Title Page

Discovery and pharmacological characterization of succinate receptor (SUCNR1/GPR91) agonists

Running title: *cis*-Cyclic dicarboxylic acids as SUCNR1 agonists

Pierre Geubelle[1,2], Julie Gilissen[1, 2], Sébastien Dilly[2,3], Laurence Poma[4], Nadine Dupuis[1], Céline Laschet[1], Dayana Abboud[1], Asuka Inoue[5,6], François Jouret[4], Bernard Pirotte[2] and Julien Hanson[1,2*]

[1] Laboratory of Molecular Pharmacology, GIGA-Molecular Biology of Diseases, University of Liège, Avenue de l'Hôpital, 11, Liège, 4000, Belgium

[2] Laboratory of Medicinal Chemistry, Centre for Interdisciplinary Research on Medicines (CIRM), University of Liège, 15, Avenue Hippocrate, 4000 Liège, Belgium.

[3] Laboratory of Molecular Modelling for (Bio)molecule engineering, Institute of Chemistry and Biology of Membranes and Nano-objects, University of Bordeaux, Allée Geoffroy Saint Hilaire, 33600 Pessac, France

[4] Laboratory of Experimental Surgery, GIGA-Cardiovascular Sciences, University of Liège, Liège, Belgium

[5] Graduate School of Pharmaceutical Science, Tohoku University, Sendai, Miyagi, Japan

[6] Japan Science and Technology Agency (JST), Precursory Research for Embryonic Science and Technology (PRESTO), 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan

*** Correspondence:** Dr. Julien Hanson, Laboratory of Molecular Pharmacology, GIGA-Molecular Biology of Diseases, University of Liège, Quartier Hôpital, Avenue de l'Hôpital, 11, Liège, 4000, Belgium. j.hanson@ulg.ac.be

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Abstract

Background and Purpose

The succinate receptor (SUCNR1 or GPR91) has been described as a metabolic sensor that may be involved in homeostasis. Notwithstanding its implication in important (patho)physiological processes, the function of SUCNR1 has remained elusive because no pharmacological tools were available. We report on the discovery of the first family of synthetic potent agonists.

Experimental Approach

We screened a library of succinate analogues and analysed their activity on SUCNR1. In addition, we modelled a pharmacophore and a binding site for the receptor. New agonists were identified based on the information provided by these two approaches. Their activity was studied in various bioassays, including measurement of cAMP levels, $[Ca^{2+}]_i$ mobilisation, TGF-α shedding and recruitment of arrestin 3. The *in vivo* impact of SUCNR1 activation by these new agonists was evaluated on rat blood pressure.

Key Results

We identified *cis*-epoxysuccinic acid and *cis*-1,2-cyclopropanedicarboxylic acid as agonists with an efficacy similar to the one of succinic acid. Interestingly, *cis*-epoxysuccinic acid was characterized by a 10 to 20 fold higher potency than succinate on the receptor. For example, *cis*-epoxysuccinic acid reduced cAMP levels with a $pEC_{50} = 5.57 \pm 0.02$ (EC_{50} = 2.7 µM) as compared to succinate $pEC_{50} = 4.54 \pm 0.08$ (EC_{50} = 29 µM). The rank order of potency of the three agonists was the same in all bioassays tested *cis*-epoxysuccinic and cis-1,2-cyclopropanedicarboxylic acid increased rat blood pressure to the same extent as succinate did.

Conclusions and Implications

We provide new agonist tools for SUCNR1 that should facilitate further research on this understudied receptor.

Table of Links

TARGETS
GPCRs[b]
Succinate Receptor

LIGANDS
Succinic Acid

These Tables of Links list key protein targets and ligands in this article that are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan et al., 2016), and are permanently archived in The Concise Guide to PHARMACOLOGY 2015/16 ([a,b,c,d,e]Alexander et al., 2015a,b,c,d,e).

Abbreviations

AH: Acceptor Hydrogen Bound
BrSA: BromoSuccinic Acid
cCBDA: *cis*-CycloButane-1,2-Dicarboxylic acid
cCPDA: *cis*-CycloPropane-1,2-Dicarboxylic Acid
cESA: *cis*-EpoxySuccinic Acid
ClSA: ChloroSuccinic Acid
EMA: EthylMalonic Acid
GRK: G protein-coupled receptor kinase
MA: Malonic Acid
mDMSA: meso-DiMethylSuccinic Acid
MMA: MethylMalonic Acid
MOSA: MethoxySuccinic Acid
MSA: MethylSuccinic Acid
N: Negative Charge
OAA: Oxalocetic Acid
p-NPP: para-NitroPhenylPhosphate
PTX: Pertussis Toxin
SA: Succinic Acid
SAR: Structure-Activity Relationship
SDH: Succinate dehydrogenase
SUCNR1: Succinate Receptor-1
TACE: Transcatheter arterial chemoembolization
tCBDA: *trans*-CycloButane-1,2-Dicarboxylic acid
tCPDC: *trans*-CycloPropane-1,2-Dicarboxylic acid
TGF-α: Transforming Growth Factor-Alpha
WT: Wild Type

Introduction

G Protein-coupled receptors or GPCR constitute the most broadly targeted proteins by drugs in human medicine (Overington, Al-Lazikani & Hopkins, 2006). These receptors are characterized by seven transmembrane domains and are implicated in nearly all physiological processes. GPCRs signal through various intracellular partners that affect cell function. Four main families of G proteins have been described, including G_i, G_s, $G_{q/11}$ and $G_{12/13}$ (Wettschureck & Offermanns, 2005). G_i and G_s subunits are respectively able to negatively and positively regulate the activity of AC and thus decrease and increase the cAMP levels in cells. Following activation, the receptor is usually desensitized by phosphorylation of intracellular sites by specific GPCR kinases called GRK (Lefkowitz & Shenoy, 2005). Particular scaffold proteins named arrestins (for non-visual GPCR, β-arrestin 1 and 2 or arrestins 2 and 3) strengthen the desensitization and generally induce receptor internalization (Lefkowitz & Shenoy, 2005). It was recently proposed that arrestins adopt receptor-dependent conformation and activate specific signalling pathways in a manner similar to G proteins (Lee et al., 2016; Nuber et al., 2016).

Succinate Receptor (SUCNR1 or GPR91) is a member of the rhodopsin-like GPCR family and was initially identified as an orphan receptor (Wittenberger, Schaller & Hellebrand, 2001). In a landmark study, He *et al.* paired it with its natural ligand succinate (SA) (He et al., 2004). SUCNR1 displays some homology with the purinergic receptor family, although it does not bind nucleotide ligands (He et al., 2004). Few studies have addressed SUCNR1 signalling pathways. It is coupled to G_i and its activation negatively modulates cAMP levels (Gilissen, Geubelle, Dupuis, Laschet, Pirotte & Hanson, 2015; He et al., 2004). In addition, SUCNR1 activation promotes transient $[Ca^{2+}]_i$ mobilization (Gilissen, Geubelle, Dupuis, Laschet, Pirotte & Hanson, 2015; He et al., 2004). Although it has been suggested that G_q was mediating this effect (He et al., 2004; Robben et al., 2009), more recent investigations in native and heterologous systems could not detect G_q coupling and proposed the Gβγ dimer as the protein responsible for PLC-β activation (Gilissen, Geubelle, Dupuis, Laschet, Pirotte & Hanson, 2015; Hakak et al., 2009; Hogberg et al., 2011; Sundström, Greasley, Engberg, Wallander & Ryberg, 2013). Notwithstanding, there is also the possibility that coupling to G_q is tissue-dependent. Upon activation, SUCNR1 induces PTX sensitive ERK phosphorylation and is rapidly desensitized and/or internalized (Gilissen, Geubelle, Dupuis, Laschet, Pirotte & Hanson, 2015; Hakak et al., 2009; He et al., 2004; Robben et al., 2009). It is currently not

clear if arrestins or phosphorylation of the receptor take an active part in the process of desensitization/internalization.

SA is an intermediate of the citric acid (or Krebs) cycle that takes place in the mitochondria. In case of oxygen deprivation, SA may accumulate and be released in the extracellular space. SA and its receptor have been linked to several (patho)physiological processes such as hypertension (He et al., 2004; Toma et al., 2008), diabetes and obesity (McCreath et al., 2015; Sadagopan et al., 2007; Toma et al., 2008), activation of the immune system (Rubic et al., 2008), platelet aggregation (Hogberg et al., 2011; Spath, Hansen, Bokemeyer & Langer, 2012) and retinal angiogenesis (Sapieha et al., 2008).

Very few active ligands for SUCNR1 have been described. Maleate is a confirmed agonist with lower potency (Gilissen, Geubelle, Dupuis, Laschet, Pirotte & Hanson, 2015; He et al., 2004). In 2011, Bhuniya et al. published the discovery of potent antagonists from a high-throughput screening campaign (Bhuniya et al., 2011). There is currently no full pharmacological characterization of the compounds, although radiotracers based on these scaffolds have been described (Klenc, Lipowska & Taylor, 2015). The paucity of pharmacological tools for the receptor restricts current research and precludes a more thorough understanding of SUCNR1 function.

The present study reports on the discovery and characterization of the first potent and highly efficacious SUCNR1 agonists, namely *cis*-epoxysuccinic Acid (*c*ESA) and *cis*-1, 2-cyclopropanedicarboxylic acid (*c*CPDA). In addition, the identification of several other SUCNR1 ligands led to the definition of a precise pharmacophore for agonistic activity on SUCNR1. We refined the SA binding pocket by implementing a homology model and validated it by site-directed mutagenesis. *c*ESA showed higher potency compared to SA in SUCNR1-mediated $[Ca^{2+}]_i$ mobilization, arrestin binding, TGF-α shedding and depletion of basal cAMP levels. In addition, *c*ESA and *c*CPDA have no activity on the mitochondrial SDH and can be utilized to specifically assess the impact of SUCNR1 activation without interfering with citric acid cycle. Furthermore, *c*ESA and *c*CPDA demonstrated activity *in vivo* on rat blood pressure. Both agonists are commercially available, which may open new possibilities for the characterization of SUCNR1 and its validation as a drug target.

Methods

Materials

The drug/molecular target nomenclature conforms to the BJP's Concise Guide to Pharmacology (Alexander et al., 2015). All chemicals used were from Sigma-Aldrich (St Louis, Missouri, USA) unless otherwise stated. *cis*-epoxysuccinic acid, Citraconic acid, acetylenedicarboxylic acid, (R)-methylsuccinic acid, (S)-methylsuccinic acid, (S)-malic acid, (R)-malic acid, (S)-aspartic acid and (R)-aspartic acid were from Tokyo Chemical Industry (Tokyo, Japan). (S)-Bromosuccinic acid and adipic acid were from Santa Cruz Biotechnology (Dallas, Texas, USA). *cis*-1,2-Cyclopropanedicarbocylic acid was from Diverchim (Roissy, France). *trans*-1,2-Cyclopropanedicarbocylic was from Enamine (Kiev, Ukraine). *trans*-1,2-Cyclobutanedicarbocylic was from abcr (Karlsruhe, Germany). Antibiotics for cell culture were from InvivoGen (San Diego, California, USA). Except for (S)-bromosuccinic acid (Sigma-Aldrich), Halogeno succinic acids have been synthesized by us according to published procedures (see Supplementary Fig. 1, supplementary methods and (Zurwerra et al., 2012)). The pGloSensor^{TM}-22F cAMP plasmid was obtained from Promega Corporation (Fitchburg, Wisconsin, USA). Stable cell lines expressing WT human SUCNR1 and the GloSensor system have been described previously (Gilissen, Geubelle, Dupuis, Laschet, Pirotte & Hanson, 2015). pcDNA3.1 was from Thermo Fisher Scientific (Waltham, Massachusetts, USA). HEK 293 cells were from ATCC (Manassas, Virginia, USA).

Cell culture

Cells were cultured at 5% CO_2 and 37°C in DMEM adjusted to contain 10% FBS (Biochrom AG, Berlin, Germany), 1% penicillin and streptomycin (Lonza, Verviers, Belgium), 1% L-glutamine (Lonza, Verviers, Belgium).

Site-directed Mutagenesis

Wild-type (WT) human SUCNR1 with a Flag epitope at the N-terminal end has been cloned into pcDNA3.1 vector bearing a neomycine resistance cassette. All mutagenesis was carried out using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs, Massachusetts, USA) according to the manufacturer instructions. Stable cell lines have been established for each clone after selection with G418 (600 $mg.L^{-1}$) and the expression at the membrane has been verified by FACS measurements (See supplementary Fig. 2 and supplementary methods).

GloSensor cAMP Assay

The assay has been conducted with a protocol previously described (Gilissen, Geubelle, Dupuis, Laschet, Pirotte & Hanson, 2015). Briefly, HEK293 cells stably expressing cAMP Glosensor with or without stable expression of human SUCNR1 were starved for 5 h with 1% FBS, detached and incubated 1 h in the dark at RT in assay buffer HBSS (120 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 10 mM HEPES; pH 7.4, 10 mM glucose) containing IBMX (300 µM) and Luciferin (GloSensor reagent, Promega, Madison, Wisconsin, USA). Then, cells were distributed into 96-well plates (150 000 cells per well, white lumitrac®, Greiner Bio-One, Kremsmünster, Austria) containing the tested compound at different concentrations. After 1 min agitation at 1200 rpm and 9 min additional incubation basal luminescence level was recorded by using a microplate luminometer (Fluoroskan Ascent FL equipped with 2 dispensers, ascent software version 2.6, Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Intracellular Calcium mobilization assay

The assay has been conducted according to previous description (Gilissen, Geubelle, Dupuis, Laschet, Pirotte & Hanson, 2015). Briefly, cells from a confluent T175 flask were detached and incubated in assay buffer (HBSS: 120 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO4, 10 mM HEPES; pH 7.4, 10 mM glucose) containing 5 mM coelenterazine h (Regis Technologies, Morton Grove, Illinois, USA) and 1.8 mM $CaCl_2$ for 1 h in the dark at 37°C. Luminescence was followed for 20s (40 measures; 500 ms integration) immediately upon ligand addition. Measurements were acquired with a microplate luminometer (Fluoroskan Ascent FL equipped with 2 dispensers, ascent software version 2.6, Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Arrestin complementation assay

Stable cell lines for the measurement of arrestin 3 recruitment by complementation of Firefly Luciferase have been described previously (Gilissen, Geubelle, Dupuis, Laschet, Pirotte & Hanson, 2015). Cells in suspension in the buffer (HBSS with 20 mM HEPES, pH 7.4, 10 mM glucose) were incubated into 96-well plates (100 000 cells per well) containing the ligands at different concentrations for 10 min at RT. Following injection of 50 µM luciferin (Synchem, Felsberg, Germany), luminescence was recorded for 30 min using a high sensitivity

luminometer (Centro XS³ LB 960, MicroWin 2000 software, equipped with 2 dispensers, Berthold technologies, Bad Wilbad, Germany).

TGF-α shedding assay

The procedure and plasmids have been described previously (Inoue et al., 2012). Briefly, expression vectors (a mixture of 2.5 µg AP-TGFα, 1 µg receptor and 0.5 µg promiscuous Gα protein per 100 mm dish; 24h before the assay) were transfected in HEK293 cells using 12 µl per 100 mm dish of x-tremegene 9 (Sigma-Aldrich, St Louis, Missouri, USA). Transfected cells were pelleted by centrifugation and resuspended in PBS, followed by incubation for 10 min at room temperature. After centrifugation, cells were suspended in Hank's Balanced Salt Solution (HBSS) containing 5 mM HEPES (pH 7.4) and plated in 90 µl per well (40 000 cells per well) in a 96-well plate and placed in a 37 °C incubator with 5% CO_2. Thirty minutes later, 10 µl per well of 10× concentration of compounds were added and incubated for 1 h at 37 °C under 5% CO_2. Plates were centrifuged and conditioned medium (80 µl per well) was transferred into another 96-well plate. Solution containing p-NPP (10 mM p-NPP, 40 mM Tris-HCl (pH 9.5), 40 mM NaCl, 10 mM $MgCl_2$) was added at 80 µl per well into both a conditioned medium and a cell plate. Absorbance at 405 nm of both plates was read before and after a 1 h incubation for 37 °C using a microplate reader (Infinite m200, TECAN, Zurich, Switzerland). We calculated relative percentage of alkaline phosphatase activity in conditioned medium = ΔOD_{405} CM / (ΔOD_{405} CM + ΔOD_{405} Cell), where ΔOD_{405} CM and ΔOD_{405} Cell denote changes in OD_{405} in the conditioned medium and on the cell surface, respectively, before and after a 1h incubation in the presence of p-NPP.

SDH activity

SDH activity was measured with the Succinate Dehydrogenase Activity Colorimetric Assay Kit (BioVision, San Francisco, California, USA) following the manufacturer instructions. HEK293 cells were lysed with SDH Assay Buffer (100µL for 10^6 cells) to isolate mitochondrial SDH. 20µL of lysate and 1µL of disodium salts of the compounds (OAA, *c*ESA and *c*CPDA) dissolved in PBS were added in 96 well plate 5 min before adding the probe. The absorbance was measured using a microplate reader (Infinite m200, TECAN, Zurich, Switzerland).

Compliance with requirements for studies using animals

The studies reported in this paper comply with the ARRIVE guidelines (McGrath & Lilley, 2015). The University of Liège Animal Ethic committee approved all animal procedures, including experimental design and statistical determination of group size (Approval number #1651). The protocol and procedures are compliant with Belgian and European regulations on protection of animals used for scientific purposes (Arrêté Royal du 29 mai 2013 and EU Directive 2010/63/UE). In total, 32 male Wistar rats (250-350g, 3 months of age) originating from University of Liège animal facility (Federal authorization for breeding animals LA1610002) were kept in a pathogen free (SPF) facility at least two per ventilated cages in a controlled temperature and regular light/dark cycle. The non-invasive tail-cuff method used to measure blood pressure has been extensively described as an adequate method for the estimation of blood pressure and routinely used in our lab (Bialy et al., 2015; Dogne, Rath, Jouret, Caron, Dessy & Flamion, 2016).

Rat blood pressure measurements

One week before the experiment, rats were acclimated to the experimental procedure and contention devices to prevent stress. They were randomized according to weight in four groups of 8 animals to receive vehicle (NaCl 0.9% m/v) or saline solutions of disodium salts of the compounds (SA, *c*ESA, *c*CPDC and *t*CPDC). BP was measured by the tail-cuff method using CODA system (Kent Scientific Corporation; NIBP-CODA8-PACK). The animals were injected via the tail vein. Immediately after injection, systolic, diastolic and mean BP were recorded 10 times every 20 seconds after an acclimation of 5 runs (CODA software). The method being non-invasive, no surgical procedure, anaesthesia, analgesia, euthanasia has been implemented. General welfare assessment was conducted prior to and following the experiments.

Randomization and blinding

The whole cohort of rats was constituted of animals of similar age and weight. They were attributed a group randomly (number generated by computer program). The homogeneity of weight in each group was not significantly different. The scientist in charge of preparing the solutions was different than the one operating the blood pressure measuring device. The solutions and their vessels did not differ in volume, colour or shape. The operator of the blood pressure device was not aware of the nature of the injected solution. The results were analysed by a third person that did not know which groups of animals received the solutions containing active ligands.

Pharmacophore Model

The pharmacophore model was built using the program phase 3.3. implemented in the Maestro 9.2 software package (Schrödinger, LLC, New-York, New-York, USA, 2011) and based on the results obtained from the primary SAR study. The 3D structures were initially built using the program Ligprep 2.5 of Maestro 9.2 (Schrödinger, LLC, New-York, New-York, USA, 2011). Structural conformers were generated with the thorough sampling option. The top-ranked hypothetical pharmacophore including two hydrogen bond donors (AH) and two negative charges (N) was selected for the subsequent virtual screening. Excluded volumes were also added according the superimposition of inactive ligands.

Binding site and docking

The SUCNR1 receptor model was built by means of the SYBYL 8.0 molecular modelling package (SYBYL, version 8.0; Tripos Inc.: St. Louis, Missouri, 2008). First, the human sequence of the SUCNR1 receptor obtained from the Universal Protein Resource (code entry: Q9BXA5) were aligned with the sequence of the human β2 adrenergic GPCR (code entry P07550) by using the FUGUE module (Shi, Blundell & Mizuguchi, 2001). The next step was the copy of a set of constraints derived from the crystal structure of the human β2 adrenergic GPCR to the corresponding residues of the sequences to be modelled using the ORCHESTRAR protein structure modelling module (Dilly & Liegeois, 2011). Since SA is a SUCNR1 agonist, the SUCNR1 receptor model was built in its active form from a crystal structure of the activated turkey β2 adrenergic receptor (PDB code 3P0G) (Rasmussen et al., 2011).

The binding mode of SA into the SUCNR1 receptor was then investigated by molecular docking using the GOLD 5.2 program (Jones, Willett, Glen, Leach & Taylor, 1997). The binding pocked was defined from the one proposed by He et al. (He et al., 2004). The structure of the resulting SA-SUCNR1 complex was finally refined by a 2ns molecular dynamics simulation using the MMFF94 force field implemented in SYBYL 8.0 (Halgren, 1996), a temperature of 310 K, and a time step of 1 fs.

Data and statistical analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2015). Statistical analysis and plotting of concentration-response curves were performed with GraphPad Prism version 5.0. The EC_{50}

were calculated by the software following nonlinear regression (curve fit) with 4 parameters. In the screening experiments and *in vitro* determination all the compounds have been tested at least in three independent experiments ($n \geq 3$). Determinations of assay performance have previously demonstrated the robustness and variability of the procedure, which is sufficient for this number of independent experiments. For the *in vitro* cAMP determinations, the results have been normalized to the activity of SA to be able to compare the activity of compounds from different experiments, the absolute (but not the relative) response being influenced by the number of receptors expressed by the cells, with slight variations from one day to another. No statistical analysis has been performed in datasets of less than 5 independent experiments. For the evaluation of significant differences in the *in vivo* determination of BP, a one way ANOVA followed by Bonferroni's multiple comparison test was performed. The post-hoc tests have been run only when F achieved $P<0.05$. There was no significant variance in homogeneity. P values less than 0.05 between means were considered as statistically significant and are marked with * in the figures.

Results

Screening of a library of succinic acid analogues

In the search for novel SUCNR1 agonists, we tested a small library of 32 analogues of succinic acid (Fig. 1A). The library was built using three parameters: 1) the substituent borne by the backbone, 2) the size of the carbon backbone and 3) the influence of negative or positive charges. For ease of the presentation, we refer to the acidic form of the tested analogues even if the carboxylic acid moieties of the interacting molecules are deprotonated at physiological pH. The chemical properties and formulas of all tested compounds in this report are summarized in Table 1. Each compound was tested at a single concentration of 500 µM on a HEK293 cell line stably expressing SUCNR1 together with the cAMP GloSensor (Gilissen, Geubelle, Dupuis, Laschet, Pirotte & Hanson, 2015). We evaluated their ability to inhibit the basal cAMP levels according to a procedure established in the laboratory (Gilissen, Geubelle, Dupuis, Laschet, Pirotte & Hanson, 2015). None of the evaluated analogues was more active than SA at 500 µM (Fig. 1A). Compounds characterized by a backbone of more than 4 as well as less than 3 carbon atoms were completely inactive (Fig. 1A and Table 1). Compounds with no negative charges were also unresponsive at 500 µM. Succinamic acid and monomethylsuccinate, both having a single negative charge at physiological pH (Table 1), showed partial response (Fig. 1A). Besides, we evaluated all the compounds as antagonists to make sure that inactive analogues were not binding to the receptor without activating it. We did not detect antagonistic activity of inactive compounds toward SUCNR1 (Fig. 1B). We performed complete concentration-response curves on compounds that displayed at 500 µM an activity higher than 10% (Normalized to SA activity set at 100%, Fig. 1A). We chose 10% because we determined it was a significant level compared to the background of the assay (Gilissen, Geubelle, Dupuis, Laschet, Pirotte & Hanson, 2015). None of the tested compounds showed significant activity on cells not transfected with SUCNR1.

Impact of the nature and stereochemistry of substituents

We analysed the impact of the nature of substituents and their stereochemistry on potency and maximal efficacy. In the screening, methylsuccinic acid (MSA) showed some activity at 500µM (Fig. 1A). The methyl substituent introduces a chiral centre and two stereoisomers exist. The compound evaluated in the screening was a racemic mixture of two stereoisomers. Thus, we performed concentration-response curves on both R and S enantiomers (Fig. 2A).

Interestingly, these two analogues were characterized by an important difference in their activity, (S)-MSA being inactive in the range of tested concentrations. We followed a similar strategy for the evaluation of bromosuccinic acid (BrSA). In this case, we observed an opposite response with regard to stereochemistry compared to MSA. The (S)-BrSA enantiomer could not reach its maximal efficacy in the range of tested concentrations whereas the R enantiomer was inactive at the same concentrations (Fig. 2B). We reasoned that the size of the bromine atom could preclude an efficient interaction in the binding pocket of the receptor. Therefore, we synthesized (R)- and (S)-chlorosuccinic acid (ClSA), the chlorine atom being smaller than the bromine atom (for the synthesis route, see supplementary Fig. S1). The S derivative was also the most potent, being an agonist with potency and efficacy close to the one of SA in our assay (SA pEC_{50} = 4.54 ± 0.08, EC_{50} = 29 µM; S-ClSA pEC_{50} = 4.14 ± 0.04, EC_{50} = 72 µM, Fig. 2B). We investigated other types of substituents such as the hydroxy group (malic acid, Fig. 2C) that is able to establish hydrogen bond (as a donor). In a similar fashion than with the BrSA, the (S)-malic (or (L)-malic) acid was the only one showing an agonist behaviour although weaker compared to SA. We also addressed the impact of positively charged substituents such as amines by evaluating aspartic acid (Fig. 2D). Both S and R isomers were inactive (Fig. 2D). Oxaloacetic acid (OAA) is characterized by a carbonyl group on the succinic acid backbone (see Table 1) that is able to form hydrogen bonds as an acceptor. This compound behaved as an agonist with similar efficacy but lower potency compared to succinic acid (SA pEC_{50} = 4.85 ± 0.08, EC_{50} = 14 µM; OAA pEC_{50} = 4.15 ± 0.04, EC_{50} = 70 µM, Fig. 2D).

cis-Conformation of the negative charges is an essential feature for SUCNR1 agonism

In the screening results (Fig. 1A), we noticed that although maleic acid showed activity as expected, fumaric acid, a very close derivative, was inactive at this concentration. We confirmed with full concentration-response curves that fumaric acid was lacking activity on the receptor even at the highest concentration tested whereas maleic acid was an agonist with lower potency but similar efficacy compared to SA (Maleic acid pEC_{50} = 4.24 ± 0.07, EC_{50} = 57 µM, Fig. 3A). Surprisingly, the only difference between the two derivatives is the *cis* (maleate) or *trans* (fumarate) configuration of the carboxylic acids. We reasoned that the orientation of the negative charges had to be in close vicinity to interact with two adjacent positively charged amino acids in the binding pocket. We tested this hypothesis with *meso*-dimethylsuccinic acid (*m*DMSA) that has two bulky substituents (Table 1) that orient the carboxylic acids in a pseudo *trans* configuration. *m*DMSA was inactive in the range of tested

concentrations (Fig. 3B). We evaluated malonic acid (MA) and its substituted analogues methyl (MMA) and ethylmalonic acid (EMA) (see Table 1 for chemical structures). Consistent with the idea that promoting a conformation where the negative charges are closer will produce compounds with increased potency, MMA (pEC_{50} = 3.77 ± 0.05, EC_{50} = 169 µM, Fig. 3B) was more potent than MA (pEC_{50} > 3.15, EC_{50} > 700µM, Fig. 3B). EMA was inactive in the range of tested concentrations (Fig. 3B). With all these results, we developed an *in silico* model of the SUCNR1 receptor. We achieved a docking of SA into the binding pocket proposed by He et al. (Fig. 3C) (He et al., 2004). The binding results predicted that SA negatively charged oxygen atoms (carboxylate functions) would establish ionic interactions with positively charged nitrogen atoms (guanidinium functions) of arginines 252 ($R252^{6.55}$, superscripts indicates the residue topology according to the Ballesteros-Weinstein numbering system (Ballesteros & Weinstein, 1995)) and 281 ($R281^{7.39}$) (Fig. 3C). In our model, arginine 99 ($R99^{3.29}$) and histidine 103 ($H103^{3.33}$), although involved in the interaction with SA, were not critical. To confirm our model, we generated mutants for the 4 AA presumably involved in SA binding. We generated stable cell lines expressing the mutants and evaluated the ability of the different receptors to be activated by SA (Fig. 3D). R252A and R281A mutants were unresponsive to SA at concentrations up to 100 mM whereas R99A and H103A could be activated by SA although at very high concentrations (SA pEC_{50} on R99A < 1.50, EC_{50} on R99A > 30 mM ; SA pEC_{50} on H103A < 0.85, EC_{50} on H103A > 140 mM, Fig. 3D).

cis-Cyclic derivatives of succinic acid are SUCNR1 agonists

Based on the active and inactive compounds that we identified so far, we developed a pharmacophore for activity at SUCNR1 (Fig. 4A). The yellow spheres represent exclusion volume. Pink and red spheres indicate the hydrogen bond acceptor and negative sites, respectively. We screened the ZINC library and obtained as hits cyclic compounds such as *c*CPDA (Fig. 4B). We evaluated this compound with our cAMP assay on SUCNR1 and found that *c*CPDA was an agonist of similar potency and efficacy compared to SA (*c*CPDA pEC_{50} = 3.31 ± 0.02, EC_{50} = 49 µM, Fig. 4C). Consistent with our binding site model, the *trans* analogue of 1, 2-cyclopropanedicarboxylic acid was inactive (Fig. 4C). We evaluated the *cis*-1, 2-cyclobutanedicarboxylic (*c*CBDA) acid and measured an activity for the *cis* isomer (pEC_{50} < 3.60, EC_{50} > 250 µM, Fig. 4C), although it did not reach maximal efficacy in the range on tested concentrations, whereas the *trans* isomer was inactive (Fig. 4C). The *cis* and *trans* 1, 2-cyclopentanedicarboxylic acid were both inactive (data not shown). Given the good potency obtained with OAA (pEC_{50} = 4.15 ± 0.04, EC_{50} = 70 µM, Fig. 2D), we

reasoned that a hydrogen bond acceptor in the cycle could have beneficial effect on the activity of the cyclic dicarboxylic acids. This led to the evaluation of *cis*-epoxysuccinic acid (*c*ESA) on SUCNR1. This compound displayed a 100% efficacy and was more potent than SA (*c*ESA pEC_{50} = 5.57 ± 0.02, EC_{50} = 2.7 µM, Fig. 4D).

cis-epoxysuccinic acid and cis-1, 2-cyclopropanedicarboxylic acid are agonists for all known SUCNR1 pathways and do not interfere with succinate dehydrogenase

We performed a complete validation of *c*ESA and *c*CPDA pharmacology on SUCNR1. First, we analysed the agonist behaviour of the compounds on a recently described TACE induced TGFα shedding assay (Inoue et al., 2012). We detected activation of the receptor with the transient transfection of the alpha subunit of the chimeric G_{qi1} that couples to G_{i1} receptors and induces the G_q pathway. In this assay, *c*ESA was the most potent agonist and *c*CPDA displayed a similar potency and efficacy compared to SA (*c*ESA pEC_{50} = 4.38 ± 0.04, EC_{50} = 42 µM; *c*CPDA pEC_{50} = 3.05 ± 0.04, EC_{50} = 887 ± 80 µM; SA pEC_{50} = 3.46 ± 0.03, EC_{50} = 350 µM, Fig. 5A). A similar experiment with the chimeric G_{qi3} that couples to G_{i3} receptors and induces the G_q pathway gave similar results (data not shown). Next, we used a firefly luciferase complementation assay (Gilissen, Geubelle, Dupuis, Laschet, Pirotte & Hanson, 2015) to evaluate the arrestin 3 recruitment to the receptor. *c*ESA and SA, but not *c*CPDA, activated the receptor with similar efficacies at the highest concentrations tested (*c*ESA pEC_{50} = 4.13 ± 0.04, EC_{50} = 74 µM; SA pEC_{50} = 3.06 ± 0.07, EC_{50} = 865 µM; *c*CPDA pEC_{50} = 2.97 ± 0.07, EC_{50} = 1076 µM, Fig. 5B). SUCNR1 is able to elicit $[Ca^{2+}]_i$ mobilization and the three tested compounds elicited an activation of this pathway in the range of tested concentrations (*c*ESA pEC_{50} = 3.72 ± 0.01, EC_{50} = 191 µM; *c*CPDA pEC_{50} = 2.98 ± 0.01, EC_{50} = 1040 µM; SA pEC_{50} = 3.23 ± 0.01, EC_{50} = 581 µM, Fig. 5C). We tested the activity of the two synthetic agonists on SDH activity. At concentrations up to 500 µM neither *c*ESA nor *c*CPDA had an effect on SDH activity (Fig. 5D). OAA was used as a positive control for SDH inhibition. None of the agonists displayed activity on cells not expressing SUCNR1.

cis-1, 2-cyclopropanedicarboxylic acid and cis-epoxysuccinic acid are active in vivo

We finally tested SUCNR1 agonists in an *in vivo* model. We noninvasively measured BP in rats following intravenous perfusion of SA and observed a significant increase in BP, as previously reported (He et al., 2004; Vargas, Toma, Kang, Meer & Peti-Peterdi, 2009) (Fig. 6). We observed a similar increase in response to the injection of *c*CPDA (10 $mg.kg^{-1}$) and

SA (10 mg.kg^{-1}). At the dose of 1 mg.kg^{-1}, *c*ESA injection was followed by an increase in BP that was not statistically different from the one induced by SA or *c*CPDA (Fig. 6).

Discussion

Although the human genome sequence has been published in 2001 (Venter et al., 2001), many proteins continue to be understudied, presumably because of a lack of research tools such as small molecule ligands (Edwards, Isserlin, Bader, Frye, Willson & Yu, 2011). Despite extensive knowledge on the function and pharmacology of some receptors, and despite their potential as drug targets, the majority of the GPCR family is actually understudied (Roth & Kroeze, 2015). Thus, an important number of GPCRs are poorly characterized or even devoid of known ligands and labelled as "orphans". Recently, Roth and Kroeze proposed that it was the availability of good ligands that made some GPCR popular and not the other way around (Roth & Kroeze, 2015). The receptor for SA, SUCNR1, belongs to this category of attractive drug target (Gilissen, Jouret, Pirotte & Hanson, 2016) whose pharmacology is poorly defined. Notwithstanding its demonstrated implication in immune responses and inflammation (Littlewood-Evans et al., 2016; Rubic et al., 2008), retinal angiogenesis (Sapieha et al., 2008) and regulation of renin release (Peti-Peterdi, Gevorgyan, Lam & Riquier-Brison, 2013; Toma et al., 2008), surprisingly few research tools are available and only scarce information about its molecular structure has been published.

In the initial report describing its pairing with SA, He *et al.* evaluated 200 carboxylic acids, of which SA was the more potent (He et al., 2004). They disclosed the activity for a couple of them including the weaker agonists maleate and methylmalonate. They identified one important feature for agonist activity, the mandatory dicarboxylic nature of active molecules (He et al., 2004). Here, we aimed to go several step further and propose a full structure-activity relationship analysis. Compared to previous work, our investigation adds novel essential features required for activation of SUCNR1. In summary, succinate derivatives with an agonist profile must have: i) 2 negative charges at physiological pH; ii) a distance from 3 to 5 carbon atoms between these two negatively charged atoms and iii) the possibility for the molecule to adopt a *cis* conformation of the two charges. The pharmacophore for agonist ligands is schematically summarized on Fig. 6B. This refined pharmacophore led to the establishment of an improved model for the SA binding pocket. It was initially proposed that

four AA were critical for SA binding to the receptor (He et al., 2004). However, the published data showed only the results at one concentration of SA (200µM) on the mutant (He et al., 2004). Having defined the three criteria for SUCNR1 activation, we speculated that the most important interactions should be between two positively charged AA located in close proximity and on the same side inside the binding pocket. We performed docking simulation in an improved binding pocket model (Fig. 3B) and identified $R252^{6.55}$ and $R281^{7.39}$ as the probable candidates. Mutagenesis experiments confirmed that these two AA are indeed critical for the interaction whereas $R99^{3.29}$ and $H103^{3.33}$ were important but not crucial. Interestingly, residues 3.33, 6.55 and 7.39 are topologically involved in agonist binding in several other class A GPCRs (Venkatakrishnan, Deupi, Lebon, Tate, Schertler & Babu, 2013). A better delineation of the SA binding mode is important for future modelling of the receptor to identify new ligands. Subsequently, we identified *cis*-cyclic dicarboxylic acids as potent agonists, in particular *c*ESA that is characterized by a 10- to 20-fold improved potency compared to SA in various assays. The presence of an oxygen atom in its cycle, together with the fact that OAA is an agonist with good potency suggest that a putative AA able to establish an hydrogen bond is ideally positioned inside the binding pocket.

In all bioassays that were used, the ligands showed the same rank order of potencies. This observation suggests that the ligands are not biased for the measured response although more thorough experiments would be required to analyse biased signalling (or its absence). Interestingly, the potency of the ligands was lower compared to cAMP related pathways (Fig. 5B). This is consistent with previous reports by us (Gilissen, Geubelle, Dupuis, Laschet, Pirotte & Hanson, 2015) and others (Southern et al., 2013). The luciferase complementation assay utilized to monitor arrestin recruitment is very different from the physiological environment of the receptor, which may impact the observed potency. The discrepancy between the assays could also be the consequence of a receptor reserve for the G_i mediated inhibition of AC. This raises the interesting possibility that in native tissues, in a given (patho)physiological context, the activation of some pathway might occur only in certain conditions of very high SA concentrations whereas the other pathways (such as G_i-related ones) are activated at lower SA concentrations due to receptor reserve and amplification.

The other synthetic ligands that have been described as antagonists are reported as being able to inhibit succinate mediated $[Ca^{2+}]_i$ mobilization in CHO-K1 cells overexpressing human SUCNR1 in the nanomolar range for the best compounds (2c and 4c) (Bhuniya et al., 2011; Gilissen, Jouret, Pirotte & Hanson, 2016). Their selectivity has been evaluated and they

represent valuable tools to characterize the receptor (Klenc, Lipowska & Taylor, 2015). Interestingly and in stark contrast with the compounds described here, these ligands have no clear structural relationship to succinate. In addition, they display no negative charges at physiological pH. It would be interesting to test whether these antagonists can block the action of *c*ESA and *c*CPDA with the same potency. Although the compounds reported by Bhuniya *et al.* are not available commercially, the novel agonists that we have identified will permit to investigate more precisely the mechanism of action and the binding mode (competitive vs. irreversible orthosteric or negative allosteric modulators...) of the SUCNR1 antagonists.

We also addressed two important characteristics in the perspective of the use of our compounds as pharmacological tools: specificity and *in vivo* efficacy. Historically, several SA analogues such as oxaloacetate, malonate, or L-malate have been described as mitochondrial complex II (or SDH) inhibitors. Although they show agonist activity on SUCNR1, they would not be useful as tools because they also disrupt cellular respiration and could lead to artefacts. Similarly, there are some limitations in using SA to address SUCNR1 roles, the endogenous ligand being also an important cellular metabolite. Our results clearly show that *c*ESA and *c*CPDA are devoid of activity on SDH activity and suggest that they can be used to specifically address the consequences of SUCNR1 activation in cells, native tissues and organs, although we cannot exclude other off-target effects. Another important aspect for chemical tools is their usefulness *in vivo*. More specifically, *c*ESA has an "epoxy" (see Table 1) function that may be metabolically unstable. SA has been shown to increase blood pressure through SUCNR1 and release of renin in mice (He et al., 2004; Vargas, Toma, Kang, Meer & Peti-Peterdi, 2009). Therefore, we assessed in rats the ability of our compound to recapitulate the effects of SA *in vivo* after intravenous injection. Both compounds were able to significantly increase BP with similar amplitude compared to SA. Although it is tempting to speculate that it is the consequence of an activation of SUCNR1, additional experiments are needed (with antagonist or knock-out animals) to substantiate the direct link between *c*ESA- and *c*CPDA- mediated increase in blood pressure and SUCNR1. The oxoglutarate receptor (OXGR1) and the purinergic $P2Y_1$ receptor are the two most closely SUCNR1 related receptors (Gilissen, Jouret, Pirotte & Hanson, 2016). Although SA has been shown previously to be unable to activate these receptors in various assays (He et al., 2004), future investigation should address the activity of SA and other SUCNR1 ligands with regard to these receptors.

In conclusion, this is the first study, to the best of our knowledge, reporting on a synthetic agonist for SUCNR1/GPR91 that is more potent that the endogenous ligand, SA. The clinical relevance of these results is the possibility that SUCNR1 might represent an innovative drug target, for instance in hypertension-related diseases. However, it needs thorough preclinical validation first. We expect that the emergence of a properly characterized tool, which is readily commercially available, will spur new research on the understudied SUCNR1 that should advance its validation as a drug target.

Author Contributions

JH designed and supervised the study. PG, JG, ND, CL and DA performed the in vitro experiments and acquired the data. SD modelled the pharmacophore and receptor, identified privileged structures and docked succinate. LP and FJ designed and performed the in vivo experiments. PG, FJ, BP and JH analysed the data, interpreted the results, created the figures and wrote the paper. AI designed and set up the shedding assay and provided research tools.

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Conflicts of Interest Statement

The authors declare no conflicts of interest.

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Table 1. Chemical structure of tested compounds

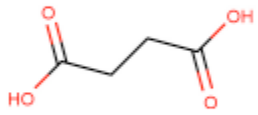
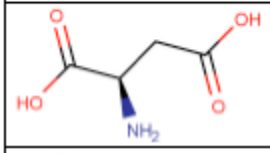
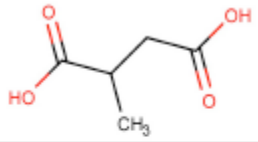
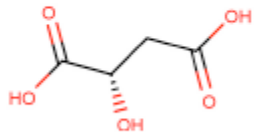
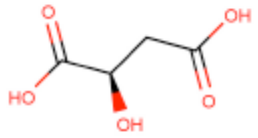
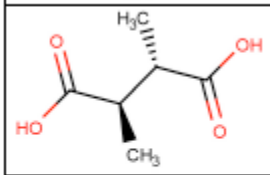
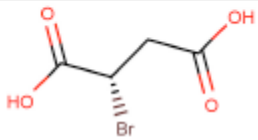
Structure	Name	Backbone Length	Number of Charges		Activity
			Neg	Pos	
	Succinic acid	4	2	0	Natural Ligand
	S-Aspartic acid L-Aspartic acid	4	2	1	Inactive
	Methylsuccinic acid 2-Methylsuccinic acid	4	2	0	**Active**
	L-Malic acid (2S)-2-Hydroxysuccinic acid	4	2	0	**Active**
	D-Malic acid (2R)-2-Hydroxysuccinic acid	4	2	0	Inactive
	meso dimethyl Succinic acid (2R,3S)-2,3-Dimethylsuccinic acid	4	2	0	Inactive
	S-Bromo Succinic acid (2S)-2-Bromosuccinic acid	4	2	0	**Active**

Figure 1

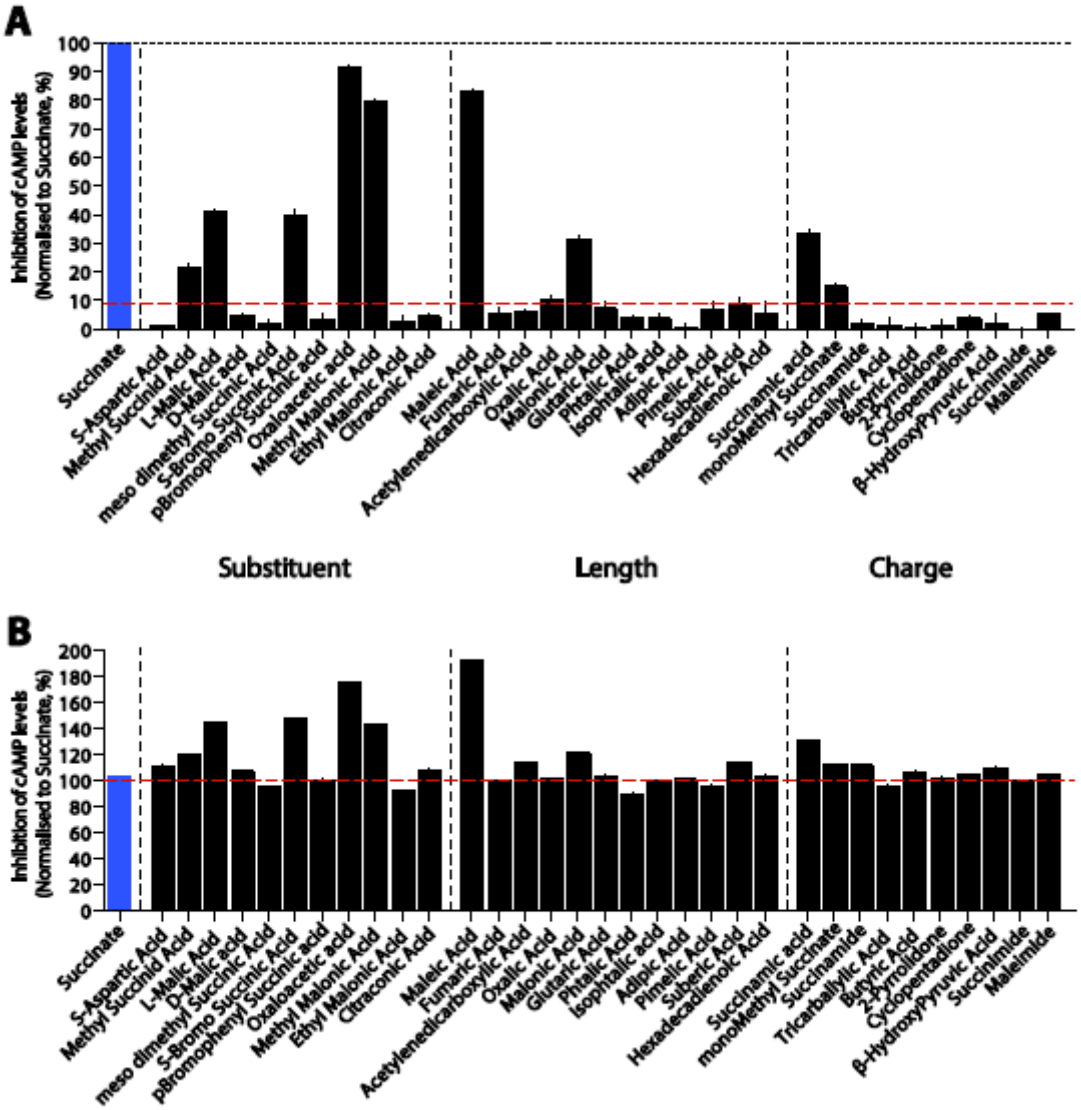


Figure 1. Screening of library of succinic acid analogs

We selected diverse compounds that share some characteristics with SA. The compounds were grouped on three criteria: 1) nature of substituent 2) length of carbon backbone and 3) charge of the molecule. See Table 1 for a complete list of molecular structures. **A.** The different compounds were tested at 500µM on SUCNR1. The agonist nature was evaluated by measuring the levels of cAMP in the presence of compounds compared with Vehicle control and succinate response. No compounds showed activity on cell line without SUCNR1. **B.** 100µM of compounds were added prior to the addition of 500µM SA to evaluate their antagonistic activity. Results are given as percentage of SA activity for ease of comparison and as Mean ± SEM of 3 independent experiments.

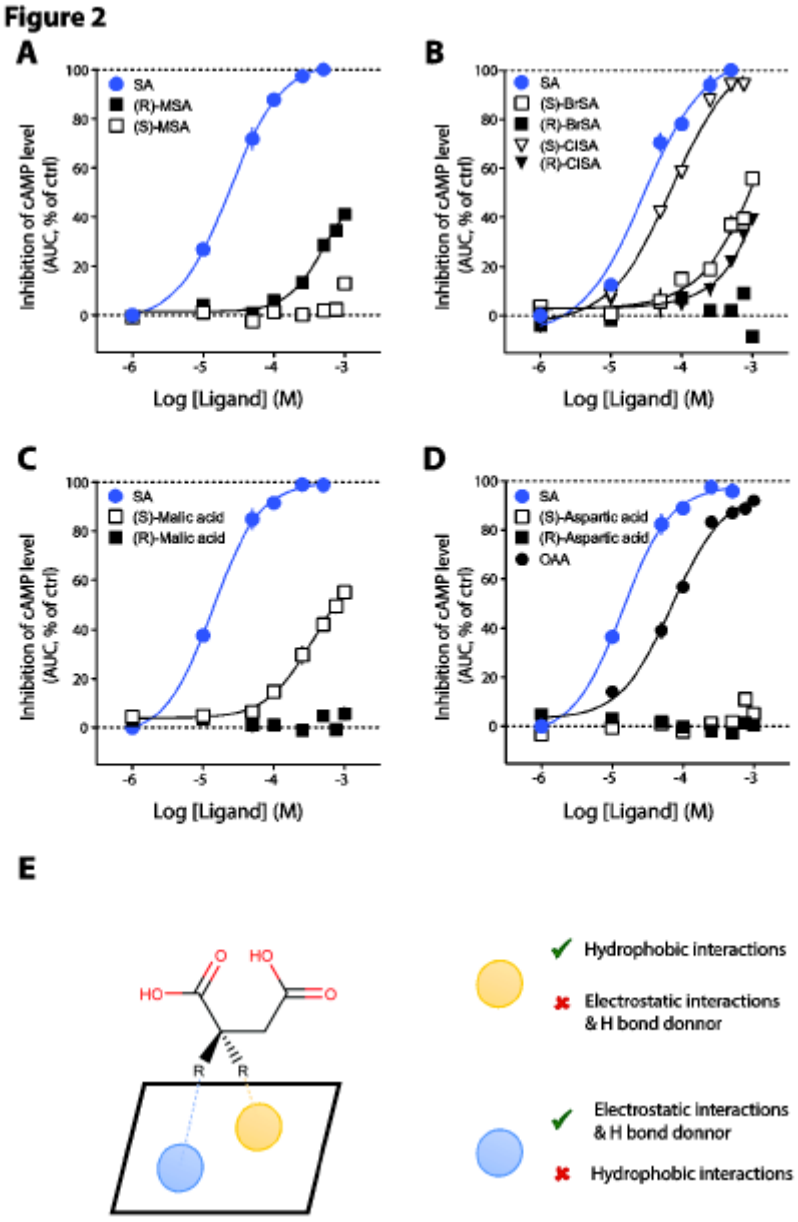


Figure 2. Impact of the nature and stereochemistry of substituents

Some of the compounds showing activity in screening at one concentration were evaluated with full concentration-response curves. Concentration-response curve for the effect on cAMP of **A.** (R)- and (S)-methylsuccinic acid; **B.** Bromo- and chlorosuccinic acid; **C.** Malic acid; **D.** Aspartic and oxaloacetic acid. In all experiments, SA has been used as a reference compound and the data normalized accordingly. Data are expressed as Mean ± SEM of independent 3 experiments. **E.** Model for the interaction of substituents with the binding pocket.

Figure 3

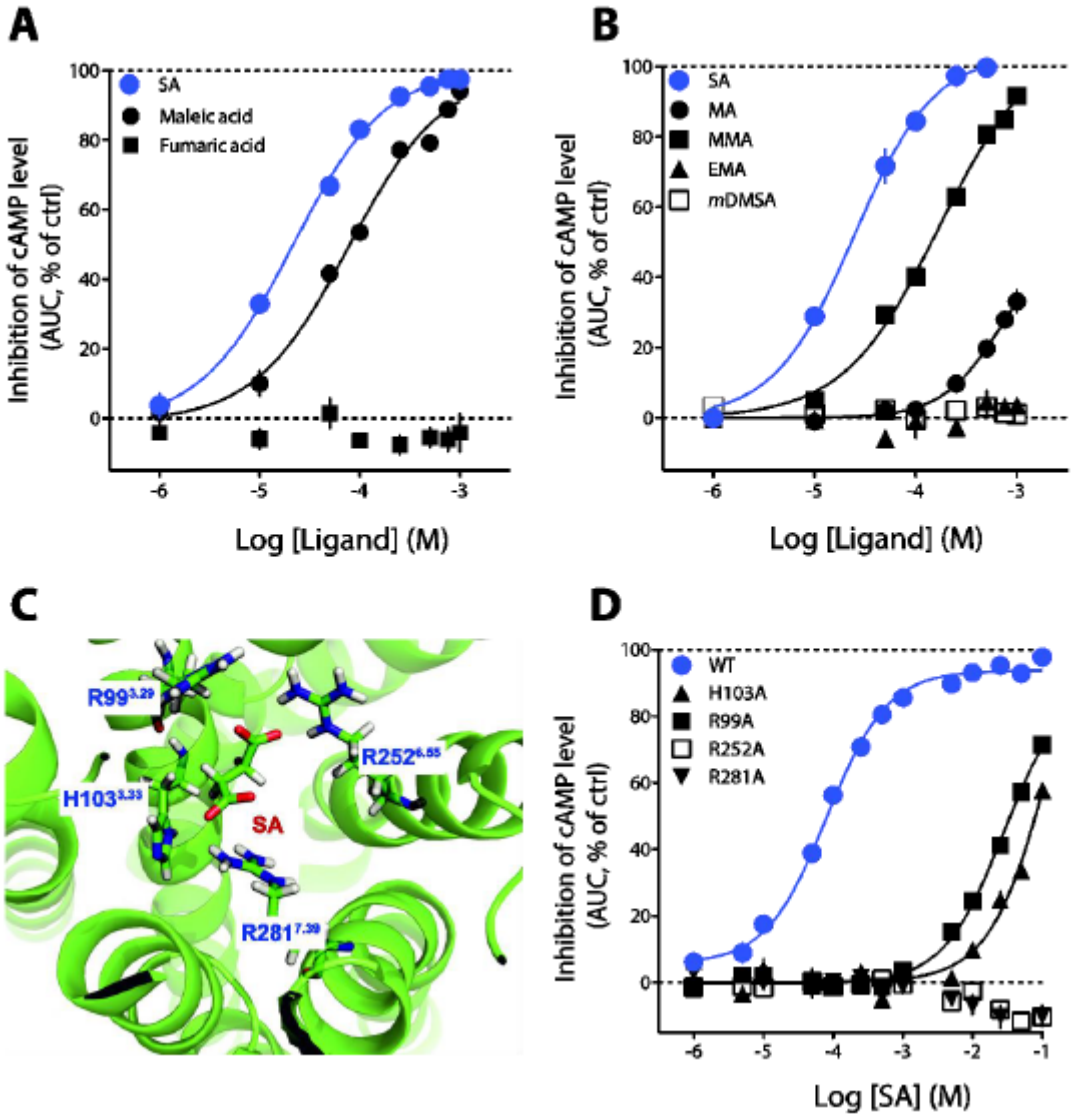


Figure 3. ***cis*-Conformation of the negative charges is an essential feature for SUCNR1 agonism. A.** Maleic acid is a full agonist for SUCNR1, albeit with a lower potency compared with SA. Fumaric acid is completely inactive **B.** Malonic, methylmalonic, ethylmalonic and *meso*-dimethylsuccinic acid concentration-response curves on basal cAMP levels. **C.** Homology modeling of SA binding pocket. SA is positioned in a pseudo *cis* conformation. **D.** Evaluation of the effect of SA on basal cAMP level in several HEK293 cell lines stably transfected by GloSensor system and SUCNR1 mutants (H103A, R99A, R252A, R281A). Data are presented as Mean ± SEM of three independent experiments.

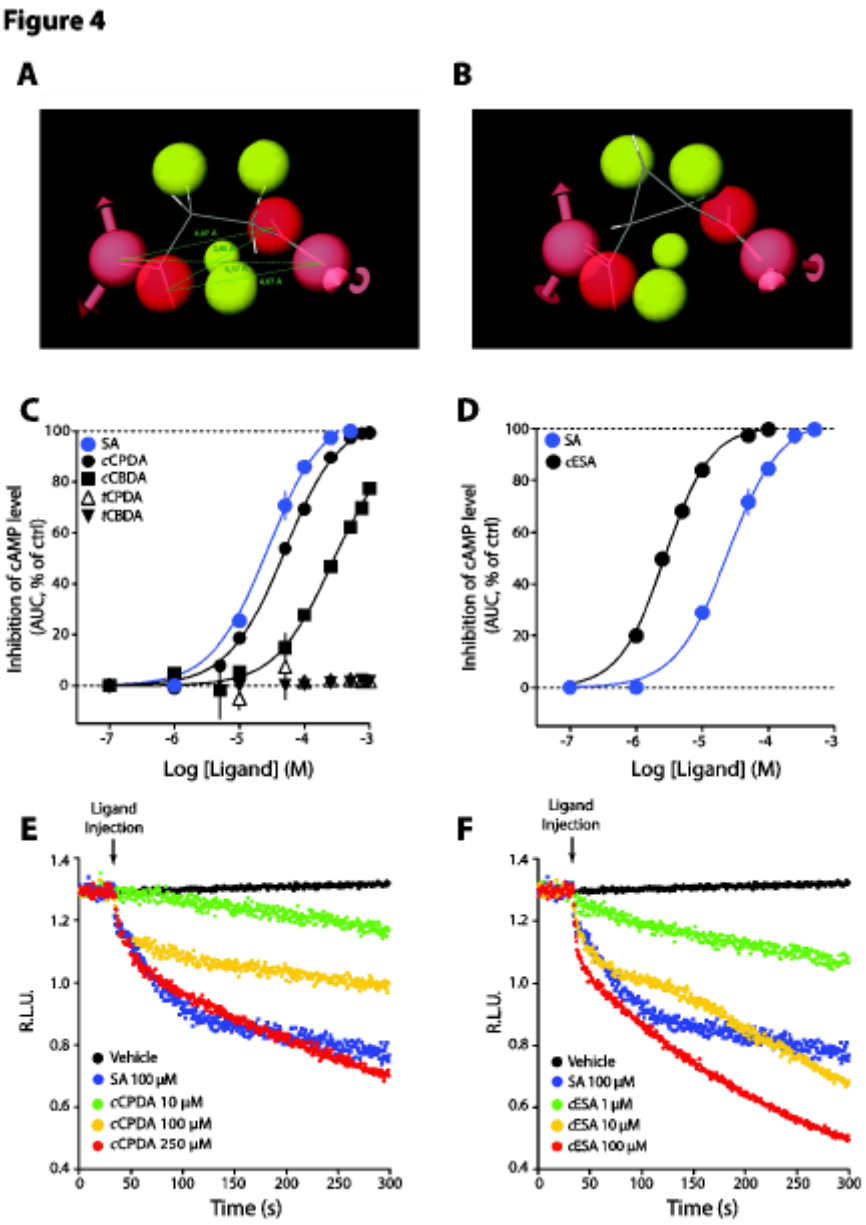


Figure 4. *cis*-Cyclic derivatives of succinic acid are SUCNR1 agonists

A. Model for the SUCNR1 agonist pharmacophore. Yellow spheres represent exclusion volume and red spheres negative charges. **B.** *cis*-1, 2-cyclopropanedicarboxylic acid fit with the pharmacophore model. **C.** Evaluation of *cis* and *trans* cyclic compounds on the inhibition of basal cAMP levels. **D.** *cis*-epoxysuccinic acid concentration-response curve on the inhibition of basal cAMP level. **E&F.** Kinetic measurement of the inhibition of cAMP levels followed in HEK293 cells stably expressing cAMP Glosensor and SUCNR1 upon addition of the SUCNR1 agonists *cis*-1, 2-cyclopropanedicarboxylic (**E**) and *cis*-1, 2-

cyclopropanedicarboxylic (**F**). Succinic acid has been used as a positive control. Data are expressed as Mean ± SEM of 3 independent experiments.

Figure 5

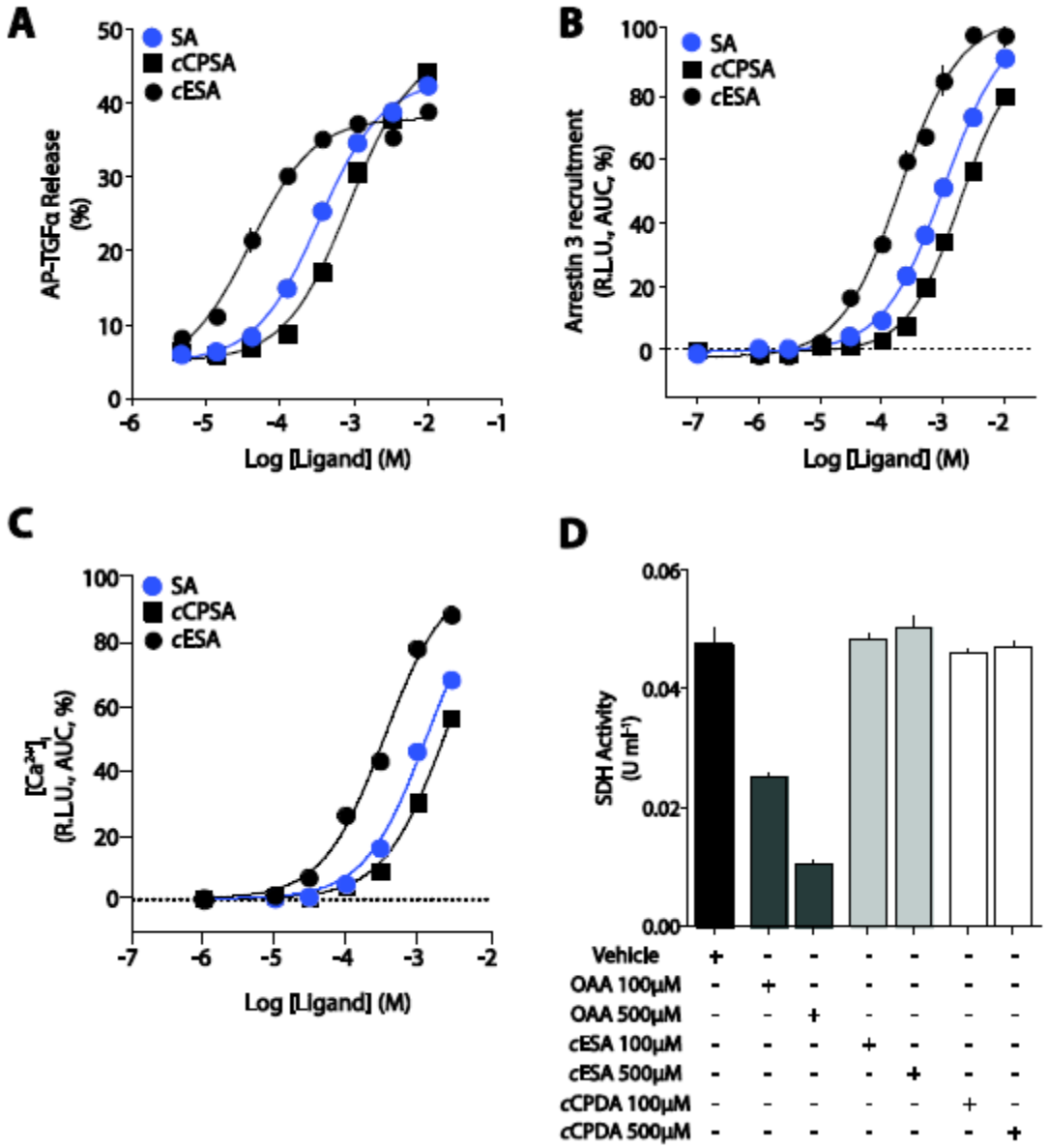


Figure 5. *cis*-epoxysuccinic acid and *cis*-1, 2-cyclopropanedicarboxylic acid are agonists for all known SUCNR1 pathways and do not interfere with succinate dehydrogenase

Concentration-response curves for SA, *cis*-epoxysuccinic acid and *cis*-1, 2-cyclopropanedicarboxylic acid on **A.** TGF-α shedding; **B.** Arrestin 3 recruitment; **C.** $[Ca^{2+}]_i$ mobilization and **D.** Succinate dehydrogenase activity. Data are presented as Mean ± SEM of 6 independent experiments.

Figure 6

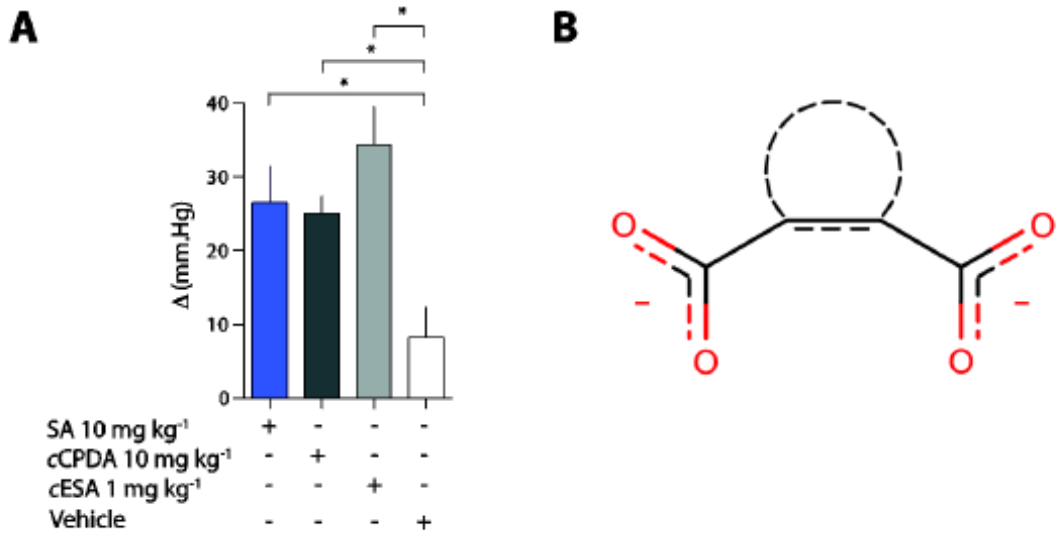


Figure 6. ***cis*-epoxysuccinic acid and *cis*-1, 2-cyclopropanedicarboxylic acid are active *in vivo***

A. Repeated noninvasive measures of blood pressure in rats (n=8 in each group) injected intravenously with a saline solution of various test compounds. These experiments have been performed at least three times for each condition on different animal cohorts. Data are expressed as the difference between mean blood pressure before injection and 15 min post-injection. Data are presented as Mean ± SD. * P value < 0.05 based on 1 way ANOVA. **B.** Proposed pharmacophore for succinate derivatives agonists.