which contain a pyrazoline core structure provided a strong rationale to explore pyridazine-based core to generate peripherally acting CB1R antagonists. Important physicochemical parameters like lipophilicity (ClogP), polar surface area, (tPSA) leading to multiparameter optimization (MPO) scores are descriptors that play important roles in the design of compounds with potential BBB permeability. Inclusion of polar moieties increases PSA and decreases lipophilicity, thereby helping limit BBB penetration. Aligned with our previous approach, introduction of polar imidamide group into the 1,4,5,6-tetrahydropyridazines central core allowed the

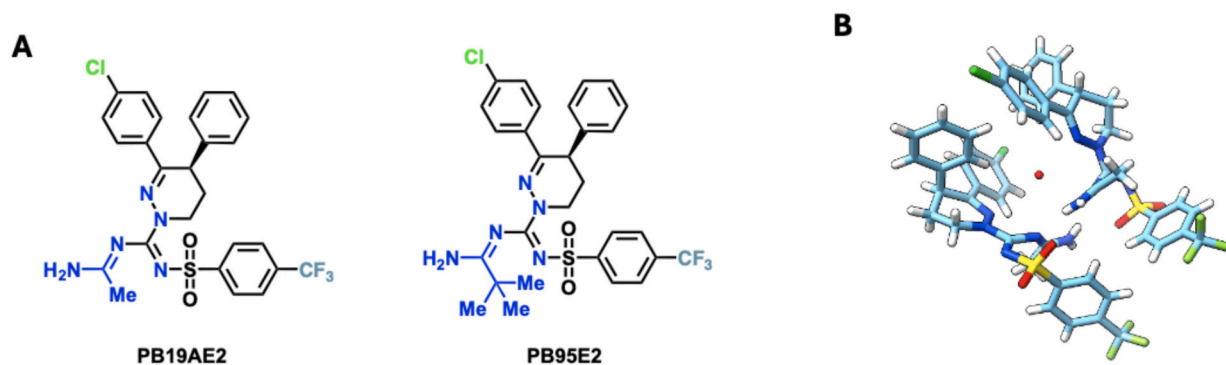


Fig. 2. (A) Eutomers of PB19A and PB95 (B) Single crystal X-ray structure of PB19AE1.

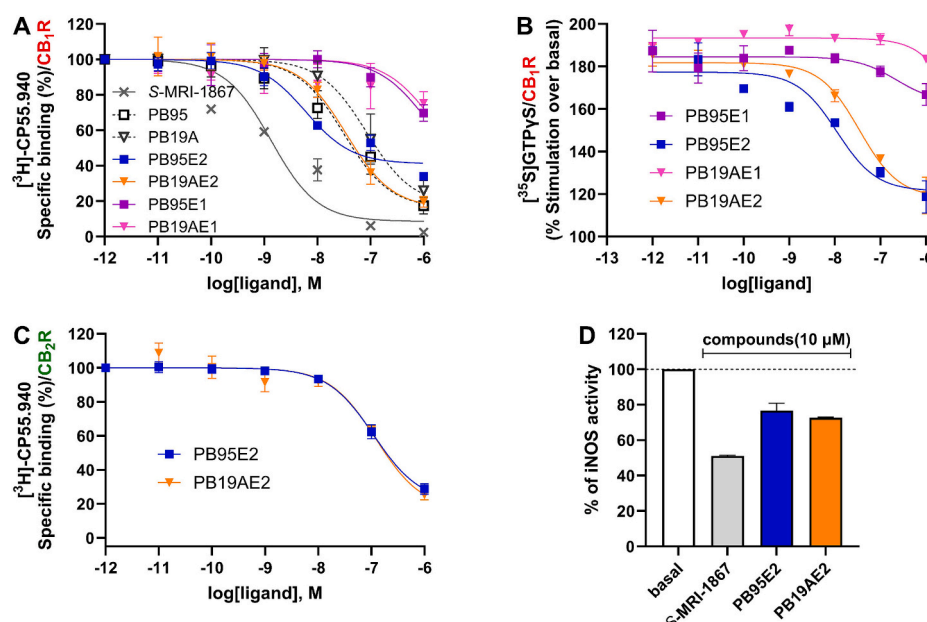


Fig. 3. (A) Binding affinity for compounds and the enantiomers of PB19A and PB95 (determined from binding displacement isotherms using [³H]-CP55940 as the labeled ligand and plasma membranes from CB₁R-expressing CHO-K1 cells.) (B) Functional CB₁R antagonism was determined using the agonist-stimulated [³⁵S]-GTPγS binding assay. The compounds concentration-dependently antagonized the effect of CP55940 (1 μM). (C) Binding affinity of active enantiomers were determined from binding displacement isotherms using [³H]-CP55940 as the labeled ligand and plasma membranes from human CB₂R expressing cells. (D) iNOS inhibition on iNOS mouse recombinant enzyme. Statistical significance of differences between groups was determined by one-way analysis of variance followed by Dunnett's post hoc test. $p < 0.0001$; ****, $p < 0.001$; ***, $p < 0.01$; **.

generation of novel four-arm antagonists PB19A and PB95 (Fig. 1 and Scheme 1A) [29]. As shown in Table 1, initially PB19A and PB95 were designed as racemic compounds and both compounds showed CB₁R binding in the nanomolar range had higher PSA than rimonabant (tPSA 100 vs 47). Further, we evaluated their binding efficacy and functional potency with the CB₁R. The molecules share a common scaffold of pyridazine, with a *p*-CF₃ phenyl sulfonyl group in the south part, and the only difference is the presence of a different imidamide moiety. PB19A has a methyl group, whereas PB95 has a bulkier tert-butyl group. We first evaluated the CB₁R binding affinity of racemic PB19A and PB95. Then proceeded to evaluate their CB₂R binding in at 1 μM concentration (SI Fig. S15). They demonstrated a lower binding affinity for CB₂R compared to CB₁R. Initial functional characterizations of the tested ligands using the CB₁R-based GRAB_{CB2.0} sensor showed, that PB95 and PB19A, behaved as antagonists (IC₅₀ = 73.8 nM and 154.7 nM respectively), responses that mirror their predicted potencies at cannabinoid 1 receptors (CB₁R) [40]. We found that both PB19A and PB95, behave as inverse agonists (SI Fig. S16). Subsequently, we also performed a GTPγS functional assay, and the results were consistent with the GRAB_{CB2.0}

sensor-based results. Obesity and weight gain are associated with the overactivation of CB₁R, and literature precedent suggests that inducible nitric oxide synthase (iNOS) plays a key role in the relationship between obesity associated metabolic syndrome disorders. Chronic iNOS activation can lead to insulin resistance in muscles. This is because higher levels of nitric oxide (NO) from iNOS can negatively affect insulin signaling. Therefore, we first conducted a cell-based assay to assess iNOS inhibition using mouse RAW 264.7 cells, presenting the results as a percentage of specific binding or activity inhibition. Racemic PB19A and PB95 both showed iNOS inhibition 64 and 66 %, respectively (Table 1). PB19A and PB95 have a chiral center at the C4 position. Promising in vitro data from racemic compounds provided the impetus to carry out the chiral separation into component enantiomers for both these compounds. As shown in Scheme 1B, the separated component enantiomers identified as PB19AE1/E2 and PB95E1/E2 were then reevaluated. Interestingly enantiomer 1 of PB19A crystalized rapidly allowing us to determine the absolute configuration. Through careful investigation of the crystal structure and optical rotation values we were able to determine that *S* configuration to enantiomer 1 (E1) and *R* configuration to

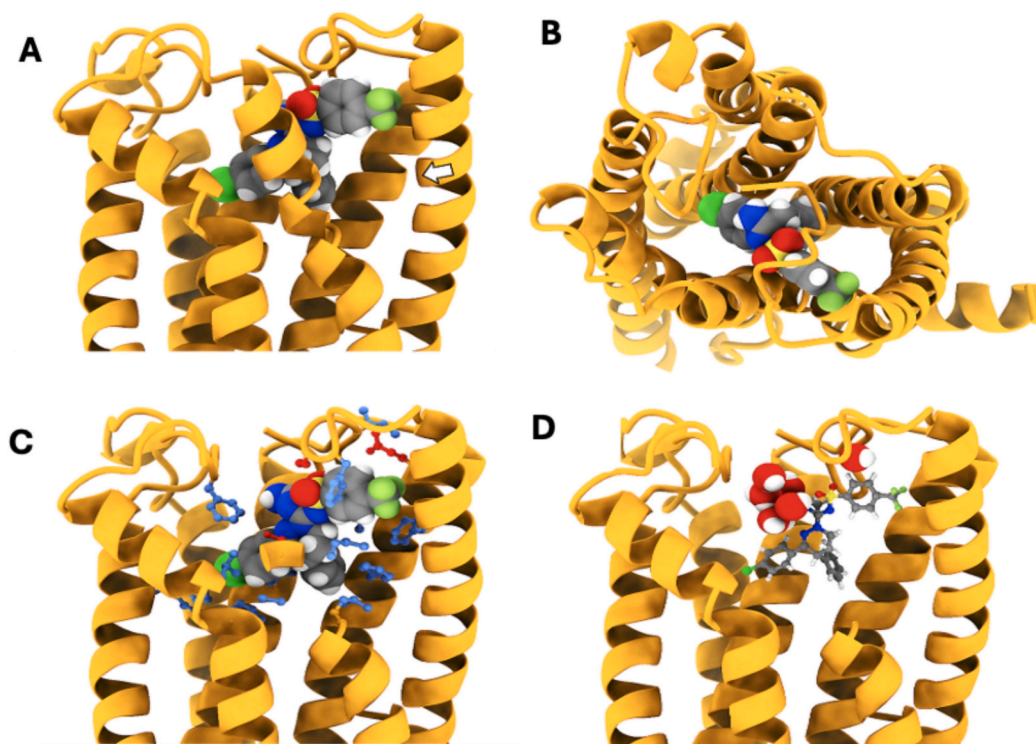


Fig. 4. Main binding mode of PB19AE2 to CB1R identified in this study, with the ligand in the pc_1 conformation (SI Fig. S12.): Transmembrane view (A) and view from the extracellular side (B) of the bound ligand rendered as atom-based vdW spheres, with TMH1 on the right of each panel (F: light green, C: gray; H: white; S: yellow; O: red; N: blue; Cl: bright green). (C) Statistically prevalent interactions between the ligand and the receptor: residues engaged in nonpolar or hydrophobic interactions are shown in light blue (F102, M103, I105, F108, I169, F170, F174, V196, F268, W279, W356, L359, M384, C386, L387), while residues involved in polar or H-bond interactions are shown in red (C107, N116, S383); the position of S123 is indicated by white arrow in panel A. (D) Rehydration of the hydrophilic arms 3 and 4 appears to determine the affinity and orientation of the arms; water molecules interacting with the polar moieties of PB19AE2 are shown as atom-based vdW spheres (from a representative snapshot at the end of the simulation). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

enantiomer 2 (E2) providing a valuable insight into the stereochemical properties of the molecules. Single molecule X-ray structure of PB19AE1 indicated the (+)-isomer had *S*-stereochemistry at the benzylic carbon C4 position (Fig. 2A/B). CB1R radioligand binding and GTP γ S functional assay of the component enantiomers indicated that the CB1R binding affinity resided with (–) enantiomer i.e., PB19AE2 and PB95E2 which are in *R* configuration. As assessed from the *in vitro* biochemical assay, both PB19AE2 and PB95E2 showed negligible iNOS inhibitory properties arising from the amidine pendant which is depicted in the Table 1 (Fig. 3D). The compounds were also assessed in β -arrestin-2 recruitment assays where they showed CB1R antagonism comparable to GTP γ S (SI Fig. 17).

3.2. *In silico* analysis

The availability of the crystal structure of *h*CB1R in its inactive state, bound to four-arm antagonists, allowed us to simulate the newly synthesized compounds and analyze their critical interactions with residues in the receptor [43]. To gain a deeper understanding of their binding modes and provide a rationale for their affinities for CB1R, we created models of the complexes and characterized ligand binding through MD simulations.

The main differences in the structural scaffold between rimonabant and zevaquenabant is that they have a rigid five-membered central heterocyclic ring, whereas the six-membered ring of PB19AE2 is marginally less rigid, and the additional carbon forces Arm 3 to adopt a slightly different relative orientation. By contrast, taranabant lacks a central core, conferring the most flexibility. To understand how these differences affect the binding modes of PB19AE2 and how its interactions with CB1R modulate its affinity and efficacy, we modeled the

complex using the structure of CB1R bound to zevaquenabant. Geometry optimization (see Methods and SI Table S1) shows that the conformation of the six-membered central ring can undergo inversion, with the more favorable conformation corresponding to Arm 2 in an axial position (labeled pc_2). However, docking of the pc_2 conformer is unfavorable due to clashes between Arm 2 and TMH1 and the C(5)H2 group with TMH7, leading early in the dynamics to a pseudo-chair inversion with Arm 2 in the equatorial position (labeled pc_1). Using the same criteria previously used to predict the binding mode of MRI-1891 (see Methods), we identify the most likely binding mode of PB19AE2, as shown in Fig. 4. The general orientation of the arms is similar to those seen in taranabant and zevaquenabant, but with Arm 3 pointing slightly towards the extracellular side of the receptor, keeping the trifluoromethyl group away from S123 in TMH1, an interaction preserved in other two compounds. It is worth mentioning that the binding mode of the reverse pc_1 conformer (labeled $pc_1(r)$; see Methods and SI Figs. S12–S14) leads to more favorable interactions between water and the hydrophilic moieties of Arms 3 and 4 than pc_1 , although the polar and nonpolar interactions with the receptors are slightly less favorable. Rehydration by water accessing from the extracellular side is about 60 % higher for the reverse conformer, which may be enough to make it the preferred binding mode if Arms 3 and 4 can switch positions inside the pocket by flipping the central dihedral angle ψ (SI Fig. S12); this switch, however, was not observed in any of the simulations. This observation may be relevant for designing compounds with higher affinity and similar peripheral restriction.

3.3. *In vitro* and *in vivo* ADME studies

The racemic compounds PB19A, and PB95 were subjected to ADME

Table 2

Metabolic stability in mouse and human liver microsomes for PB19A and PB95.

Test compounds	Metabolic stability in mouse liver microsomes		Metabolic stability in human liver microsomes	
	$t_{1/2}$, min	Cl_{int} , $\mu\text{L}/\text{min}/\text{mg}$	$t_{1/2}$, min	Cl_{int} , $\mu\text{L}/\text{min}/\text{mg}$
PB19A	48.8	34	762.9	2
PB95	73.8	23	64.2	26

investigations based on their strong affinity for CB1R, dual-target synergism, and high functional potency in the GTP γ S binding assay. Compound PB19A had a half-life of 48.8 min and a moderate clearance rate ($Cl = 23 \mu\text{L}/\text{min}/\text{mg}$) in mouse liver microsomes. In addition, it showed exceptional stability against human liver microsomes with a half-life of 762.9 min and a clearance limit of 2 mL/min/kg (Table 2). As a result of the compound's exceptional stability, its half-life was substantial. The compounds exhibited a strong nonspecific affinity ($\geq 99\%$) for human and mouse plasma proteins, most likely attributed to their high lipophilicity. In the Caco-2 permeability assay experimental conditions, compounds PB19A and PB95 demonstrate intermediate permeability in the apical-basal (A-B) direction and were not subject to active efflux (Table 3) [46].

To estimate the brain permeability of the active enantiomers of the most effective compounds, we utilized the Madin-Darby canine kidney

(MDCK)-MDR1 (SI Table S2). Under the experimental conditions, all test compounds demonstrate limited permeability in the A-B direction. Active efflux is observed in the PB19AE2. An observed reduction in the efflux ratio when an inhibitor is present suggests that this substance functions as a substrate for P-gp activity. PB95E2 does not undergo active efflux and does not function as a P-gp substrate.

3.4. In vivo pharmacokinetic study and tissue distribution of chiral enantiomers of PB19A and PB95

The compounds PB95E2 and PB19AE2 exhibit structural similarity, differing solely in the alkyl substitution in the amidine portion. The compounds showed good oral exposure at a dose of 10 mg/kg (Fig. 5A). The oral plasma half-life of the enantiomeric pure compounds (PB19AE2, PB95E2) was determined to be 4 h and 8 h respectively at a dosage of 10 mg/kg. PB95E2 showed C_{max} values 3.4 μM whereas C_{max} for PB19AE2 was 9.67 μM .

Previous literature suggests that rimonabant's ability to penetrate the brain and induce neuropsychiatric adverse effects led to its market discontinuation; considering this, we conducted a tissue distribution analysis of enantiomer PB19AE2 and PB95E2 to assess their brain penetration. Chiral enantiomer PB19AE2 and PB95E2 demonstrated brain/plasma ratios of 4.3 % and 5.2 %, respectively (Fig. 5B). This result attests to the design principles that validated peripheral

Table 3

Caco-2 permeability (apical-basal, A-B and basal-apical, B-A permeability data) for PB19A and PB95.

Test compounds	P_{app} (AB), 10^{-6} cm/s				P_{app} (BA), 10^{-6} cm/s				Efflux ratio*
	1	2	Mean	SD	1	2	Mean	SD	
PB19A	2.4	2.5	2.5	0.1	1.9	1.8	1.9	0.1	0.8
PB95	1.9	2.4	2.2	0.4	0.5	0.5	0.5	0.1	0.2

* Efflux ratio is expressed as the quotient of $P_{app}(\text{BA})$ to $P_{app}(\text{AB})$.

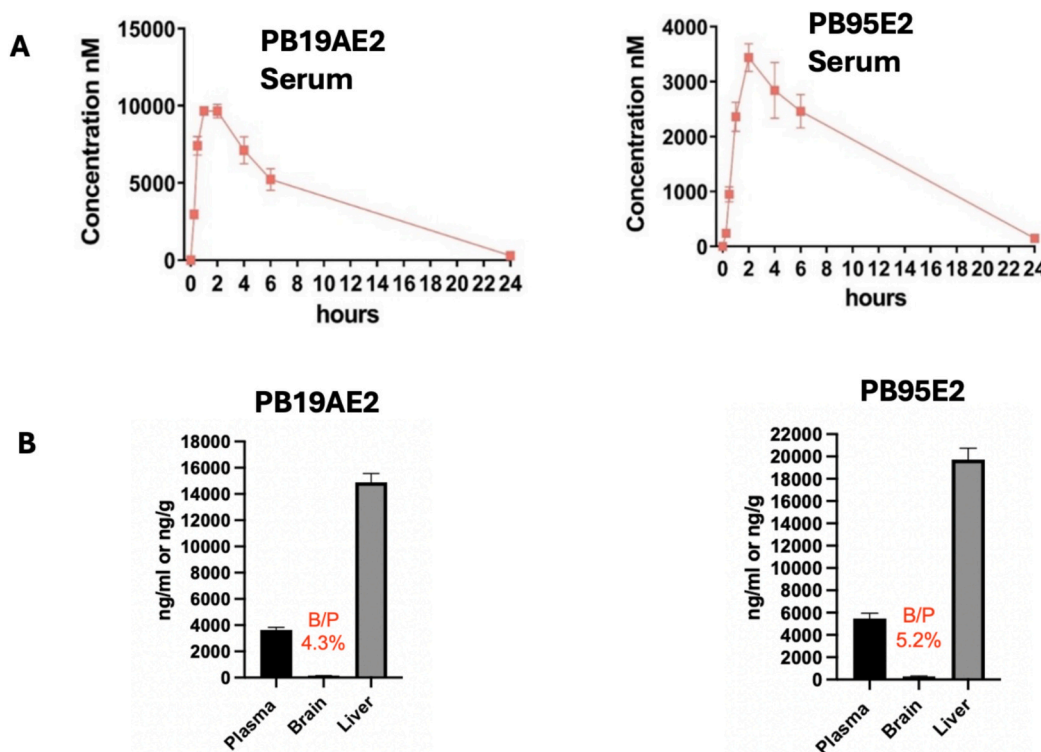


Fig. 5. (A) Oral exposure (10 mg/kg) for compounds PB19AE2 and PB95E2 in C57BL6/J. (B) Tissue distribution showing peripheral restriction of compounds PB19AE2, PB95E2, at T_{max} in C57BL6/J mice at 1 h or 2 h after acute oral administration of 10 mg/kg dose, respectively. (Data represent the mean $\pm$ SEM from 3 mice per group).

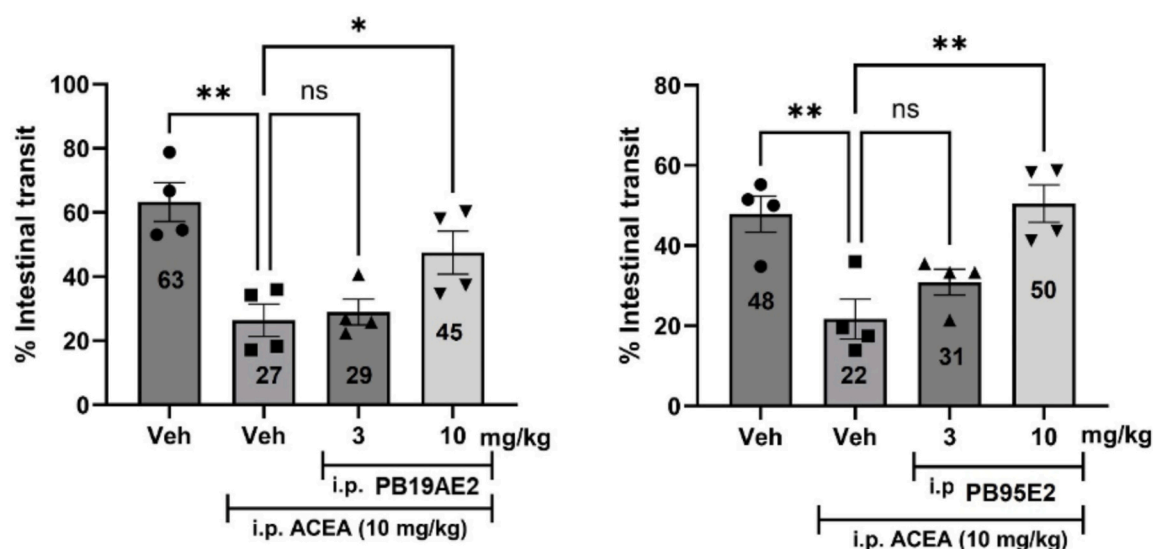


Fig. 6. In vivo peripheral CB1R blockade by compounds PB19AE2 and PB95E2. Both compounds (3–10 mg/kg po) dose-dependently reversed the ACEA (CB1R agonist)-induced reduction in upper GI motility. Data represent the mean $\pm$ SEM from 4 mice per group. Statistical significance of differences between groups was determined by one-way analysis of variance followed by Dunnett's post hoc test. $p < 0.01$; **, $p < 0.05$; *, $p < 0.001$.

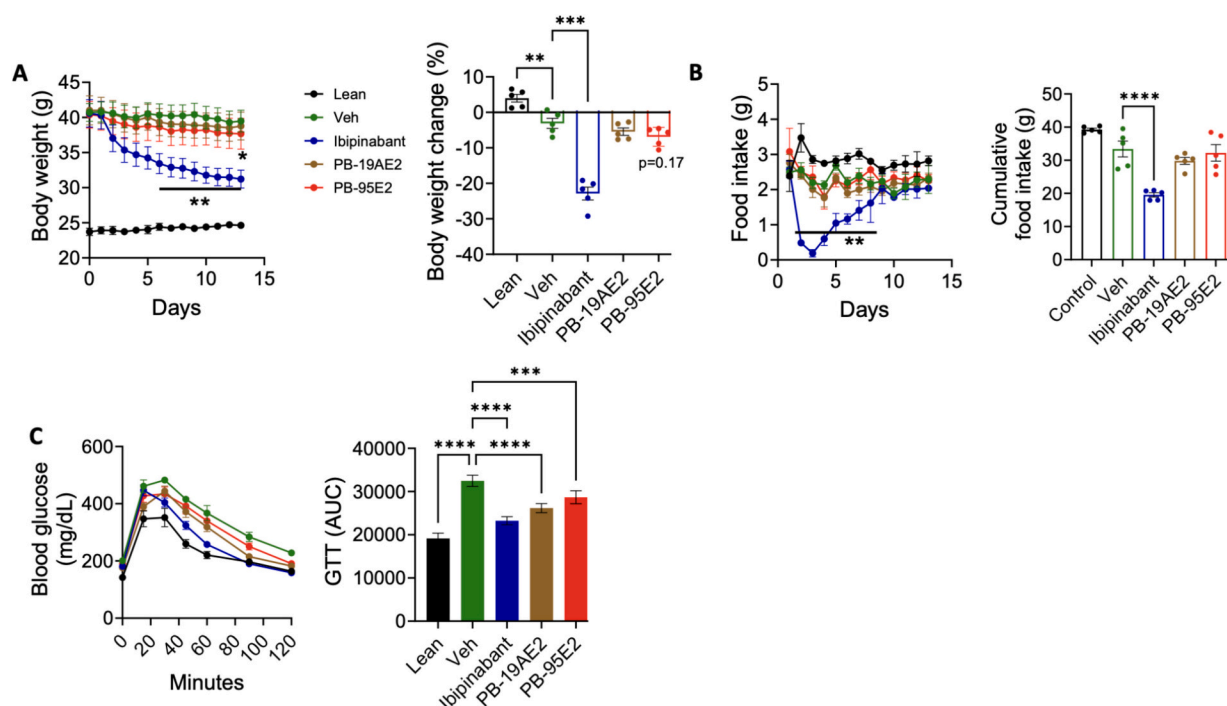


Fig. 7. Metabolic effects of compounds on body weight and food intake regulation. (A) Body weights were measured daily during 14 days with oral dosing of 10 mg/kg of compounds and percentage of body weight change in that period. (B) Daily food intake and 14-day cumulative food intake evaluation (C) Intraperitoneal glucose tolerance test and area under the curve (AUC) analysis. Black circles and bars represent the lean vehicle-treated control mice ($n = 5$), the green circles and bars represent the vehicle-treated DIO mice ($n = 5$), the blue circles and bars represent the Ibipinabant treated DIO mice ($n = 5$), the brown circles and bars represent the PB19AE2-treated DIO mice ($n = 5$). The red circles and bars represent the PB95E2-treated DIO mice ($n = 5$). Data represent mean $\pm$ SEM. Statistical significance versus vehicle-treated DIO mice: ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

restriction. The high functional potency towards CB1R and the lower brain/plasma ratio motivated the evaluation of the in vivo properties using the active enantiomers.

3.5. In vivo upper GI motility studies

Further upper gastrointestinal motility (GI) assay used to evaluate

functional CB1R antagonism of test compounds in vivo. Both compounds PB95E2 and PB19AE2 were able to neutralize the effects of a 10 mg/kg dosage of the CB1R agonist arachidonoyl 2-chloroethylamide on GI motility at oral doses ranging from 3 mg/kg to 10 mg/kg (Fig. 6) which demonstrated in vivo CB1R antagonism.

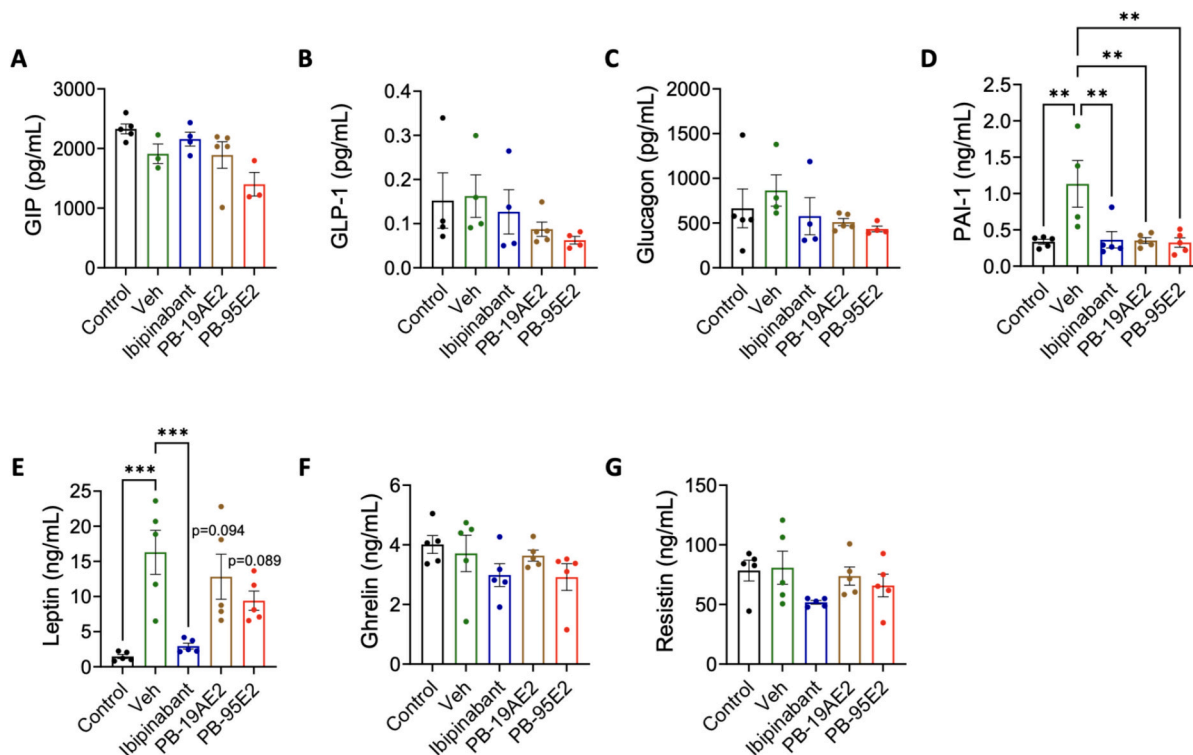


Fig. 8. Effects of compounds on circulating levels of diabetes protein biomarkers. (A) Gastric Inhibitory Polypeptide (GIP). (B) Glucagon-Like Peptide-1 (GLP-1). (C) Glucagon. (D) Plasminogen Activator Inhibitor-1 (PAI-1). (E) Leptin. (F) Ghrelin. (G) Resistin. Black dots represent the lean vehicle-treated control mice ($n = 5$), the green dots represent the vehicle-treated DIO mice ($n = 5$), the blue dots represent the ibipinabant treated DIO mice ($n = 5$), the brown dots represent the PB19AE2-treated DIO mice ($n = 5$). The red dots represent the PB95E2-treated DIO mice ($n = 5$). Data represent mean $\pm$ SEM. Statistical significance versus vehicle-treated DIO mice: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.6. Effects of PB19AE2 and PB95E2 on metabolic parameters in C57BL/6J mice

Subsequently, we conducted in vivo efficacy studies utilizing the active enantiomer PB95E2 and PB19AE2. Further, 14-day treatment regimens of daily oral of PB95E2, and PB19AE2 in DIO mice showed that both the compounds resulted in a modest and non-significant decrease in body weight respectively. This is attributable to its CB1R antagonist MoA via the periphery with 6.88 ± 1.32 and 5.44 ± 1.16 % body weight reduction respectively while vehicle treated mice lost 3.11 ± 1.57 % of their initial body weight (Fig. 7A). Interestingly, PB19AE2 modestly reduced cumulative food intake while PB19AE2 was not statistically significant at the dose tested (Fig. 7B). Additionally, both PB95E2 and PB19AE2 significantly improved glucose tolerance (Fig. 7C).

PB95E2 was able to reduce the circulating levels of both Gastric Inhibitory Polypeptide (GIP) (Fig. 8A) while PB19AE2 was ineffective. Compounds had no effects on GLP-1 and glucagon serum concentration (Fig. 8C-D). Interestingly, both PB95E2 and PB19AE2 were able to normalize the plasminogen activator inhibitor-1 (PAI-1) circulating levels (Fig. 8D). Given that elevated PAI-1 level is a core feature of insulin resistance, this effect is of particular importance [47]. Given the well-known reversal of hyperleptinemia usually observed with this class of compounds, it is important to note that both compounds tends to indeed reduce this parameter (Fig. 8E). We did not observe any effect of the compounds on ghrelin, and resistin levels (Fig. 8F-G).

Since CB1R antagonism is known to impact hepatic metabolism, we investigated potential regulation of hepatic *Cnr1* expression (gene coding for CB1R). Interestingly, PB95E2 was able to reverse the obesity-induced increase in *Cnr1* expression in a similar extend as ibipinabant while PB19AE2 did not show marked effects (Fig. 9A). Interestingly, while none of the compounds were able to reduce the

hypertriglyceridemia (Fig. 9B) and hypercholesterolemia (Fig. 9C), we observed a marked increase in HDL circulating levels after PB95E2 treatment (Fig. 9D). This was associated with a marked decrease in LDL concentration (Fig. 9E) and resulted in a strong increase in the HDL/LDL cholesterol ratio (Fig. 9F) which suggest a positive effect of dyslipidemia. This is further reinforced by the observed reduction in Alanine Aminotransferase (ALT) circulating levels which suggests an hepatoprotective effect of this compound (Fig. 9G). Aspartate Aminotransferase (AST), another transaminase enzyme is usually evaluated in a similar context, and we did not find any difference between the various treatments (Fig. 9H). An interesting observation with the compounds on HDL/LDL ratio and the decrease in LDL is that we did observe the same earlier indicating potential involvement of iNOS [48].

4. Conclusion

To exploit the therapeutic potential of CB1R receptor antagonism in addressing metabolic syndrome diseases, peripherally restricted molecules that minimize the neuropsychiatric side effects is currently a viable approach. In pursuit of this objective, we present herein our efforts to evaluate novel pyridazine-based four-arm antagonists featuring an amidine pendant. We hypothesized that the newly synthesized compounds would function as antagonists of the CB1R while also putatively inhibiting inducible nitric oxide synthase (iNOS). The design of these compounds incorporates features that limit brain penetration, thereby potentially reducing the neuropsychiatric adverse effects associated with central CB1R engagement. The amidine-fused molecules described in this study demonstrated nanomolar binding affinity and potent CB1R functional antagonism both in vitro and in vivo. Tissue distribution studies exhibited brain penetration levels of 4–5 %. Furthermore, the enantiomers of the synthesized compounds displayed stereoselective

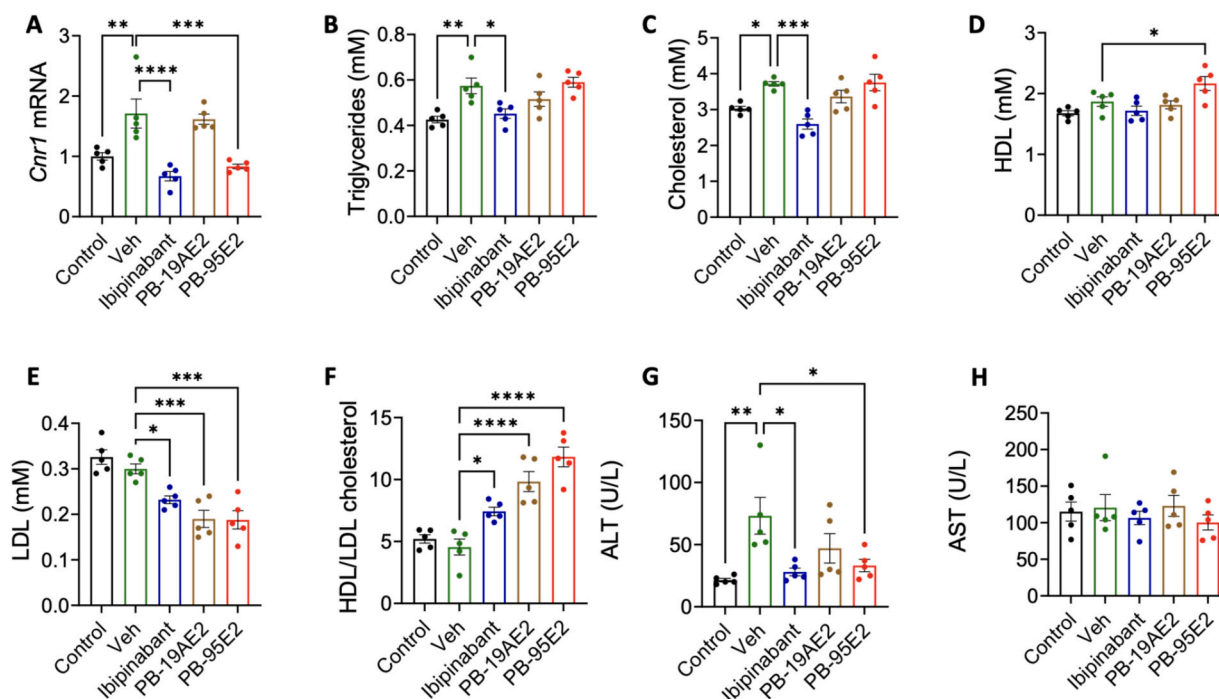


Fig. 9. Effects of compounds on (A) hepatic *Cnr1* mRNA expression. (B) fasted circulating triglycerides levels. (C) fasted circulating total cholesterol levels. (D) fasted circulating HDL levels. (E) fasted circulating LDL levels. (F) Serum LDL/HDL cholesterol ratio. (G) fasted circulating ALT levels. (H) fasted circulating AST levels. Black dots represent the lean vehicle-treated control mice ($n = 5$), the green dots represent the vehicle-treated DIO mice ($n = 5$), the blue dots represent the ibipinabant treated DIO mice ($n = 5$), the brown dots represent the PB19AE2-treated DIO mice ($n = 5$). The red dots represent the PB95E2-treated DIO mice ($n = 5$). Data represent mean $\pm$ SEM. Statistical significance versus vehicle-treated DIO mice: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

affinity and efficacy towards the CB1R. Daily oral administration (10 mg/kg) of the active enantiomers PB19AE2, PB95E2, yielded modest effects on body weight reduction and glucose tolerance and varied CB1R mediated effects on hepatic parameters. Despite their moderate in vivo half-lives, the enantiomeric compounds, including PB19AE2, PB95E2, exhibited good metabolic stability. These findings have now culminated in this present work to identify a new class of orally available, dual-target molecules with interesting compound specific pharmacological properties and promising activity against MetS. However, this study has limitations in that the role of iNOS engagement in vivo in the presented molecules needs to be studied in detail. Furthermore, an important factor that is also worth considering from the translation perspective is the low CB1/CB2 receptors selectivity seen in binding assays. Although the role of CB2R in feeding behavior and body weight is inconclusive [49], the in vivo effects seen with the compounds presented here cannot be unequivocally attributed to selectivity index. Barring significant off-targets effects that could counteract, though which were not readily apparent, the study points to the possibility for testing a potentially higher-dose (<10 mg/kg) or twice daily formulation for the molecules in the present class. Interestingly, a two-day study of racemic PB19A (60 mg/kg, i.p.) indicated that the compound showed CB1 antagonism-mediated in vivo efficacy in food intake and body weight reduction (SI Fig. S18). Optimistically, the pharmacological profile, combined with negligible brain penetrance, positions these compounds as solid hits to harness the therapeutic potential of peripheral CB1R antagonists. These novel pyridazine-based compounds represent a preliminary evaluation of two compounds PB19AE2 and PB95E2 as therapeutics in the pursuit of treatment for metabolic diseases.

5. Summary

Our present work identifies a new class of orally available, dual-target pyridazine-based, four-arm antagonists containing an amidine

pendant. The compounds have attractive pharmacological properties and promising activity against MetS. The strength of this work lies in a detailed study of two compounds which could serve as CNS-sparing CB1 antagonists. Certain caveats remain in that the role of iNOS in vivo in the presented molecules needs to be delved in detail. The study also points to the possibility for testing a potentially higher-dose or twice daily formulation for the molecules PB19AE2 and PB95E2. From the translation perspective, the moderate CB1/CB2 selectivity ratio could point to a need for further optimization. Nonetheless, the pharmacological profile revealed that these compounds are solid starting points that could further help exploit the promised therapeutic potential of peripheral CB1R antagonists against metabolic disorders.

CRedit authorship contribution statement

Pinaki Bhattacharjee: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Szabolcs Dvorácskó:** Writing – review & editing, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Océane Pointeau:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Biswajit Kundu:** Writing – review & editing, Methodology. **Nicholas Rutland:** Writing – review & editing, Methodology. **Henry Puhl:** Writing – review & editing, Methodology. **Jie Liu:** Writing – review & editing, Methodology. **Grzegorz Godlewski:** Writing – review & editing, Methodology, Data curation. **Sergio A. Hassan:** Writing – review & editing, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation. **Tony Jourdan:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation. **Resat Cinar:** Writing – review & editing, Supervision, Funding acquisition, Formal analysis, Data curation. **Malliga R. Iyer:** Writing – review & editing, Writing – original draft, Supervision, Resources, Investigation,

Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

P.B, S.D, R.C and M.R.I are listed as co-inventors on patent application filed based on the compounds in this publication.

Acknowledgment

This work was supported by intramural funds from the National Institute on Alcohol Abuse and Alcoholism (NIAAA) to M.R.I (ZIA AA000360) and R.C (ZIA AA000355). For help with HRMS data John Lloyd is acknowledged. We acknowledge Logan Johnson* for his help with PK studies. Dr. Walter Teague is acknowledged for help with NMR experiments. We thank Judy Harvey-White for technical assistance with mass spectrometry experiments. This study utilized the high-performance computational capabilities of the Biowulf HPC cluster at the NIH. SD is currently supported by the grant PD-139012 of National Research, Development and Innovation Office and the János Bolyai Research Scholarship of the Hungarian Academy of Sciences. TJ is supported by INSERM and Université Bourgogne intramural funds, the Société Francophone du Diabète, by a French government grant managed by the French National Research Agency under the program Investissements d'Avenir with the reference ANR-11-LABX-0021-01-LipSTIC LabEx and by the French National Research Agency (ANR-22-CE14-0055-01).

*Logan Johnson is no longer with us.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.metabol.2025.156291>.

References

- [1] Lu H-C, Mackie K. An introduction to the endogenous cannabinoid system. *Biol Psychiatry* 2016;79:516–25.
- [2] de Rodríguez Fonseca F, Del Arco I, Bermudez-Silva FJ, Bilbao A, Cippitelli A, Navarro M. The endocannabinoid system: physiology and pharmacology. *Alcohol Alcohol* 2005;40:2–14.
- [3] Freitas HR, Isaac AR, Malcher-Lopes R, Diaz BL, Trevenzoli IH, De Melo Reis RA. Polyunsaturated fatty acids and endocannabinoids in health and disease. *Nutr Neurosci* 2018;21:695–714.
- [4] Pacher P, Bátkai S, Kunos G. The endocannabinoid system as an emerging target of pharmacotherapy. *Pharmacol Rev* 2006;58:389–462.
- [5] Pacher P, Kunos G. Modulating the endocannabinoid system in human health and disease: successes and failures. *FEBS J* 2013;280:1918–43.
- [6] Vandevoorde S. Overview of the chemical families of fatty acid amide hydrolase and monoacylglycerol lipase inhibitors. *Curr Top Med Chem* 2008;8:247–67.
- [7] Pertwee RG. Cannabinoid receptor ligands: clinical and neuropharmacological considerations, relevant to future drug discovery and development. *Expert Opin Investig Drugs* 2000;9:1553–71.
- [8] Howlett AC, Barth F, Bonner TI, Cabral G, Casellas P, Devane WA, et al. XXVII. Classification of cannabinoid receptors. *Pharmacol Rev* 2002;54:163–4.
- [9] Howlett AC, Abood ME. CB1 and CB2 receptor pharmacology. *Adv Pharmacol* 2017;80:169–206.
- [10] Cabral GA, Raborn ES, Griffin L, Dennis J, Marciano-Cabral F. CB2 receptors in the brain: role in central immune function. *Br J Pharmacol* 2008;153:240–51.
- [11] Chen D-J, Gao M, Gao F-F, Su Q-X, Wu J. Brain cannabinoid receptor 2: expression, function and modulation. *Acta Pharmacol Sin* 2017;38:312–6.
- [12] Pagotto U, Marsicano G, Cota D, Lutz B, Pasquali R. The emerging role of the endocannabinoid system in endocrine regulation and energy balance. *Endocr Rev* 2006;27:73–100.
- [13] Piliatsi E, Farr OM, Polyzos SA, Perakakis N, Nolen-Doerr E, Papanthanasios A-E, et al. Pharmacotherapy of obesity: available medications and drugs under investigation. *Metab Clin Exp* 2019;92:170–92.
- [14] Després J-P, Golay A, Sjöström L, Rimonabant in Obesity-Lipids Study Group. Effects of rimonabant on metabolic risk factors in overweight patients with dyslipidemia. *N Engl J Med* 2005;353(20):2121–34. <https://doi.org/10.1056/NEJMoa044537>.
- [15] Patel PN, Pathak R. Rimonabant: a novel selective cannabinoid-1 receptor antagonist for treatment of obesity. *Am J Health Syst Pharm* 2007;64:481–9.
- [16] Ward SJ, Raffa RB. Rimonabant redux and strategies to improve the future outlook of CB1 receptor neutral antagonist/inverse-agonist therapies. *Obesity (Silver Spring)* 2011;19:1325–34.
- [17] Sharma MK, Murumkar PR, Kanhed AM, Giridhar R, Yadav MR. Prospective therapeutic agents for obesity: molecular modification approaches of centrally and peripherally acting selective cannabinoid 1 receptor antagonists. *Eur J Med Chem* 2014;79:298–339.
- [18] Han JH, Kim W. Peripheral CB1R as a modulator of metabolic inflammation. *FASEB J* 2021;35(4):e21232. <https://doi.org/10.1096/fj.202001960R>.
- [19] Nguyen T, Thomas BF, Zhang Y. Overcoming the psychiatric side effects of the cannabinoid CB1 receptor antagonists: current approaches for therapeutics development. *Curr Top Med Chem* 2019;19:1418–35.
- [20] Lange JHM, Coolen HKAC, van Stuivenberg HH, Dijkman JAR, Herremans AHJ, Ronken E, et al. Synthesis, biological properties, and molecular modeling investigations of novel 3,4-diarylpyrazolines as potent and selective CB(1) cannabinoid receptor antagonists. *J Med Chem* 2004;47:627–43.
- [21] Lange JHM, van Stuivenberg HH, Veerman W, Wals HC, Stork B, Coolen HKAC, et al. Novel 3,4-diarylpyrazolines as potent cannabinoid CB1 receptor antagonists with lower lipophilicity. *Bioorg Med Chem Lett* 2005;15:4794–8.
- [22] Lange JHM, den Hartog AP, van der Neut MAW, van Vliet BJ, Kruse CG. Synthesis and SAR of 1,4,5,6-tetrahydropyridazines as potent cannabinoid CB1 receptor antagonists. *Bioorg Med Chem Lett* 2009;19:5675–8.
- [23] Janero DR, Makriyannis A. Cannabinoid receptor antagonists: pharmacological opportunities, clinical experience, and translational prognosis. *Expert Opin Emerg Drugs* 2009;14:43–65.
- [24] Cinar R, Iyer MR, Kunos G. The therapeutic potential of second and third generation CB1R antagonists. *Pharmacol Ther* 2020;208:107477.
- [25] Klumpers LE, Fridberg M, de Kam ML, Little PB, Jensen NO, Kleinloog HD, et al. Peripheral selectivity of the novel cannabinoid receptor antagonist TM38837 in healthy subjects. *Br J Clin Pharmacol* 2013;76:846–57.
- [26] Chorvat RJ. Peripherally restricted CB1 receptor blockers. *Bioorg Med Chem Lett* 2013;23:4751–60.
- [27] Liu Z, Iyer MR, Godlewski G, Jourdan T, Liu J, Coffey NJ, et al. Functional selectivity of a biased cannabinoid-1 receptor (CB1R) antagonist. *ACS Pharmacol Transl Sci* 2021;4:1175–87.
- [28] Cinar R, Iyer MR, Liu Z, Cao Z, Jourdan T, Erdelyi K, et al. Hybrid inhibitor of peripheral cannabinoid-1 receptors and inducible nitric oxide synthase mitigates liver fibrosis. *JCI Insight* 2016:1.
- [29] Iyer MR, Cinar R, Katz A, Gao M, Erdelyi K, Jourdan T, et al. Design, synthesis, and biological evaluation of novel, non-brain-penetrant, hybrid cannabinoid CB1R inverse agonist/inducible nitric oxide synthase (iNOS) inhibitors for the treatment of liver fibrosis. *J Med Chem* 2017;60:1126–41.
- [30] Kato Y, Miura Y, Yamamoto N, Ozaki N, Oiso Y. Suppressive effects of a selective inducible nitric oxide synthase (iNOS) inhibitor on pancreatic beta-cell dysfunction. *Diabetologia* 2003;46:1228–33.
- [31] Zheng L, Du Y, Miller C, Gubitosi-Klug RA, Kern TS, Ball S, et al. Critical role of inducible nitric oxide synthase in degeneration of retinal capillaries in mice with streptozotocin-induced diabetes. *Diabetologia* 2007;50:1987–96.
- [32] Heemskerk S, Masereeuw R, Russel FGM, Pickkers P. Selective iNOS inhibition for the treatment of sepsis-induced acute kidney injury. *Nat Rev Nephrol* 2009;5:629–40.
- [33] Aktan F. iNOS-mediated nitric oxide production and its regulation. *Life Sci* 2004;75:639–53.
- [34] Bogdan C. The multiplex function of nitric oxide in (auto)immunity. *J Exp Med* 1998;187:1361–5.
- [35] Perreault M, Marette A. Targeted disruption of inducible nitric oxide synthase protects against obesity-linked insulin resistance in muscle. *Nat Med* 2001;7:1138–43.
- [36] Cinar R, Park JK, Zawatsky CN, Coffey NJ, Bodine SP, Abdalla J, et al. CB1 R and iNOS are distinct players promoting pulmonary fibrosis in Hermansky-Pudlak syndrome. *Clin Transl Med* 2021;11:e471.
- [37] Udi S, Hinden L, Ahmad M, Drori A, Iyer MR, Cinar R, et al. Dual inhibition of cannabinoid CB1 receptor and inducible NOS attenuates obesity-induced chronic kidney disease. *Br J Pharmacol* 2020;177:110–27.
- [38] Zawatsky CN, Park JK, Abdalla J, Kunos G, Iyer MR, Cinar R. Peripheral hybrid CB1R and iNOS antagonist MRI-1867 displays anti-fibrotic efficacy in bleomycin-induced skin fibrosis. *Front Endocrinol (Lausanne)* 2021;12:744857.
- [39] Iyer MR, Cinar R, Liu J, Godlewski G, Szanda K, Puhl H, et al. Structural basis of species-dependent differential affinity of 6-Alkoxy-5-Aryl-3-pyridinecarboxamide cannabinoid-1 receptor antagonists. *Mol Pharmacol* 2015;88:238–44.
- [40] Shivshankar S, Nimely J, Puhl H, Iyer MR. Pharmacological evaluation of cannabinoid receptor modulators using Grabc2.0 sensor. *Int J Mol Sci* 2024;25(9):5012. <https://doi.org/10.3390/ijms25095012>.
- [41] Iyer MR, Cinar R, Wood CM, Zawatsky CN, Coffey NJ, Kim KA, et al. Synthesis, biological evaluation, and molecular modeling studies of 3,4-diarylpyrazoline series of compounds as potent, nonbrain penetrant antagonists of cannabinoid-1 (CB1R) receptor with reduced lipophilicity. *J Med Chem* 2022;65:2374–87.
- [42] Dvoráček S, Herrerías A, Oliverio A, Bhattacharjee P, Pommerolle L, Liu Z, et al. Cannabinoformins: designing biguanide-embedded, orally available, peripherally selective cannabinoid-1 receptor antagonists for metabolic syndrome disorders. *J Med Chem* 2023;66:11985–2004.
- [43] Kumari P, Dvoráček S, Enos MD, Ramesh K, Lim D, Hassan SA, et al. Structural mechanism of CB1R binding to peripheral and biased inverse agonists. *Nat Commun* 2024;15:10694.
- [44] Iyer MR, Cinar R, Coffey NJ, Kunos G. Synthesis of ¹³C₆-labeled, dual-target inhibitor of cannabinoid-1 receptor (CB1R) and inducible nitric oxide synthase (iNOS). *J Labelled Comp Radiopharm* 2018;61(10):773–9. <https://doi.org/10.1002/jlcr.3639>.

- [45] Cinar R, Godlewski G, Liu J, Tam J, Jourdan T, Mukhopadhyay B, et al. Hepatic cannabinoid-1 receptors mediate diet-induced insulin resistance by increasing de novo synthesis of long-chain ceramides. *Hepatology* 2014;59:143–53.
- [46] Trapa PE, Troutman MD, Lau TY, Wager TT, Maurer TS, Patel NC, et al. In vitro-in vivo extrapolation of key transporter activity at the blood-brain barrier. *Drug Metab Dispos* 2019;47:405–11.
- [47] Altalhi R, Pechlivani N, Ajjan RA. PAI-1 in diabetes: pathophysiology and role as a therapeutic target. *Int J Mol Sci* 2021;22.
- [48] Roger C, Buch C, Muller T, Leemput J, Demizieux L, Passilly-Degrace P, et al. Simultaneous inhibition of peripheral CB1R and iNOS mitigates obesity-related dyslipidemia through distinct mechanisms. *Diabetes* 2020;69:2120–32.
- [49] Rodríguez-Serrano LM, Chávez-Hernández ME. Role of the CB2 cannabinoid receptor in the regulation of food intake: a systematic review. *Int J Mol Sci* 2023; 24.