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ORIGINAL ARTICLE

# Novel Fubinaca/Rimonabant hybrids as endocannabinoid system modulators

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## Abstract

The discovery of novel modulators of the cannabinoid system is a current topic in medicinal chemistry. In this paper, we report nine novel carboxamides designed as hybrids of Fubinaca family compounds and Rimonabant. These hybrids were obtained by linking the 1-benzyl-2,5-dichloroindazole-3-carboxylic acid to different amino acids bearing a hydrophobic side chain and three different *C*-terminus. The new chemical entities were tested in vitro to evaluate their bioactivity by means of receptor binding assays and [$^{35}$S]GTPγS stimulation assays to reveal their affinity and potency. We found that all compounds were able to bind to the cannabinoid receptors in the low nanomolar range with a marked selectivity towards the CB1 cannabinoid receptor. Some of them are full agonists, whereas the others act as partial agonists. These molecules could be potentially used as anti-obesity agents, antiemetic and analgesics.

**Keywords** Cannabinoids · Lonidamine · Amino acids · Cannabinoid receptors · Selectivity

## Abbreviations

| | |
|---|---|
| MCT | Monocarboxylate transporter |
| MPC | Mitochondrial pyruvate carrier |
| CB | Cannabinoid receptor |
| SC | Synthetic cannabinoid |
| SAR | Structure activity relationships |
| EDC·HCl | *N*′-Ethylcarbodiimide hydrochloride |
| HOBt | Hydroxybenzotriazole |
| NMM | *N*-Methylmorpholine |
| LRMS | Low resolution mass spectrometry |
| $^{1}$H-NMR | Proton nuclear magnetic resonance |
| $^{13}$C-NMR | Carbon nuclear magnetic resonance |
| RP-HPLC | Reverse phase high performance liquid chromatography |
| DMF | Dimethylformamide |
| THF | Tetrahydrofurane |
| TFA | Trifluoroacetic acid |
| DCM | Dichloromethane |
| DMSO | Dimethyl sulfoxide |
| BSA | Bovine serum albumin |
| EGTA | Ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid |

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## Introduction

Recently, a number of new synthetic psychoactive substances acting on the cannabinoid system have appeared worldwide (Lewin et al. 2014; Banister et al. 2015). Synthetic cannabinoids (SCs) are a major class of abused drugs that elicit cannabimimetic effects similar to $\Delta^9$-tetrahydrocannabinol by interacting with the CB1 and CB2 cannabinoid receptors (Aung et al. 2000; Huggman et al. 2005; Makriyannis and Deng 2005).

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*N*-(1-amino-3-methyl-1-oxobutan-2-yl)-1-(4-fluorobenzyl)-1*H*-indazole-3-carboxamide (AB-FUBINACA) is an indazole-type SC containing a 4-fluorobenzyl substituent at the N-1 position (Uchiyama et al. 2013a, b; Castanet et al. 2015). AB-FUBINACA has much higher affinity for the CB1 receptor ($K_i = 0.9$ nM) than tetrahydrocannabinol ($K_i = 41$ nM) and acts as an agonist of the CB1 receptor (Buchler et al. 2009).

As SCs are undergoing a period of proliferation and diversification, there is a corresponding increase in the challenges faced by emergency and critical care deriving by the substance abuse. New psychoactive substances provide users with alternatives to older and better-characterized drugs of abuse, such as amphetamines, heroin, cocaine, and cannabis. More severe clinical features, including psychosis, delirium, cardiotoxicity, seizures, acute kidney injury, hyperthermia, and death, have also been noted (Schwartz et al. 2015; Trecki et al. 2015). Depression of the central nervous system (CNS) is consistent with the potent CB1 agonist activity that has been reported for many SCs, cardiotoxicity may be due to inhibition of the alpha subunit of potassium channels in cardiomyocytes, and autonomic symptoms may be due to the affinity of some cannabinoids for serotonin 2B receptors (Wiley et al. 2016). Some SCs also possess agonist activity at dopamine receptors in vitro (Wiley et al. 2016); substantial changes in dopamine signaling and a severe withdrawal syndrome have been observed among cannabinoid users (Rominger et al. 2013).

Due to its expression and localization in the CNS, the CB1 receptor together with its endogenous ligands, i.e., endocannabinoids (eCBs) and the enzymes involved in their synthesis and degradation, are implicated in multiple pathophysiological events ranging from memory deficits to neurodegenerative disorders, energetic homeostasis and feeding behaviors. Thus, novel synthetic cannabinoid system modulators may be of great interest as pharmacological tools. The compound AB-FUBINACA (Fig. 1) was developed by Pfizer and described in a patent (Buchler et al. 2009). Later, it was first identified in illegal synthetic cannabinoid products in Japan in 2012 (Uchiyama et al. 2013a, b).

It came to our attention because there is a structural similarity between FUBINACA analogs and the drug Lonidamine.

Lonidamine is a derivative of the indazole-3-carboxylic acid that is the core of the 1-benzyl-indazole-3-carboxylic scaffold of AB-FUBINACA. It is known for its antispermatogenic effect and as male contraceptive (Gunda et al. 2009); recently, it was also studied as an adjuvant to radiotherapy, chemotherapy, photodynamic therapy and hyperthermia in the management of advanced tumors such as lung carcinoma, breast, kidney, prostate, brain, colorectal and liver metastases (Floridi et al. 1981; Floridi and Lehninger 1983; Caputo and Silvestrini 1984).

The heterocyclic scaffold of Lonidamine is present in the cannabimimetic carboxamide derivatives of FUBINACAs (Fig. 1) (Uchiyama et al. 2013a, b) and partially in Rimonabant, a CB1 receptor inverse agonist.

FUBINACA family of SCs has an indazole scaffold joined to the amino acid derivative L-*tert*-leucinamide; its parent compound AB-FUBINACA possesses the same indazole scaffold featuring an L-valinamide residue. Preliminary SAR investigations suggest that L-*tert*-leucinamide derivatives have greater affinity for CB1 receptors in vitro than the L-valinamide analogs. AB-FUBINACA is able to dose-dependently decrease body temperature and heart rate, indicating that this SC behaves as a cannabimimetic in vivo (Wiley et al. 2016). On the other hand, Rimonabant is a potent, selective, CB1 receptor neutral antagonist that reverses CB1-mediated cannabinoid agonist effects in rodents and humans (Husteis et al. 2001; Rinaldi-Carmona et al. 1994, 1995). Considering the common heterocyclic scaffold enclosed in FUBINACA compounds and that of Lonidamine, with the only difference on the halo-substitution on the benzylic portion, which is present in the CB1 antagonist Rimonabant (Fig. 1), we designed and synthesized a series of indazole-based compounds as AB-FUBINACA/Rimonabant hybrids, containing the Lonidamine

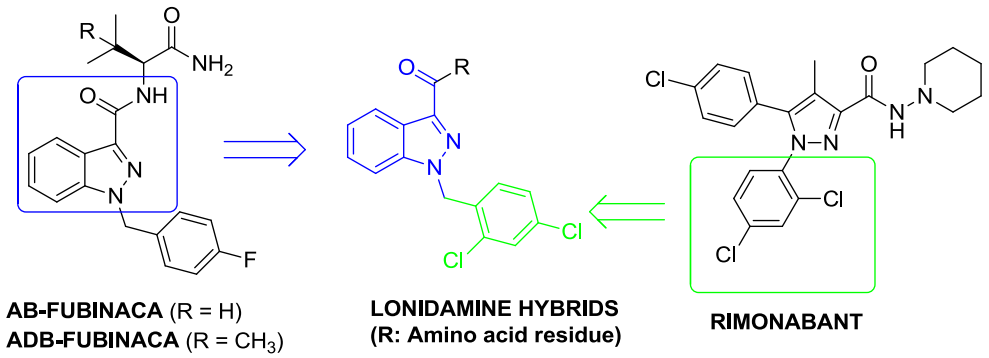



**Fig. 1** Structures of Lonidamine and related compounds ADB-FUBINACA, AB-FUBINACA

scaffold coupled to hydrophobic amino acid residues (*tert*-Leu, Leu, Val). Moreover, we also explored the *C*-terminal function of the novel derivatives as acids, amides or methyl esters, in order to understand if this portion is relevant in the biasing of the potency and efficacy of the novel hybrid compounds (Fig. 1). A further study has also been conducted on the Lonidamine scaffold and the *C*-terminal modified derivatives **LONI A-C** in order to verify if the Lonidamine itself and its derivatives could possess any initial cannabimimetic activity.

## Results

### Chemistry

Compounds **LONI 1-9** were obtained in good yields by standard solution phase peptide synthesis via EDC/HOBt-mediated condensation in the presence of NMM as a base, using Boc strategy (Mollica et al. 2013); *N*-Boc deprotection was carried out by TFA treatment (Scheme 1). The Lonidamine amide (**LONI B**) and methyl ester (**LONI C**) derivatives were also prepared according to the general procedures D and A, respectively (Matteucci et al. 2007), for the biological assays. Compounds **LONI2, 3, 5, 6, 9** have been purified by column chromatography on silica gel and the final products have been characterized by LRMS, $^{1}$H- and $^{13}$C-NMR; purity of the final products was determined by analytical RP-HPLC and resulted to be > 95% (see supporting data). The enantiomeric excess of all novel compounds has been checked by chiral HPLC on CHIRALPAK IA stationary phase, as previously reported (Mollica et al. 2011).

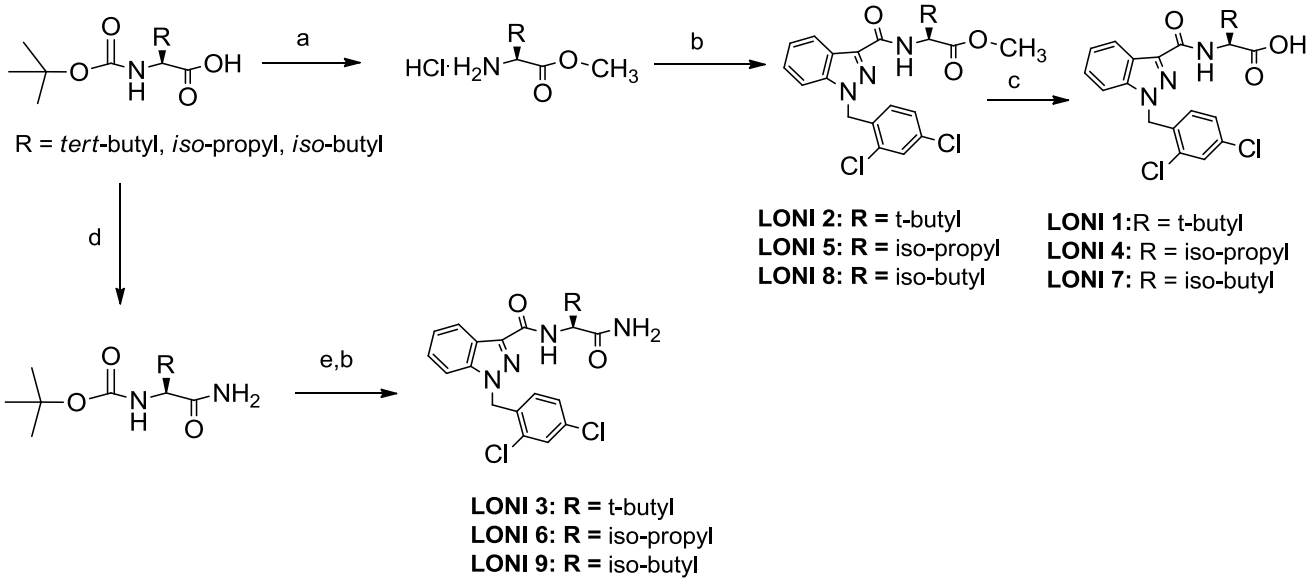
### Receptor binding assays

#### Competition binding experiments

Competition binding experiments were performed to compare the ability of the parent ligand Lonidamine (**LONI A** for the sake of clarity in the biological assays), **LONI B, C** derivatives and the novel compounds **LONI 1-9**, to inhibit the binding of [$^{3}$H]WIN55212-2 to a rat brain membrane homogenate. The binding affinities of **LONI A-C** were found to be very low ($K_i$ > 1 μM) while the Lonidamine analogs **LONI 1-9** showed a wide range of binding to CB receptors from subnanomolar ($K_i$ = 0.8 nM) to low nanomolar ($K_i$ = 88 nM) affinity. **LONI 1**, **LONI 2**, **LONI 4** and **LONI 5** showed the highest affinity for CB receptors (Fig. 2).

#### [$^{35}$S]GTPγS binding experiments

In order to determine whether the parent ligand and its derivatives are able to stimulate the CB receptor-associated G proteins, these compounds were subjected to ligand-stimulated [$^{35}$S]GTPγS binding assays in rat brain membrane homogenate. In in vitro functional assays **LONI A-C** did not show G protein stimulation below the micromolar concentration range. **LONI 2**, **LONI 5** and **LONI 8** efficiently stimulated the G proteins, demonstrating high potency ($EC_{50}$ from 8 to 33 nM) and high stimulatory activity ($E_{max}$ from 122 to 143%). **LONI**




**Scheme 1** Synthesis of **LONI 1-9**. a) $SOCl_2$, MeOH, 0 °C for 10 min., then r.t. overnight. b) Lonidamine, HOBt, EDC·HCl, NMM, DMF at r.t. overnight. c) 1 M NaOH in MeOH at r.t. d) isobutyl chloroformate, NMM, $NH_3$ in THF at −20 °C, then r.t. overnight. e) TFA:DCM = 1:1 at r.t., 1 h

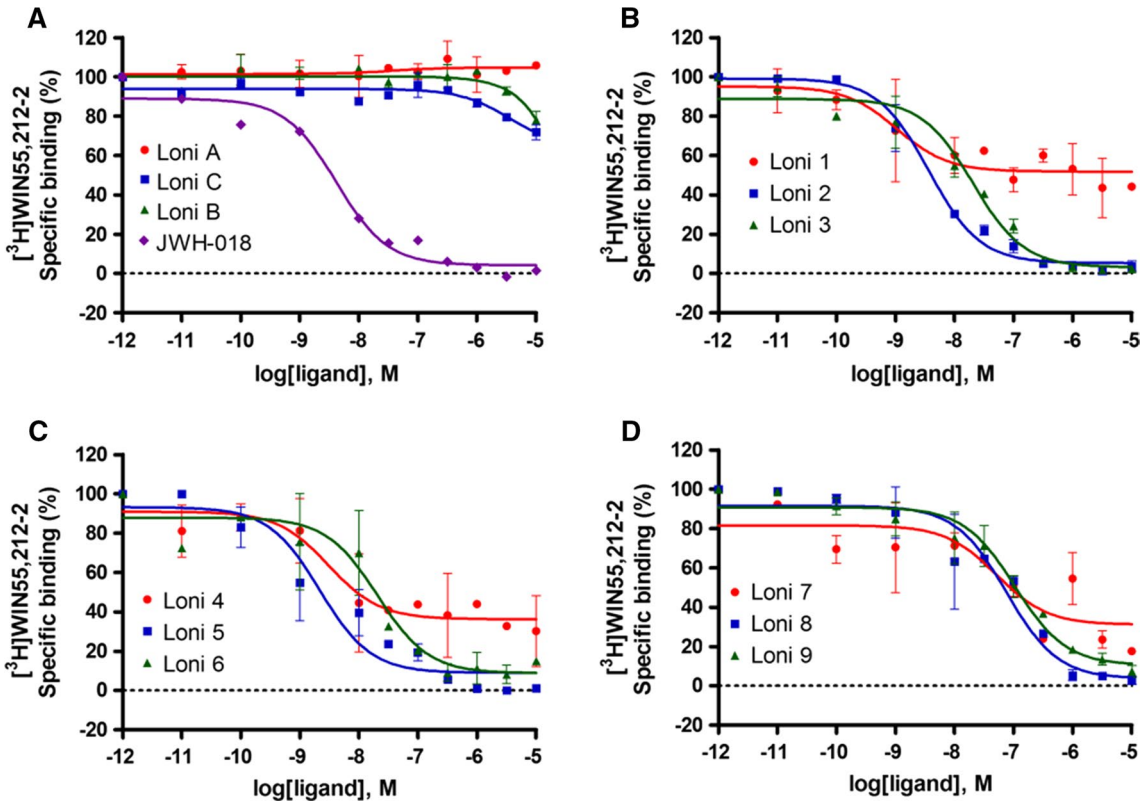



**Fig. 2** Cannabinoid receptor binding affinity of **LONI A-C** and **LONI 1-9** in [$^3$H]WIN55,212-2 competition binding assays on rat whole brain membrane homogenates. Figures represent the specific binding of the radioligand in percentage in the presence of increasing concentrations (from $10^{-11}$ to $10^{-5}$ M) of the indicated ligands. Data are expressed as percentage of mean specific binding ± S.E.M. ($n \geq 3$). The affinity values of the unlabeled compounds are indicated in Table 1

**3**, **LONI 6** and **LONI 9** were partial agonists ($E_{max}$ from 120 to 139%) with low potency ($EC_{50}$ from 12 6 nM to 317 nM), while the introduction of the carboxyl function in **LONI 1**, **LONI 4** and **LONI 7** resulted in weak inverse agonist activity with similar parameters: the maximum efficacy was between 82 and 86%, and their potency was between 1.8 and 3.2 μM (Fig. 3a–d, Table 1).

In the next step, the compounds were evaluated for CB1/CB2 receptor selectivity by [$^{35}$S]GTPγS binding assays. The G protein stimulatory effect of compounds **LONI 1–9** (10 μM) was reduced by the CB1 receptor antagonist/inverse agonist rimonabant (10 μM) and by the CB2 receptor antagonist/inverse agonist AM630 (10 μM). **LONI 3** and **LONI 9** exhibited 6-fold selectivity for CB1 receptor, while **LONI 4** demonstrated only 3-fold selectivity for CB1 receptor. The other compounds have 4-5-fold selectivity for the CB1 receptor (Fig. 4).

## Discussion

Rimonabant is an anti-obesity drug approved in Europe in 2006 but withdrawn worldwide in 2008 due to serious psychiatric side effects. The novel molecules here presented come from the hybridization of the indazol-carboxylic portion of the AB/ADB-FUBINACA with the 2,4-dichloro benzyl moiety present in Rimonabant, with the aim to discover novel SCs. It is worth noting that the scaffold of final compounds obtained by the combination of these two features; it is enclosed in the structure of the drug Lonidamine. Thus, we initially demonstrated that Lonidamine and its *C*-terminus derivatives do not possess any CB receptor affinity. In light of the binding assay data, the following SAR have been defined:

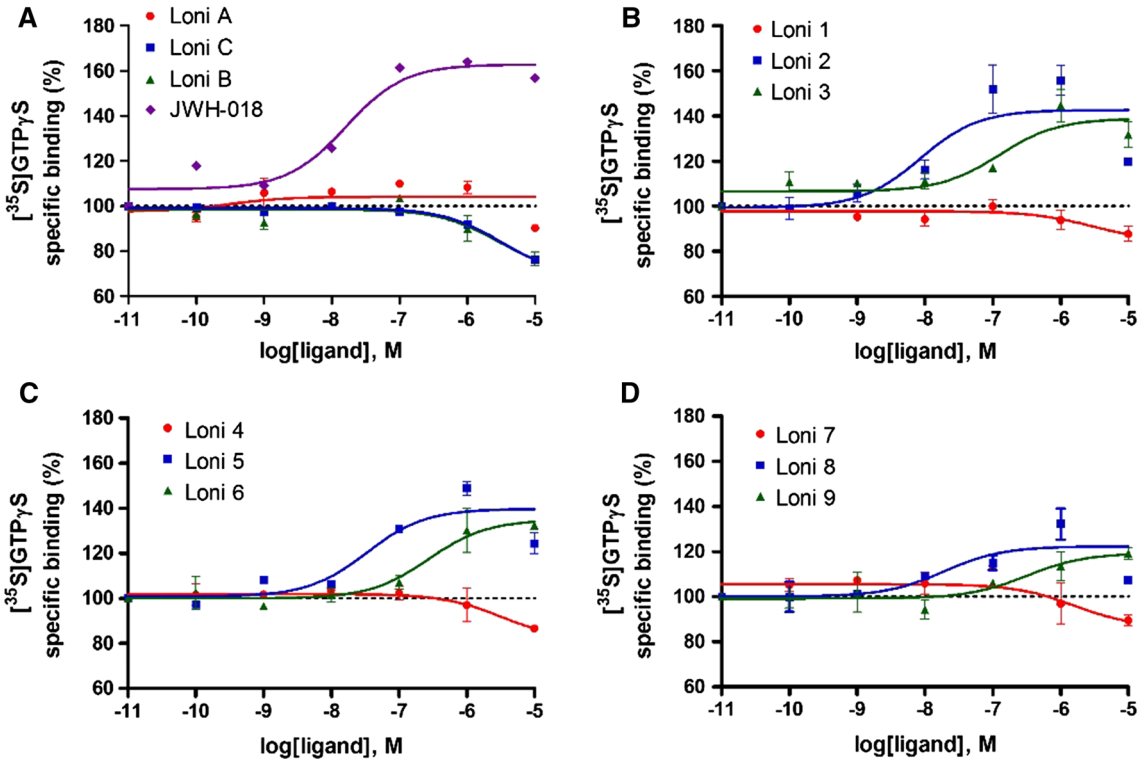



**Fig. 3** G protein stimulatory effects of the indicated ligands in [$^{35}$S]GTPγS binding assays in rat brain membrane homogenates. Figures represent relative specific binding of [$^{35}$S]GTPγS in the presence of increasing concentrations (from $10^{-10}$ to $10^{-5}$ M) of the indicated compounds. Data are expressed as percentage of mean specific binding ± S.E.M. ($n \geq 3$). The maximum efficacy and potency values of the unlabeled compounds are indicated in Table 1

**Table 1** Inhibitory constant values and signaling properties of Lonidamine-based compounds

| Compounds | Sequence | Binding assay | GTP stimulation assay | |
|---|---|---|---|---|
| | | $K_i$ (nM)* | $E_{max}$ (%) ± SEM | $EC_{50}$ (nM) |
| JWH-018 | | 3.5 | 163 ± 5.2 | 16 |
| **LONI A** | Lonidamine | – | – | – |
| **LONI B** | Lonidamine-$NH_2$ | > 1 μM | 70 ± 8.7 | > 1 μM |
| **LONI C** | Lonidamine-$OCH_3$ | > 1 μM | 69 ± 2.6 | > 1 μM |
| **LONI 1** | Lonidamine-*tert*-Leu-OH | 0.8 | 84 ± 6.6 | > 1 μM |
| **LONI 2** | Lonidamine-*tert*-Leu-$OCH_3$ | 3.1 | 143 ± 5.7 | 8.4 |
| **LONI 3** | Lonidamine-*tert*-Leu-$NH_2$ | 17 | 139 ± 4.5 | 126 |
| **LONI 4** | Lonidamine-Val-OH | 2.6 | 82 ± 10.6 | > 1 μM |
| **LONI 5** | Lonidamine-Val-$OCH_3$ | 1.8 | 140 ± 3.7 | 33 |
| **LONI 6** | Lonidamine-Val-$NH_2$ | 17 | 135 ± 4.9 | 273 |
| **LONI 7** | Lonidamine-Leu-OH | 43 | 86 ± 9.3 | > 1 μM |
| **LONI 8** | Lonidamine-Leu-$OCH_3$ | 63 | 122 ± 4.6 | 19 |
| **LONI 9** | Lonidamine-Leu-$NH_2$ | 88 | 120 ± 5.9 | 317 |

$K_i$ values were calculated as $K_i = EC_{50}/(1 + [\text{ligand}]/K_d)$, where $K_d([^3H]WIN55212\text{-}2) = 10.1$ nM. The corresponding displacement curves are shown in Fig. 2. The $E_{max}$ and $EC_{50}$ values were calculated from the dose–response curves of Fig. 3. *Mean of three independent measurements (S.D. values are in the range of 5–10%)

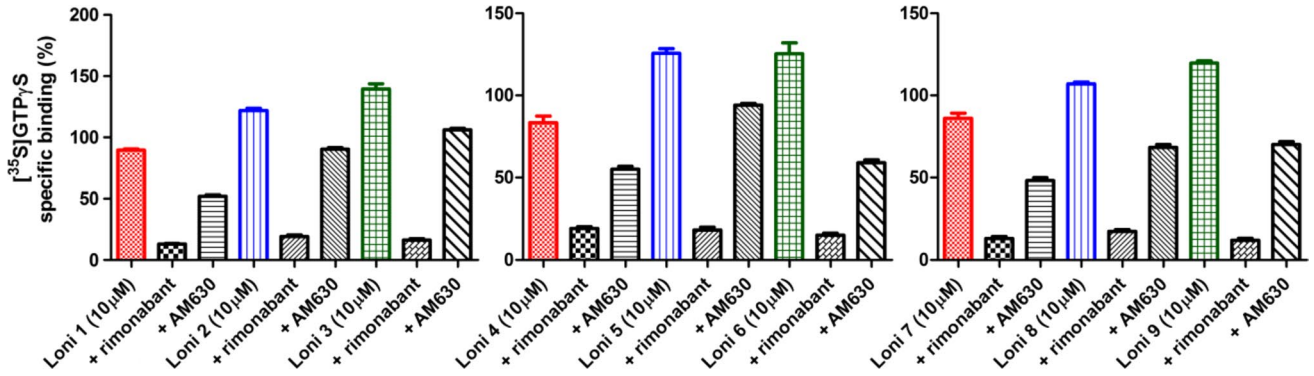



**Fig. 4** The CB1/CB2 cannabinoid receptors selectivity of indicated ligands in [$^{35}$S]GTPγS binding assays in rat brain membrane homogenates. The specific binding of [$^{35}$S]GTPγS is represented in percentage in the presence or absence of 10 μM rimonabant or AM630. Data are expressed as percentage of mean specific binding ± S.E.M. (n ≥ 3)

(1) Lonidamine derivatives **LONI A-C** are not able to bind to CB receptors; thus, the 2,4-dichlorobenzyl indazole scaffold alone does not possess any cannabimimetic potential; (2) **LONI B** and **C** may stimulate the receptor-associated G proteins as weak inverse antagonists, but the mechanism is aspecific and not related to CB receptors; (3) In our in vitro experimental model, the investigated ligands competed for [$^{3}$H]WIN55212-2 binding sites with the following order of affinity: **LONI 1 > LONI 5 > LONI 4 > LONI 2 > LONI 3**, **LONI 6 > LONI 7 > LONI 8 > LONI 9** (Table 1, Fig. 2a–d). The presence of the terminal carboxylic function and *tert*-butyl side chain or *iso*propyl containing amino acids seems to be essential for high CB receptor binding affinity; (4) **LONI 2**, **LONI 5** and **LONI 8** incorporating the methyl ester function possess high potency ($EC_{50}$ from 8 to 33 nM) and high stimulatory activity ($E_{max}$ from 122 to 143%). **LONI 3**, **LONI 6** and **LONI 9** incorporating the amide function behave as partial agonists ($E_{max}$ from 120% to 139%) with low potency ($EC_{50}$ from 126 to 317 nM). The introduction of the carboxyl function in **LONI 1** and **LONI 7** determines weak inverse agonist activity on CB1 receptor; (5) The presence of different hydrophobic groups on the amino acid side chains influences the G protein stimulation efficacy; (6) From the [$^{35}$S]GTPγS binding assays, a range of 3-6 fold CB1 receptor selectivity was observed for **LONI 1-9**. Two compounds incorporating the amide terminal group (**LONI 3** and **LONI 9**) and one bearing the methyl ester group (**LONI 5**) show the highest selectivity for CB1 receptor, while lower selectivity was revealed for **LONI 2**, **4, 6**, **7**, **8**. By the analysis of the results shown in Fig. 4, the free carboxylic-terminus seems to determine the lowest selectivity among the whole series; (7) The *C*-terminus amide group appears to be the major responsible for CB1 receptor selectivity of **LONI 3** and **LONI 9** being the side chain of the amino acidic residues not determinant, as for the SC ADB-FUBINACA. Only amide **LONI 6,** bearing the isopropyl side chain, appears to be the lowest selective compound on the contrary, the isopropyl group does not seem to be responsible for such loss of selectivity for the methyl ester compounds, where the most unselective compound is **LONI 8,** bearing the isobutyl side chain. Thus, the presence of a different hydrophobic function on the amino acid side chain has an unclear role in determining the loss of receptor selectivity of compounds; however, the substitution at the C-terminus appears to be the main player to determine the receptor affinity and efficacy indeed all the free carboxylic acids has the lowest binding activity and the worst capacity to stimulate the G protein activation, whereas the methyl esters have a good receptor affinity and the best efficacy to stimulate the G protein activation; finally, the amide terminus showed intermediate properties; (8) The presence of two chlorine atoms on the benzyl group does not influence the CB1 receptor profile, considering that high affinity found for **LONI 3, 9** similar to that of AB-FUBINACA and ADB-FUBINACA containing a fluorine atom shows low nanomolar CB1 receptor affinity in vitro and CB1 receptor selectivity (Uchiyama et al. 2013a, b; Nath et al. 2016).

## Conclusion

In summary, the hybrids ADB/AB-FUBINACA/Rimonabant possess a broad range of activities depending on the amino acid side chain and the *C*-terminus substitution. **LONI 1, 2, 4** and **5** showed the highest affinity for CB receptors with low nanomolar inhibitory constant ($K_i$) values; **LONI 2, 5** and in **less extent LONI 8** efficiently stimulated the G proteins, demonstrating high potency ($EC_{50}$ from 8 to 33 nM) and high stimulatory activity ($E_{max}$ from 122 to 143%). **LONI 3, 6** and **9** are partial agonists ($E_{max}$ from 120 to 139%) with low potency ($EC_{50}$ from 126 to 317 nM), while the introduction of the carboxyl function in **LONI 1, 4** and **7** gave inverse

agonists with very similar parameters. **LONI 3** and **LONI 9** exhibited the best (6-fold) selectivity for CB1 receptor, while **LONI 4** demonstrated the lowest 3-fold selectivity for CB1 receptor. The other compounds have 4/5-fold selectivity for the CB1 receptor. The similarity among the SAR studies of FUBINACA family of compounds and that of the novel indazole-based compounds highlights the presence of common key features responsible for CB1 receptor selectivity and potency. Similarly, with the classic agonist cannabinoid drugs such as $\Delta^9$-THC, compounds with agonist activity toward CB1 receptor (**LONI 2, 5, 8**) could be involved in the treatment of pain and act as anorectic drugs to control the loss of appetite and weight in AIDS patients, and for palliative cares of untreatable cancer patients, while compounds with inverse agonist activity (**LONI 1,4,7**) similarly to Rimonabant could be used to treat chemotherapy-related nausea, vomiting, and as anti-obesity drugs. Although promising preliminary data have been found, compounds **LONI 2,5,8** bear the C-terminal methyl ester group, which is considered to be highly labile; thus, pharmacokinetic and toxicological properties of our novel compounds must be addressed by further in vitro and in vivo studies taking in consideration the SAR studies herein reported.

## Experimental protocols

### Materials and methods

Lonidamine, solvents, reagents and amino acids, Boc-*tert*-Leu-OH, Boc-Val-OH, Boc-Leu-OH, are commercially available and were purchased from Sigma-Aldrich (Milano, Italy). The structures of the intermediates and the final compounds were confirmed by $^1$H-NMR and $^{13}$C-NMR spectra recorded on a 300 MHz Varian Inova spectrometer (Varian Inc., Palo Alto, CA). Chemical shifts are reported in parts per million (δ) downfield from the internal standard tetramethylsilane ($Me_4Si$). Purifications via column chromatography if required were performed on Merck 60, 70-230 mesh ASTM silica gel column. Homogeneity was confirmed by analytical thin-layer chromatography TLC on silica gel Merck 60 F254 (Merck, Darmstadt, Germany). Visualization was carried out under UV irradiation (254 and 365 nm) and by iodine staining. Where mixtures of solvents are specified, the ratios are given as volume/volume. Unless otherwise indicated, all aqueous solutions were saturated. The purity of each final product has been established by analytical RP-HPLC (C18-bonded 4.6 mm × 150 mm) at a flow rate of 1 mL/min, using as eluent a gradient of $H_2O$/acetonitrile 0.1% TFA ranging from 10% acetonitrile to 90% acetonitrile in 25 min and was found to be > 95%. UV detection at 254 nm was chosen for analytical HPLC. Chiral HPLC analysis has been performed to check the enantiomeric excess of each sample following standard conditions (Zhang et al. 2005): Column CHIRALPAK IA 250 mm L 4.6 mm I.D.; detection UV at 254 nm; flow rate: 3 mL/min; injection of 20 µL of a solution containing 1 mg/mL of analyte in methanol; gradient elution with a mixture of hexane/2-butanol/0.1%TFA (2-butanol from 5 to 80% in 32 min, see the Supplementary data). All reactions involving air- or moisture-sensitive compounds were performed under a nitrogen atmosphere using dried glassware and syringes. Temperatures are reported in °C. Mass spectra were recorded on an LCQ (Finnigan-Mat) ion trap mass spectrometer (San Jose, CA) equipped with an electrospray ionization (ESI) source. The capillary temperature was set at 300 °C and the spray voltage at 3.5 kV. The fluid was nebulized using nitrogen as both the sheath gas and the auxiliary gas (Mollica et al. 2015).

### General procedures

**General procedure A: methyl ester formation** To a solution of Boc-amino acid (200 mg) or Lonidamine (200 mg, 0.62 mmol) in dry MeOH (10 mL) cooled to − 15 °C, thionyl chloride (1.24 mmol, 0.1 mL) was added dropwise. The reaction mixture was kept at − 15 °C in stirring for 30 min, then at r.t. overnight. The mixture was evaporated under reduced pressure to give the crude methyl ester derivative, which was used for the next reaction without further purification. This procedure was also applied to the synthesis of **LONI C** derivative, previously characterized in literature (Gunda et al. 2009; Angapelly et al. 2017).

**General procedure B: coupling reaction** To a stirred solution of Lonidamine (200 mg, 0.62 mmol) and HOBt anhydrous (84 mg, 0.61 mmol) in DMF (5 mL), EDC·HCl (119 mg, 0.61 mmol) and NMM (0.61 mmol, 0.07 mL) were added at 0 °C, followed by an ice-cold suspension of amino acid derivative as hydrochloride or TFA salt and NMM (0.61 mmol, 0.07 mL) in DMF (5 mL). After 10 min at 0 °C, the reaction was stirred at r.t overnight. Later, the reaction mixture was evaporated to dryness and the residue was taken up in EtOAc. The organic phase was washed with 5% citric acid, $NaHCO_3$ s.s. and NaCl s.s., dried on $Na_2SO_4$ anhydrous and evaporated to obtain the desired crude white product.

**General procedure C: saponification** To a solution of Lonidamine-based compound in MeOH (15 mL), NaOH 1 M (6 eq., 1.04 mL) was added under stirring at r.t. When the reaction reached the completeness, the solution was acidified with HCl 1 M and extracted with EtOAc. The organic phase was washed with water, dried and evaporated to yield the white powder as a sole product.

**General procedure D: amidation** Isobutyl chloroformate (1.93 mmol, 0.25 mL) and NMM (2.31 mmol, 0.26 mL)

were added dropwise to a solution of the Boc-amino acid (200 mg) or Lonidamine (200 mg, 0.62 mmol) in THF (10 mL) stirring at −20 °C for 30 min. $NH_4OH$ solution (2.1 eq., 0.37 mL) was added to the reaction stirring at −20 °C for 30 min, then the reaction was maintained at r.t. overnight. The reaction mixture was evaporated to dryness and the residue extracted with EtOAc. The organic phase was washed with 5% citric acid, $NaHCO_3$ s.s. and NaCl s.s., dried on $Na_2SO_4$ anhydrous and evaporated to obtain the desired amide derivative as a crude white product. This procedure was also applied to the synthesis of **LONI B** derivative previously characterized in the literature (Angapelly et al. 2017).

*(S)-2-(1-(2,4-dichlorobenzyl)-1H-indazole-3-carboxamido)-3,3-dimethylbutanoic acid* **(LONI 1)**. Boc-*tert*-Leu-OH was transformed in its methyl ester derivative following the general procedure A. The so-obtained product was coupled with Lonidamine following the general procedure B, to give an intermediate product (**LONI 2**), which was converted in **LONI 1**, following the general procedure C; overall yield 30%, HPLC $t_R = 20.69$ min. $^1$H NMR (300 MHz, DMSO-$d_6$) δ 8.17 (d, 1H, H5'), 7.74 (d, 1H, H4), 7.70 (s, 1H, H3'), 7.59 (d, 1H, NH), 7.47 (t, 1H, H5), 7.28–7.37 (m, 2H, H6 and H6'), 6.84 (d, 1H, H7), 5.85 (s, 2H, H1), 4.29–4.32 (m, 1H, $CH^{\alpha}$ L-*tert*-Leu), 0.98 (s, 9H, 3×$CH_3$ 3 L-*tert*-Leu). $^{13}$C NMR (300 MHz, DMSO-$d_6$) δ 174.04, 161.52, 141.51, 137.50, 133.55, 133.43, 130.90, 129.57, 128.26, 127.75, 123.42, 122.51, 122.22, 111.02, 108.87, 101.85, 60.25, 50.16, 34.69, 27.11. LRMS calcd. for $C_{21}H_{21}Cl_2N_3O_3$: 433.1; found: 456.1 $[M+Na]^+$.

*(S)-methyl 2-(1-(2,4-dichlorobenzyl)-1H-indazole-3-carboxamido)-3,3-dimethylbutanoate* **(LONI 2)**: Boc-*tert*-Leu-OH was transformed in its methyl ester derivative following the general procedure A. The so-obtained product was coupled with Lonidamine following the general procedure B, to give **LONI 2** as crude material. The residue was chromatographed on silica gel column using EtOAc/n-hexane = 20:80 as eluent. $R_f = 0.68$ (EtOAc/n-hexane 30:70), overall yield 25%, HPLC $t_R = 22.24$ min. $^1$H-NMR (300 MHz, DMSO-$d_6$) δ 8.13 (d, 1H, H5'), 7.76 (d, 1H, H4), 7.70 (s, 1H, H3′), 7.65 (d, 1H, NH), 7.31–7.37 (m, 2H, H6 and H6′), 6.87 (d, 1H, H7), 5.85 (s, 2H, H1), 4.43–4.47 (m, 1H, $CH^{\alpha}$ L-*tert*-Leu), 3.66 (s, 3H, $OCH_3$ L-*tert* Leu), 0.98 (s, 9H, 3×$CH_3$ L-*tert*-Leu). $^{13}$C NMR (300 MHz, DMSO-$d_6$) δ 171.71, 161.76, 141.50, 137.50, 133.81, 133.70, 133.58, 130.99, 129.57, 128.26, 127.80, 123.51, 122.49, 122.09, 111.06, 59.94, 52.28, 50.18, 34.66, 26.85. LRMS calcd. for $C_{22}H_{23}Cl_2N_3O_3$: 447.1; found: 470.2 $[M+Na]^+$.

*(S)-N-(1-amino-3,3-dimethyl-1-oxobutan-2-yl)-1-(2,4-dichlorobenzyl)-1H-indazole-3-carboxamide* (**LONI 3**). Boc-L-*tert*-Leu-OH has been converted to its amide derivative following the general procedure D. The Boc protection has been removed with a mixture of TFA/DCM = 1:1 at r.t. for 1 h, then the intermediate TFA salt was coupled with Lonidamine, following the general procedure B. The crude product was chromatographed on silica gel column using EtOAc/n-hexane from 20:80 to 40:60 as eluent, to give **LONI 3** as sole product. $R_f = 0.11$ (EtOAc/n-hexane 3:7), overall yield 90%, HPLC $t_R = 19.95$ min. $^1$H NMR (300 MHz, DMSO-$d_6$) δ 8.18 (d, 1H, H5′), 7.73 (d, 1H, H4), 7.71, 7.26 (brs, 2H, $NH_2$),7.69 (s, 1H, H3′), 7.54 (d, 1H, NH), 7.46 (t, 1H, H5), 7.30–7.36 (m, 2H, H6 and H6′), 5.84 (s, 2H, H1), 4.41–4.45 (m, 1H, $CH^{\alpha}$ L-*tert*-Leu), 0.95 (s, 9H, 3×$CH_3$ L-*tert*-Leu). $^{13}$C NMR (300 MHz, DMSO-$d_6$) δ 172.12, 161.24, 141.55, 137.90, 133.76, 133.52, 130.82, 129.57, 128.24, 127.72, 123.38, 122.52, 122.28, 110.99, 59.15, 50.16, 35.00, 27.06. LRMS calcd. for $C_{21}H_{22}Cl_2N_4O_2$: 432.1: found: 455.2 $[M+Na]^+$.

*(S)-2-(1-(2,4-dichlorobenzyl)-1H-indazole-3-carboxamido)-3-methylbutanoic acid* **(LONI 4)**. Boc-Val-OH was transformed in its methyl ester derivative following the general procedure A. The so-obtained product was coupled with Lonidamine following the general procedure B, to give an intermediate product (**LONI 5**), which was converted in **LONI 4** following the general procedure C; overall yield 88%, HPLC $t_R = 20.17$ min. $^1$H NMR (300 MHz, DMSO-$d_6$) δ 8.17 (d, 1H, H5′), 7.86 (d, 1H, H4), 7.75 (d, 1H, NH), 7.70 (s, 1H, H3′), 7.47 (t, 1H, H5), 7.28–7.36 (m, 2H, H6 and H6′), 6.79 (d, 1H, H7) 5.85 (s, 2H, H1), 4.35–4.39 (m, 1H, $CH^{\alpha}$ Val), 2.20–2.22 (m, 1H, $\underline{CH}(CH_3)_2$), 0.94 (t, 6H, 2×$CH_3$ 2 Val). $^{13}$C NMR (300 MHz, DMSO-$d_6$) δ 173.25, 161.94, 141.50, 137.90, 133.83, 133.72, 133.40, 130.68, 129.54, 128.27, 127.74, 123.38, 122.61, 122.25, 110.94, 57.39, 50.12, 30.50, 19.60, 18.72. LRMS calcd. for $C_{20}H_{19}Cl_2N_3O_3$: 419.0; found: 442.1 $[M+Na]^+$.

*(S)-methyl 2-(1-(2,4-dichlorobenzyl)-1H-indazole-3-carboxamido)-3-methylbutanoate* **(LONI 5).** Boc-Val-OH was transformed in its methyl ester derivative following the general procedure A. The so-obtained product was coupled with Lonidamine following the general procedure B to give the crude product which was chromatographed on silica gel column using EtOAc/n-hexane from 20:80 to 30:70 as eluent. **LONI 5** was obtained in 76% overall yield, $R_f = 0.67$ (EtOAc/n-hexane 30:70), HPLC $t_R = 21.64$ min. $^1$H NMR (300 MHz, DMSO-$d_6$) δ 8.14-8.18 (m, 2H, H5′ and H4), 7.74 (d, 1H, NH), 7.70 (s, 1H, H3′), 7.47 (t, 1H, H5), 7.28–7.37 (m, 2H, H6 and H6′), 6.77 (d, 1H, H7), 5.84 (s, 2H, H1), 4.36–4.40 (m, 1H, $CH^{\alpha}$ Val), 3.65 (s, 3H, $OCH_3$ Val), 2.21-2.25 (m, 1H, $\underline{CH}(CH_3)_2$), 0.90 (dd, 6H, 2×$CH_3$ 2 Val). $^{13}$C NMR (300 MHz, DMSO-$d_6$) δ 172.36, 162.16, 141.48, 137.87, 133.78, 133.73, 133.37, 130.65, 129.49, 128.24,

127.71, 123.34, 122.70, 122.26, 110.88, 57.76, 52.30, 50.12, 30.30, 19.48, 19.08. LRMS calcd. for $C_{21}H_{21}Cl_2N_3O_3$: 433.1; found: 456.1 $[M+Na]^+$.

*(S)-N-(1-amino-3-methyl-1-oxobutan-2-yl)-1-(2,4-dichlorobenzyl)-1H-indazole-3-carboxamide* **(LONI 6).** Boc-Val-OH has been converted to its amide derivative following the general procedure D. The Boc protection has been removed with a mixture of TFA/DCM = 1:1 at r.t. for 1 h, then the intermediate TFA salt was coupled to Lonidamine, following the general procedure B. The crude product was chromatographed on silica gel column using EtOAc/n-hexane = 80:20 as eluent, to give **LONI 6** in 30% overall yield. $R_f$ = 0.67 (EtOAc/n-hexane 80:20), HPLC $t_R$ = 19.69 min. $^1$H NMR (300 MHz, DMSO-$d_6$) δ 8.18 (d, 1H, H5′), 7.74 (d, 1H, H4), 7.68–7.71 (2H, m, NH and H3′), 7.22, 7.64 (brs, 2H, $NH_2$), 7.46 (t, 1H, H5), 7.27–7.36 (m, 2H, H6 and H6′), 6.79 (d, 1H, H7), 5.84 (s, 2H, H1), 4.35–4.40 (m, 1H, $CH^{\alpha}$ Val), 2.03-2.09 (m, 1H, $\underline{CH}(CH_3)_2$), 0.88 (dd, 6H, 2×$CH_3$ 2 Val). $^{13}$C NMR (300 MHz, DMSO-$d_6$) δ 172.97, 161.56, 141.53, 138.02, 133.83, 133.72, 133.41, 130.68, 129.55, 128.27, 127.71, 123.33, 122.58, 122.32, 110.94, 57.33, 50.12, 31.66, 19.82, 18.44. LRMS calcd. for $C_{20}H_{20}Cl_2N_4O_2$: 418.1; found: 441.0 $[M+Na]^+$.

*(S)-2-(1-(2,4-dichlorobenzyl)-1H-indazole-3-carboxamido)-4-methylpentanoic acid* **(LONI 7).** Boc-Leu-OH was transformed in its methyl ester derivative following the general procedure A. The so-obtained product was coupled with Lonidamine following the general procedure B. The desired product **LONI 7** was prepared following the general procedure C starting from the intermediate product (**LONI 8**); overall yield 93%, HPLC $t_R$ = 20.64 min. $^1$H NMR (300 MHz, DMSO-$d_6$) δ 12.34 (1H, s, OH), 8.33 (d, 1H, H5′), 8.18 (d, 1H, H4), 7.74 (d, 1H, NH), 7.71 (s, 1H, H3′), 7.46 (t, 1H, H5), 7.27–7.35 (m, 2H, H6 and H6′), 6.73 (d, 1H, H7), 5.84 (s, 2H, H1), 4.46–4.54 (m, 1H, $CH^{\alpha}$ Leu), 1.53–1.68 (m, 3H, $\underline{CH_2}CH(CH_3)_2$, $\underline{CH}(CH_3)_2$), 0.86–0.82 (dd, 6H, 2×$CH_3$ 2 Leu). $^{13}$C NMR (300 MHz, DMSO-$d_6$) δ 174.42, 162.13, 141.51, 138.20, 133.92, 133.66, 133.25, 130.47, 129.51, 128.29, 127.68, 123.24, 122.76, 122.41, 110.82, 50.44, 50.07, 24.97, 23.39, 21.75. LRMS calcd. for $C_{21}H_{21}Cl_2N_3O_3$: 433.1; found: 456.1 $[M+Na]^+$.

*(S)-methyl 2-(1-(2,4-dichlorobenzyl)-1H-indazole-3-carboxamido)-4-methylpentanoate* **(LONI 8).** Boc-Leu-OH was transformed in its methyl ester derivative following the general procedure A. The so-obtained product was coupled with Lonidamine following the general procedure B to give **LONI 8** in 95% overall yield as the sole product. $R_f$ = 0.61 (EtOAc/n-hexane 70:30), HPLC $t_R$ = 21.88 min. $^1$H NMR (300 MHz, DMSO-$d_6$) δ 8.57 (d, 1H, H5′), 8.17 (d, 1H, H4), 7.74 (d, 1H, NH), 7.71 (s, 1H, H3′), 7.46 (t, 1H, H5), 7.27–7.36 (m, 2H, H6 and H6′), 6.71 (d, 1H, H7), 5.84 (s, 2H, H1), 4.52–4.60 (m, 1H, $CH^{\alpha}$ Leu), 3.62 (s, 3H, $OCH_3$ Leu), 1.80–1.88 (m, 1H, $\underline{CH}(CH_3)_2$), 1.54–1.64 (m, 2H, $\underline{CH_2}CH(CH_3)_2$), 0.87 (dd, 6H, 2×$CH_3$ 2 Leu). $^{13}$C NMR (300 MHz, DMSO-$d_6$) δ 173.32, 162.28, 141.51, 138.08, 133.89, 133.67, 133.25, 130.47, 129.51, 128.29, 127.69, 123.28, 122.81, 122.38, 110.82, 52.39, 50.50, 50.08, 24.85, 23.31, 21.68. LRMS calcd. for $C_{22}H_{23}Cl_2N_3O_3$: 447.1; found: 470.0 $[M+Na]^+$.

*(S)-N-(1-amino-4-methyl-1-oxopentan-2-yl)-1-(2,4-dichlorobenzyl)-1H-indazole-3-carboxamide* **(LONI 9).** Boc-Leu-OH has been converted to its amide derivative following the general procedure D. The Boc protection has been removed with a mixture of TFA/DCM = 1:1 at r.t. for 1 h, then the intermediate TFA salt was coupled with Lonidamine, following the general procedure B to give a crude product. The residue was chromatographed on silica gel column using EtOAc/n-hexane = 60:40 as eluent to give **LONI 9** in 83% overall yield; $R_f$ = 0.58 (EtOAc/n-hexane 7:3), HPLC $t_R$ = 19.87 min. $^1$H NMR (300 MHz, DMSO-$d_6$) δ 8.19 (d, 1H, H5′), 7.93 (d, 1H, NH), 7.73 (d, 1H, H4), 7.69 (s, 1H, H3′), 7.09, 7.52 (brs, 2H, $NH_2$) 7.46 (t, 1H, H5), 7.27–7.35 (m, 2H, H6 and H6′), 6.77 (d, 1H, H7), 5.82 (s, 2H, H1), 4.50–4.52 (m, 1H, $CH^{\alpha}$ Leu), 1.55–1.63 (m, 3H, $\underline{CH_2}CH(CH_3)_2$ and $\underline{CH}(CH_3)_2$), 0.88 (dd, 6H, 2×$CH_3$ 2 Leu). $^{13}$C NMR (300 MHz, DMSO-$d_6$) δ 174.33, 161.61, 141.51, 138.20, 133.86, 133.70, 133.34, 130.64, 129.52, 128.26, 127.68, 123.25, 122.69, 122.40, 110.85, 51.14, 50.08, 41.85, 24.91, 23.48, 22.27. LRMS calcd. for $C_{21}H_{22}Cl_2N_4O_2$: 432.1, found: 455.1 $[M+Na]^+$.

#### Rat brain membrane preparation

Wistar rats were locally bred and handled according to the European Communities Council Directives (86/609/ECC) and the Hungarian Act for the Protection of Animals in Research (XXVIII.tv. Section 32). Crude membrane fractions were prepared from the brain without cerebellum. Brains were quickly removed from the euthanized rats and directly put in ice-cold 50 mM Tris–HCl buffer (pH 7.4). The collected tissue was then homogenized in 30 volumes (v/w) of ice-cold buffer with a Braun Teflon-glass homogenizer at the highest rpm. The homogenate was centrifuged at 20,000 g for 25 min and the resulting pellet was suspended in the same volume of cold buffer followed by incubation at 37 °C for 30 min to remove endogenous ligands. Centrifugation was then repeated. The final pellets were taken up in five volumes of 50 mM Tris–HCl (pH 7.4) buffer containing 0.32 M sucrose and stored at − 80 °C. Prior to the experiment, aliquots were thawed and centrifuged at 20,000 g for 25 min and then they were resuspended in 50 mM Tris–HCl (pH 7.4), homogenized with a Dounce followed by the

determination of the protein concentration by the method of Bradford.

### Competition binding experiments

Binding experiments were performed at 30 °C for 1 h in 50 mM Tris–HCl buffer pH 7.4 containing 2.5 mM EGTA, 5 mM $MgCl_2$ and 0.5 mg/mL fatty acid free BSA in plastic tubes in a total assay volume of 1 mL that contained 0.3–0.5 mg/mL membrane protein (Mollica et al. 2017).

Competition binding experiments were carried out by incubating rat brain membranes with 1.5 nM of [$^3$H] WIN55212-2 in the presence of increasing concentrations (from $10^{-11}$ M to $10^{-5}$ M) of various competing unlabeled ligands. Non-specific binding was determined in the presence of 10 µM WIN 55212-2. The incubation was terminated by diluting the samples with ice-cold wash buffer (50 mM Tris–HCl, 2.5 mM EGTA, 5 mM $MgCl_2$, 0.5% fatty acid free BSA, pH 7.4), followed by repeated washing and rapid filtration through Whatman GF/B glass fiber filters (Whatman Ltd, Maidstone, England) presoaked with 0.1% polyethyleneimine (30 min before the filtration). Filtration was performed with a 24-well Brandel Cell Harvester (Gaithersburg, MD, USA). Filters were air-dried and immersed into Ultima Gold MV scintillation cocktail and then radioactivity was measured with a TRI-CARB 2100TR liquid scintillation analyzer (Packard).

### Ligand-stimulated [$^{35}$S]GTPγS binding assay

Rat brain membranes (30 µg protein/tube) prepared as described above were incubated with 0.05 nM [$^{35}$S]GTPγS (PerkinElmer) and $10^{-10}$–$10^{-5}$ M unlabeled ligands in the presence of 30 µM GDP, 100 mM NaCl, 3 mM $MgCl_2$ and 1 mM EGTA in 50 mM Tris–HCl buffer (pH 7.4) for 60 min at 30 °C. Basal [$^{35}$S]GTPγS binding was measured in the absence of ligands and set as 100%. Non-specific binding was determined by the addition of 10 µM unlabeled GTPγS and subtracted from total binding. Incubation, filtration and radioactivity measurements of the samples were carried out as described above.

### Data analysis

Results are expressed as mean ± S.E.M. of at least three independent experiments each performed in duplicate. In competition binding studies, the inhibitory constants ($K_i$) were calculated from the inflection points of the displacement curves using non-linear least-square curve fitting and the Cheng–Prusoff equation. All data and curves were analyzed by GraphPad Prism 5.0 (San Diego, CA, USA). In [$^{35}$S]GTPγS binding studies, data were expressed as the percentage stimulation of the specific [$^{35}$S]GTPγS binding over the basal activity and are given as mean ± SEM. Each experiment was performed in triplicate and analyzed with sigmoid dose–response curve fitting to obtain potency ($EC_{50}$) and efficacy ($E_{max}$) values. Statistical comparison was done by the analysis of variance (one-way ANOVA) followed by the Bonferroni multiple comparison test of GraphPad Prism 5.0 (San Diego, CA, USA), $P < 0.05$ was chosen to indicate significant differences.

## Compliance with ethical standards

**Conflict of interest** The authors declare that no competing interests exist.

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

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