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Original article

Tricyclic pyrazoles. Part 6. Benzofuro[3,2-c]pyrazole: A versatile architecture for CB_2 selective ligands

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ABSTRACT

A new series of 1*H*-benzofuro[3,2-*c*]pyrazole-3-carboxamides was synthesized. The novel compounds (**15**–**24**) were evaluated for their affinity to CB_2 and CB_1 cannabinoid receptors. The synthesis of the title compounds takes advantage of the acid-catalysed thermal cyclization of bicyclic hydrazone ethyl 2-(2-(2,4-dichlorophenyl)hydrazono)-2-(6-methyl-3-oxo-2,3-dihydrobenzofuran-2-yl)acetate to tricyclic ethyl 1-(2,4-dichlorophenyl)-6-methyl-1*H*-benzofuro[3,2-*c*]pyrazol-3-carboxylate.

All the obtained derivatives showed high affinity to CB_2 receptors. Moreover, significant selectivity for CB_2 over CB_1 receptors was highlighted for lead derivatives amongst the novel series.

The best binding profiles were determined for homologues bearing monocyclic and bicyclic monoterpenic substituents at the carbamoyl group at 3 position of the pyrazole ring ($K_iCB_2 < 4$ nM). In particular, the isopinocampheyl-substituted derivative **22** exhibited the highest selectivity for CB_2 receptors with K_i values of 3.7 and 2398 nM for CB_2 and CB_1 receptors, respectively.

Preliminary functional assays evidenced CB_2 agonism behaviour for all the assayed novel derivatives.

1. Introduction

In the past few years the CB_2 cannabinoid (CB_2) receptor has emerged as a critical player in the regulation of inflammation and pain [1] as well as in atherosclerosis [2], osteoporosis [3] and demyelinating diseases [4].

The CB_2 receptor, isolated from HL-60 cells (the human monocytic cell line [5]), belongs to the large superfamily of G protein-coupled receptors (GPCRs).

The activation of CB_2 receptor leads to inhibition of adenyl cyclase and to stimulation of mutagen-activated protein kinase pathway [6].

Although the function of this cannabinoid receptor subtype has not been fully elucidated, CB_2 is known to be expressed almost exclusively in tissues of the immune system (spleen, tonsils and lymph nodes) and haematopoietic cells, with high levels of expression in B-cells, natural killer cells, T_4 and T_8 cells and microglial cells [7].

However, recent studies have also intriguingly demonstrated the presence of CB_2 receptors in brain [8], myocardium, cardiomyoblasts and endothelial cells of various origins [9].

The pharmacology of the CB_2 receptor has revealed significant differences compared with that of CB_1 cannabinoid (CB_1) receptor subtype. In particular, a relative absence of the unfavourable central nervous system (CNS) side effects has been observed following the administration of CB_2 cannabinoidergic compounds. In contrast, CB_1 agents acting at CNS level determined psychoactive effects, as in the case of the major component of *Cannabis sativa* L. Δ^9-tetrahydrocannabinol (Δ^9-THC) [10].

These considerations suggest that novel pharmacotherapies targeting CB_2 receptors may have considerable therapeutic potential, with particular emphasis to the treatment of inflammatory and neuropathic pain [1f,11,12], immune disorders [13], and neurodegenerative diseases [14,15].

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Various medicinal chemistry strategies have been adopted to develop selective CB_2 compounds. The identified CB_2 ligands may be classified into at least six different chemical families:(1) traditional cannabinoid ligands with a partially reduced dibenzopyran template (e.g. JWH-133 (**1**) [16] and JWH-361 (**2**) [17]), (2) indoles (e.g. AM-1241 (**3**) [18], GW405833/L768242 (**4**) [19,20], A-796260 (**5**) [21], and AM630 (**6**) [22]), (3) resorcinol compounds (e.g. HU308 (**7**) [23] and AM-1703 (**8**) [24]), (4) pyrazoles (e.g. SR144528 (**9**) [25] and NESS (**10**) [26]), (5) quinolones (e.g. JTE-907 (**11**) [27] and **12** [28]) and (6) triarylbis-sulfones (e.g. Sch. 336 (**13**) [29]) (Fig. 1).

With regard to the pyrazole-based cannabinoids, interesting pharmacological profiles have been evidenced by compounds characterized by 1,4-dihydroindeno[1,2-*c*]pyrazole planar scaffold. *N*-piperidin-1-yl-1-(2,4-dichlorophenyl)-6-methyl-1,4-dihydroindeno[1,2-*c*]pyrazole-3-carboxamide (**10**) demonstrated both marked affinity and remarkable selectivity for CB_2 receptors ($K_iCB_2 = 0.037$ nM; $K_iCB_1/K_iCB_2 = 9810{:}1$) [26]. Moreover, the lead compound as well as a series of corresponding homologues showed CB_2 agonist activity in an *in vitro* model based on the evaluation of the extracellular signal-related kinases (ERK 1/2 or p44/p42-MAPK) expression [31,32].

Structural modification of compound **10** by replacement of the carbamoyl piperidine ring with several cyclic moieties resulted in analogues expressing lower CB_2 receptor affinity, with the exception of the compound *N*-cyclohexyl-1-(2,4-dichlorophenyl)-6-methyl-1,4-dihydroindeno[1,2-*c*]pyrazole-3-carboxamide (**14**) [31]. In fact, the homologue bearing a cyclohexyl carbamoyl group at 3 position of the pyrazole ring showed a K_i value of 7.6 nM for CB_2 receptor and a selectivity expressed as K_iCB_1/K_iCB_2 of 118 (Fig. 2). Moreover, compound **14**, namely NESS400, evidenced significant antinociceptive activity in Spared Nerve Injury (SNI) neuropathic mice by alleviating both mechanical allodynia and thermal hyperalgesia [33].

To further investigate the potentiality of the planar pyrazole tricyclic scaffold to determine CB_2 affinity and selectivity and with the purpose to possibly improve CB_2 binding profile relative to **10**, in a continuing effort to identify the pharmacophoric groups in this class of potent CB_2 ligands, we designed a new series of 1,4-dihydroindeno[1,2-*c*]pyrazole analogues, namely benzofuro[3,2-*c*]pyrazoles, containing an oxygen atom at 4 position in place of a methylene unit (Fig. 3). To ascertain bioisosterism of the novel compounds relative to **10** and homologues (**series I**) we have synthesized compounds **15**–**24** belonging to the novel benzofuro[3,2-*c*]pyrazole series (Fig. 3) and we have evaluated their biological profiles (affinity to cannabinoid receptors and intrinsic activity).

Interesting profiles were determined for the new compounds, particularly in the case of derivatives containing monocyclic or bicyclic monoterpene units at the carbamoyl function at 3 position of the pyrazole ring. According to the determined cannabinoid binding affinity and CB_2 agonism behaviour, as well as with reference to previously reported data evidencing pharmaceutical properties of CB_2 agonists, selected compounds of the novel series could represent useful candidates for the development of innovative strategies for the treatment of various pathologies and disturbs involving CB_2 receptors, i.e. inflammatory and neuropathic pain, immune disorders, and neurodegenerative diseases.

2. Chemistry

Benzofuro[3,2-*c*]pyrazole analogues **15**–**24** were prepared starting from the synthesis of 6-methylbenzofuran-3(2*H*)-one **25** as obtained according to the previously reported procedure [34] (Scheme 1). The α-acylation of **25** with diethyl oxalate furnished the α,γ-diketoester **26** as a tautomeric mixture. Reaction of **26** with 2,4-dichlorophenyl hydrazine gave hydrazone **27**, then converted into the cyclic derivative **28** upon treatment with *p*-toluenesulfonic acid at reflux temperature. The hydrolysis of ester **28** with KOH in H_2O/EtOH afforded the acid precursor **29** in quantitative yield.

The final coupling reaction of **29** with the required amines in dichloromethane in the presence of BtOH/EDC gave the desired carboxamides and carbohydrazides **15**–**24** (Fig. 3).

3. Biology

3.1. Radioreceptor binding assays

Previously reported procedures based on radioreceptor binding assays were adopted to determine the affinity of the novel compounds to both CB_2 and CB_1 receptors [31]. Mouse spleen and mouse brain (minus cerebellum) were employed as starting matrixes for CB_2 and CB_1 affinity tests, respectively. [^{3}H]CP-55,940 was employed as radio-labelled ligand in both the procedures. The experimental IC_{50} data were converted into the corresponding K_i values through Cheng-Prusoff's equation [35]. The CB_2 and CB_1 affinity values of the novel derivatives, expressed as K_iCB_2 and K_iCB_1, respectively, were compared with those of 1,4-dihydroindeno[1,2-*c*]pyrazole lead compounds **10** and **14**, as well as with that of the reference CB_2 antagonist SR144528 (**9**).

3.2. Intrinsic activity by in vitro assays

CB_2 intrinsic activity of the novel derivatives was evaluated through *in vitro* tests based on the determination of phosphorylated ERK 1/2 (P-ERK 1/2) expression in human promyelocytic leukaemia HL-60 (HL-60) cell line, following the cell treatment with the cannabinoid compounds. Accordingly to previously reported results, the exposure of HL-60 cells to cannabinoid agonists induced rapid phosphorylation and activation of the ERK 1/2 which were reversed by a pre-treatment of the cells with the reference CB_2 selective antagonist SR144528 [31,32]. Moreover, no effect was highlighted by the treatment of the same cell line with CB_1 selective agonists as consequence of the expression of CB_2 but not of CB_1 receptors by HL-60 cells.

Analogue procedure was adopted to investigate CB_1 intrinsic activity of novel compounds characterized by good affinity to this cannabinoid receptor subtype. In this case, mouse neuroblastoma N1E-115 cell line was employed instead of HL-60 cell line. In fact, N1E-115 cells express CB_1 but not CB_2 receptors, and their exposure to the cannabinoid agonists ACEA and WIN55-212,2 induces a rapid phosphorylation and activation of the ERK 1/2 [36]. This effect is counteracted by a pre-treatment of the cells with CB_1 antagonists [37].

4. Results and discussion

4.1. Cannabinoid receptor affinities

The cannabinoid receptor affinities of the novel benzofuro[3,2-*c*]pyrazole derivatives are shown in Table 1. By comparison, the K_i values of the lead compounds *N*-piperidin-1-yl-6-methyl-1-(2′,4′-dichlorophenyl)-1,4-dihydroindeno[1,2-*c*]pyrazole carboxamide (**10**), *N*-cyclohexyl-6-methyl-1-(2′,4′-dichlorophenyl)-1,4-dihydroindeno[1,2-*c*]pyrazole-3-carboxamide (**14**), and reference CB_2 ligand SR144528 (**9**) are reported.

Our initial results showed that the replacement of the 1,4-dihydroindeno[1,2-*c*]pyrazole scaffold of **10** and **14** with the benzofuro[3,2-*c*]pyrazole ring system gave the analogue **15** with a nanomolar CB_2 affinity and an acceptable subtype receptor selectivity ($K_iCB_2 = 12.9$ nM; $K_iCB_1/K_iCB_2 = 138.6$). Because the analogue **15** displayed reasonable CB_2 affinity and selectivity, we conducted a

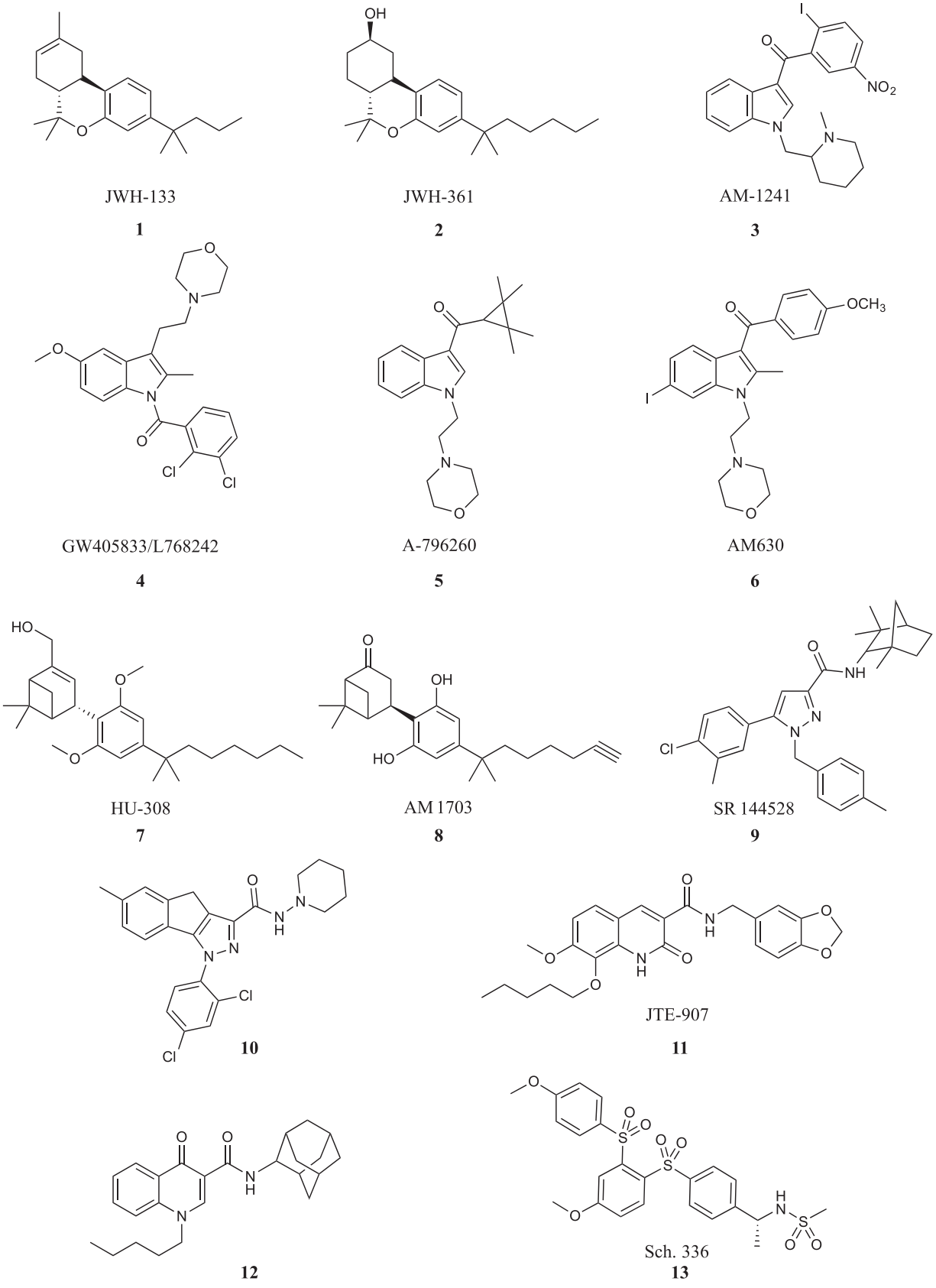
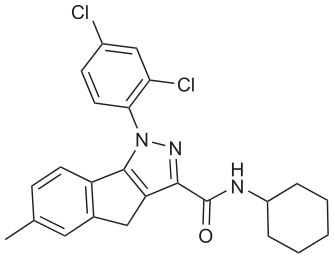


Fig. 1. Chemical structures of CB_2 selective ligands [30].



14

K_iCB_2 (nM) = 7.6 ± 0.7
K_iCB_1 (nM) = 900 ± 45
Selectivity ratio = K_iCB_1/K_iCB_2 = 118:1

Fig. 2. Structure and cannabinoid receptor profile of *N*-cyclohexyl-1-(2,4-dichlorophenyl)-6-methyl-1,4-dihydroindeno[1,2-*c*]pyrazole-3-carboxamide (**14**), namely NESS400.

detailed structure–activity relationship (SAR) investigation in order to determine the structural features influencing the affinity to cannabinoid receptors of these novel compounds.

Our strategy was to prepare analogues of **15** by stepwise introduction of various carbamoyl moieties at the pyrazole ring of the 1*H*-benzofuro[3,2-*c*]pyrazole-3-carboxamide architecture of **15**.

Compounds **16** and **17** were obtained by replacing the piperidine ring of **15** with a pyrrolidine and a morpholine ring, respectively. Both derivatives displayed acceptable affinities to CB_2 receptor (K_iCB_2 < 40.0 nM), although 2–3-fold lower than that of **15**. A comparable loss of affinity was detected for **16** and **17** also to CB_1 receptors, with consequent values of CB_2 selectivity for both derivatives in the same order of that of **15**: K_iCB_1/K_iCB_2 = 92.3 and 107.8 nM for **16** and **17**, respectively. The CB_2 affinity decreasing of **16** relative to **15** was in line with previously reported data concerning 6-chloro-1-(2′,4′-dichlorophenyl)-1,4-dihydroindeno[1,2-*c*]pyrazole carboxamide homologues [26].

Removal of the carbamoyl piperidine-nitrogen atom to give the corresponding cyclohexyl derivative **18** induced no significant variation of K_iCB_2 value and a slight increase of binding affinity to CB_1 receptors. Therefore, a lower CB_2 selectivity was determined for **18** relative to **15** (K_iCB_1/K_iCB_2 = 79.9 for **18**). These marginal changes in cannabinoid receptor affinity suggested that the nitrogen atom in the piperidine ring of the carbamoyl function at 3 position of the pyrazole ring of the novel series did not play a relevant role in binding to CB_2 receptors. As evidenced by the comparison of **18** with **14**, the 1*H*-benzofuro[3,2-*c*]pyrazole skeleton did not influence the cannabinoid binding profile of the cyclohexyl substituted carbamoyl compounds. In contrast, remarkable differences were observed by replacing 1,4-dihydroindeno[1,2-*c*]pyrazole scaffold with the novel 1*H*-benzofuro[3,2-*c*]pyrazole in the case of *N*-piperidinyl carboxamide derivatives, as suggested by K_iCB_2 and K_iCB_1 values of **15** relative to those of **10**.

Changes of the size and shape of the cyclohexyl unit of **18** (see compounds **19**–**24**) had interesting but unpredictable effects on cannabinoid binding affinity and subtype selectivity.

Indeed the adamantyl analogues **19** (K_iCB_2 = 17.5 nM) and **20** (K_iCB_2 = 28.0 nM) showed CB_2 affinity 1.1 and 1.8-fold lower than that of compound **18** and significant higher affinity to CB1 subtype compared to the same derivative. Consequently, a loss of CB_2 selectivity was recorded for both **19** and **20** relative to **18**.

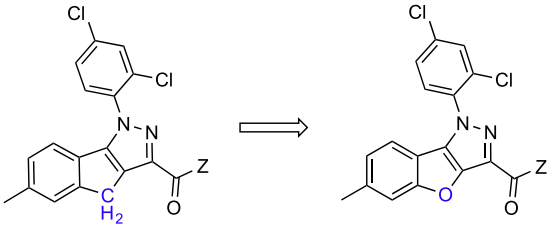
The introduction of various monoterpenic moieties in place of the cyclohexyl group of **18** to afford compounds **21**–**24** elicited K_iCB_2 values lower than 4.0 nM 4.2–6.8-fold enhancements of CB_2 receptor affinity compared to **18** were in particular determined for this sub-class of the novel 1*H*-benzofuro[3,2-*c*]pyrazole derivatives.

Menthyl homologue **21** evidenced a high affinity to CB_2 receptors (K_iCB_2 = 2.3 nM) as well as an interesting CB_1 affinity (K_iCB_1 = 38.2 nM), with a very low CB_1 to CB_2 affinity ratio (K_iCB_1/K_iCB_2 = 16.6).

Amongst the tested compounds, the isopinocampheyl derivative **22** exhibited the most suitable cannabinoid profile relative to the definition of novel selective CB_2 ligands. The compound showed in fact K_iCB_2 = 3.7 nM, K_iCB_1 = 2398 nM, and K_iCB_1/K_iCB_2 = 648. However, interesting behaviours were also determined for the bornyl (**23**) and fenchyl (**24**) compounds, which showed K_iCB_2 values of 3.5 and 2.5, respectively, and K_iCB_1/K_iCB_2 values higher than 100.

4.2. Intrinsic activity by in vitro assays

CB_2 intrinsic activity of all the synthesized compounds was ascertained by *in vitro* test based on HL-60 cells through the evaluation of P-ERK 1/2 expression induced by the novel cannabinoid ligands. CB_2 agonism behaviour was highlighted for all the novel derivatives. According to the reference cannabinoid agonist WIN55,212-2, all the assayed compounds induced in fact significant up-regulation of P-ERK 1/2. Fig. 4 reports the P-ERK 1/2 expression profiles elicited by compounds **15** and **22**, as examples of the obtained results. Analogue behaviours were recorded with other



10 **15-24**

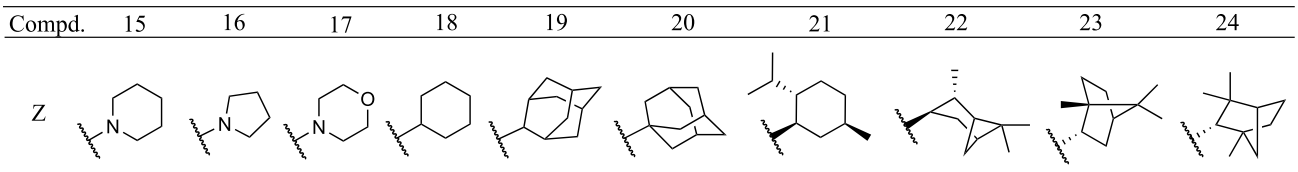
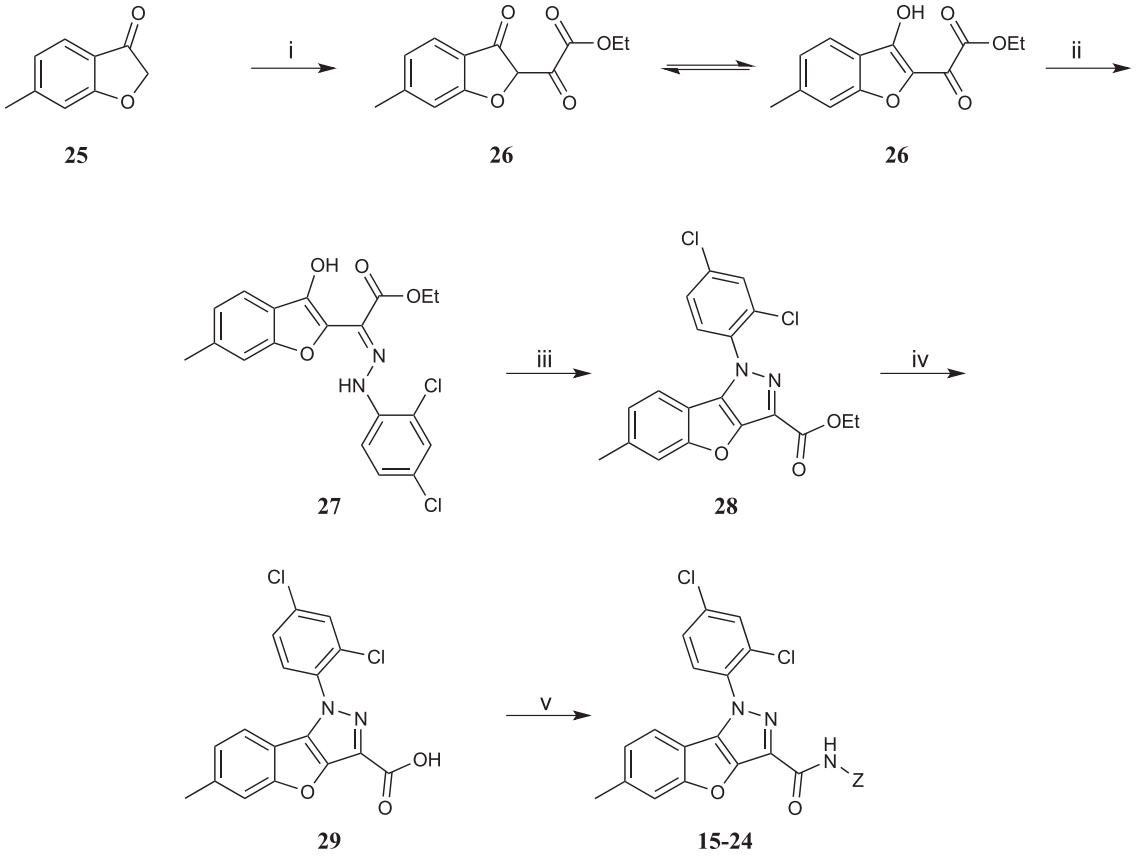


Fig. 3. Design of CB_2 cannabinoid receptor ligands: from 1,4-dihydroindeno[1,2-*c*]pyrazole scaffold (**Series I**) to 1*H*-benzofuro[3,2-*c*]pyrazole skeleton (compounds **15**–**24**).



Scheme 1. Reagents and conditions: (i) Na, $(CO_2Et)_2$, dry EtOH, rt, 20 h; (ii) $C_6H_3Cl_2NHNH_2 \cdot HCl$, dry EtOH, 78 °C, 1.5 h; (iii) *p*-TsOH·H_2O, C_6H_5Me, 110 °C, 30 h; (iv) KOH, EtOH/H_2O 80 °C, 1 h; (v) HOBt/EDC, CH_2Cl_2, rt, 1 h, then Z-NH_2, (Et_3N for **16** and **19**), CH_2Cl_2, rt, 22 h.

derivatives, which determined statistically significant increasing of P-ERK 1/2 at the concentrations reported in Table 2. As evidenced in Fig. 5, induced P-ERK 1/2 up-regulation was reversed by the pre-treatment of HL-60 cells with the reference selective CB_2 antagonist AM630, by confirming the effect elicited by the novel compounds as mediated by CB_2 receptors. The same profiles reported in Fig. 5 for **15** and **22** were determined for all other novel derivatives as well as for WIN55,212-2 (data not shown).

Considering the cannabinoid binding profile (Table 1), compound **21** characterized by $K_iCB_1 = 38.2$ nM was also assayed towards CB_1 activity. According to previously reported procedure [36], CB_1 expressing N1E-115 cell line was adopted for the test and the intrinsic activity of the novel compound was determined by evaluating the P-ERK 1/2 expression profile. As in the case of reference cannabinoid agonist WIN55,212-2, compound **21** enhanced P-ERK 1/2 expression in N1E-115 cells (Fig. 6a). CB_1 agonism behaviour of the novel cannabinoid ligand was further supported by the inhibition of its effect on P-ERK 1/2 expression by a pre-treatment step of N1E-115 cells with the reference selective CB_1 antagonist/inverse agonist rimonabant (Fig. 6b).

5. Conclusion

We have synthesized novel cannabinoid receptor ligands (**15**–**24**) that combine a new benzofuropyrazole tricyclic scaffold with various carbamoyl moieties to probe the size and shape of the ligand binding pockets of CB receptors. The novel cannabinoid ligands show good to high affinity to CB_2 receptors. The obtained results are qualitatively comparable and in agreement with those of our lead homologue **14**, as well as of corresponding analogues, characterized by 1,4-dihydroindeno[1,2-*c*]pyrazole skeleton (**Series I**) [26,31]. Differences of two orders of magnitude were instead observed by the comparison of K_iCB_2 of lead **10** and that of the closest homologue of the novel series (**15**). Further approaches will be adopted in the future to investigate the reasons of the determined differences concerning these two specific homologues.

Amongst the novel series, the compounds containing monocyclic or bicyclic monoterpene units at the carbamoyl function displayed the highest CB_2 affinities. Derivatives **22** and **23**, bearing as terpene motif the isopinocampheyl and the bornyl moiety, respectively, evidenced the best cannabinoid binding profiles. Compound **21** showed also a good CB_1 affinity.

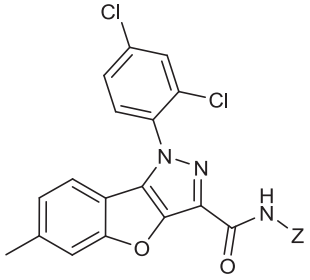
Bioisosterism of the novel 1*H*-benzofuro[3,2-*c*]pyrazole derivatives with the previously reported homologues (**Series I**) was also confirmed by *in vitro* tests aimed to evaluate CB_2 intrinsic activity. As in the case of the reference 1,4-dihydroindeno[1,2-*c*]pyrazole compounds [31,32]. All the novel synthesized derivatives evidenced CB_2 agonism profiles. CB_1 agonism behaviour was also ascertained for compound **21** by *in vitro* test.

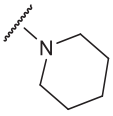
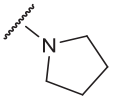
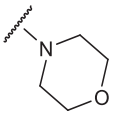
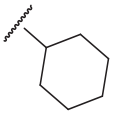
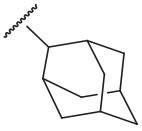
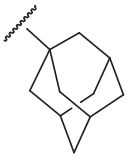
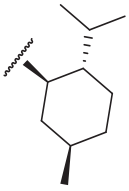
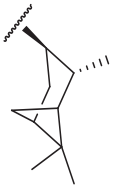
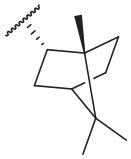
6. Experimental protocols

6.1. Chemistry. General methods

Melting points were determined using a Köfler melting point apparatus and are uncorrected. Infrared spectra (IR) were measured as Nujol mulls on NaCl plates with a Jasco FT/IR 460 plus spectrophotometer and are expressed in cm^{-1}. ^1H NMR spectra were recorded on a 400 MHz instrument, while ^{13}C NMR spectra were registered at 100 MHz, using deuterated dimethyl sulfoxide

Table 1
Structures and binding data of compounds **15**–**24**.



Compd	Z	Receptor affinity[a,b] (nM)		CB_2 selectivity
		K_iCB_2[a,c]	K_iCB_1[b,c]	K_iCB_1/K_iCB_2
15		12.9 ± 1.1	1788 ± 330	138.6:1
16		29.5 ± 8.7	2722 ± 652	92.3:1
17		37.7 ± 6.3	4064 ± 64	107.8:1
18		15.6 ± 4	1247 ± 129	79.9:1
19		17.5 ± 0.9	537 ± 132	30.7:1
20		28 ± 6.6	363 ± 92	13:1
21		2.3 ± 0.3	38.2 ± 6	16.6:1
22		3.7 ± 0.3	2398 ± 629	648:1
23		3.5 ± 0.6	469 ± 64	134:1
24		2.5 ± 0.3	257 ± 7	102.8:1
10 (comp)[26]		0.037 ± 0.03	363 ± 30	9810:1
14 (comp)[31]		7.6 ± 0.7	900 ± 45	118:1
SR144528 (comp)[25]		0.28 ± 0.04	70 ± 10	250:1

[a] Affinity of compounds to CB_2 receptor was evaluated using mouse spleen homogenate and [^{3}H]-CP-55,940.
[b] Affinity of compounds to CB_1 receptors was assayed using mouse brain (minus cerebellum) homogenate and [^{3}H]-CP-55,940.
[c] K_i values were obtained from five independent experiments run in triplicate and are expressed as the mean of the five values ± standard error.

(DMSO-d_6) or chloroform ($CDCl_3$) as solvents, and tetramethylsilane (TMS) as internal standard at room temperature. Chemical shifts are reported as δ values in parts per million (ppm) downfield from TMS. Multiplicities are described as broad singlet (br s), singlet (s), doublet (d), doublet of doublet (dd), double double doublet (ddd), triplet (t), quartet (q), multiplet (m). The coupling constants (J) are expressed in hertz (Hz). Gas chromatography–mass spectrometry measurements were performed either on a Hewlett Packard 5790A/5970A or an Agilent 6850/5973 GC/MS system by electron impact.

Thin-layer chromatography was performed on TLC silica gel 60 F_{254} aluminium and glass-plates. Visualization was carried out by UV light (254 nm), $KMnO_4$ and/or Hanessian stain. Flash chromatography was performed with Merck silica gel 60 (230–400 mesh). Specific rotation was recorded at 25 °C with a Perkin–Elmer 241 apparatus, using the sodium D line (589 nm), and chloroform as solvent.

All the solvents and reagents were obtained from Sigma Aldrich and used without further purification. Air-sensitive reactions were conducted in anhydrous solvents under dry argon atmosphere and in oven-dried glassware. 2-Bromo-2′-hydroxy-4′-methyl-acetophenone and fenchylamine were prepared according to the reported procedures [38,39]. 6-Methylbenzofuran-3(2*H*)-one was obtained by refinement of the procedure described on literature [34,40].

6.2. Preparation of compounds ***26***–***29***

6.2.1. Ethyl 2-(3-hydroxy-6-methylbenzofuran-2-yl)-2-oxoacetate (***26***)

Sodium (0.136 g, 5.9 mmol) was dissolved in absolute ethanol (3.0 mL) under argon atmosphere. To the solution were added diethyl oxalate (0.80 ml, 5.87 mmol) and a solution of the ketone **25** (0.39 g, 2.63 mmol) in absolute ethanol (30 mL). The resulting mixture was left stirring at room temperature for 20 h, poured into a mixture of ice and 2 N HCl, and extracted with chloroform. The

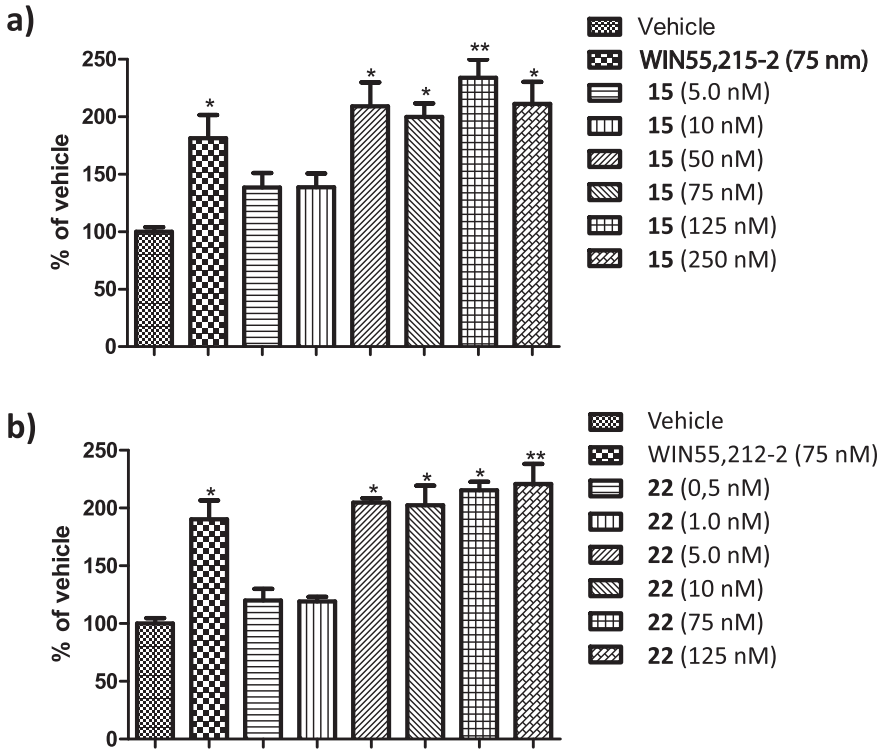


Fig. 4. Effect of different doses of the novel CB_2 receptor ligands on P-ERK 1/2 expression in HL-60 cells after 15 min of treatment: a) **15**; b) **22**. Data are expressed as mean percentage vs vehicle (100%) ± SEM and are the results of five independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$.

organic phases were dried over Na_2SO_4, and evaporated under reduced pressure. The crude residue was triturated with petroleum ether/Et_2O to give 0.47 g (73%) of the title compound as a fluorescent yellow solid. R_f 0.48 (CH_2Cl_2/acetone 7:3); mp 113.0–115.0 °C; IR (cm^{-1}): 3406, 1746, 1691, 1651; ^1H NMR (400 MHz, $CDCl_3$): δ 11.80 (br s, 1H), 7.66 (d, J = 8.0 Hz, 1H), 7.23 (s, 1H), 7.10 (d, J = 8.0 Hz, 1H), 4.55 (q, J = 6.8 Hz, 2H), 2.48 (s, 3H), 1.49 (t, J = 6.8 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 166.9, 165.3, 157.1, 153.4, 143.7, 136.6, 125.3, 122.2, 118.4, 112.6, 64.7, 22.4, 13.9; GC–MS *m*/*z* (% relative intensity, ion): 248 (25, M^+), 249 (3, M + 1).

Table 2
Effect of novel CB_2 compounds **15**–**24** on P-ERK 1/2 expression in HL-60 cells after 15 min of treatment.

Compd	Dose[a],* (nM)	Statistically significant % of P-ERK 1/2 expression vs vehicle[b],*
15	50	205 ± 12
16	75	185 ± 18
17	75	178 ± 13
18	50	196 ± 20
19	50	187 ± 20
20	75	175 ± 22
21	5	193 ± 11
22	5	205 ± 6
23	10	189 ± 11
24	10	200 ± 15
WIN55,212-2 (comp)	75	190 ± 17

[a] Lower dose of the compounds determining statistically significant increase of P-ERK 1/2 expression vs vehicle assumed as 100% ($^{*}p < 0.005$). Doses from 0.5 to 125 nM were assayed for each novel derivative. The reference CB_2 agonist WIN55,212-2 was tested at the dose of 75 nM according to previously reported results [32].

[b] Data obtained from western blots of P-ERK 1/2 expression in the case of the Lower Dose. Data are expressed as a mean percentage of vehicle ± SEM and are the results of five separate experiments.

*6.2.2. Ethyl 2-(2-(2,4-dichlorophenyl)hydrazono)-2-(6-methyl-3-oxo-2,3-dihydrobenzofuran-2-yl)acetate (**27**)*

2,4-Dichlorophenyl hydrazine hydrochloride (1.20 g, 5.60 mmol) was added to a solution of **26** (1.07 g, 4.31 mmol) in absolute ethanol (1.15 mL). After stirring at reflux temperature for 1.5 h, the mixture was cooled to room temperature and then poured into an ice bath. The resulting precipitate was filtered under vacuum, washed with cold ethanol and dried to give 1.37 g (78%) of the hydrazone as a yellow solid. R_f 0.42 (petroleum ether/EtOAc 95:5); mp 190.0–192.0 °C; IR (cm^{-1}): 3423, 1718, 1619, 1502, 1166; ^1H NMR (400 MHz, $CDCl_3$): δ 12.78 (s, 1H), 7.61–7.57 (m, 2H), 7.34 (s, 1H), 7.23 (d, J = 8.8 Hz, 1H), 6.93 (d, J = 8.0 Hz, 2H), 5.28 (s, 1H), 4.07 (q, J = 7.2 Hz, 2H), 2.45 (s, 3H), 0.84 (t, J = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 198.1, 172.8, 161.9, 149.9, 137.7, 129.1, 128.2, 127.9, 124.9, 123.9, 123.5, 120.1, 119.2, 116.0, 113.3, 116.0, 86.0, 61.4, 22.5, 13.1; GC–MS *m*/*z* (% relative intensity, ion): 134 (100, M^+ − 273).

*6.2.3. Ethyl 1-(2,4-dichlorophenyl)-6-methyl-1H-benzofuro[3,2-c] pyrazole-3-carboxylate (**28**)*

p-Toluenesulfonic acid (0.023 g, 0.123 mmol) was added to a solution of the hydrazone **27** (0.50 g, 1.23 mmol) in toluene (6.0 mL). The resulting mixture was heated at reflux temperature for 30 h and cooled to room temperature. The mixture was concentrated under reduced pressure and the crude was purified by flash-chromatography (petroleum ether/Et_2O 95:5 → 8:2) to give 0.25 g (52%) of the title compound as a white solid. R_f 0.30 (petroleum ether/Et_2O 8:2); mp 138.0–140.0 °C; IR (cm^{-1}) 3430, 2098, 1635, 1521, 1371, 1016; ^1H NMR (400 MHz, $CDCl_3$): δ 7.59–7.57 (m, 2H), 7.39–7.37 (m, 2H), 7.24 (d, J = 8.0 Hz, 1H), 7.04 (d, J = 8.0 Hz, 1H), 4.48 (q, J = 7.2 Hz, 2H), 2.43 (s, 3H), 1.41 (t, J = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 162.8, 160.8, 147.4, 138.0, 136.2, 136.1, 135.9, 130.5, 130.4, 129.6, 128.3, 126.8, 124.7, 119.3, 114.1, 113.9,

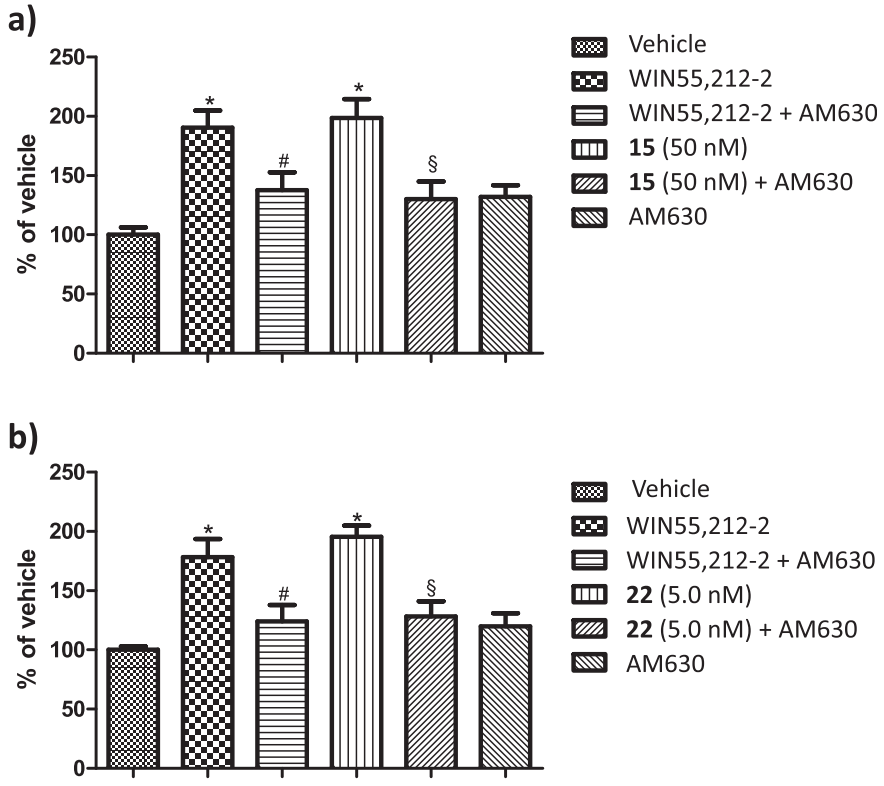


Fig. 5. Effect of the CB_2 receptor antagonist AM630 (75 nM) on P-ERK 1/2 expression induced on HL-60 cells by: a) the reference cannabinoidergic compound WIN55,212-2 (75 nM) or **15** (50 nM); b) WIN55,212-2 (75 nM) or **22** (5.0 nM). Cells were pre-treated with AM630 for 5 min, then a 15 min of exposure with WIN55,212-2 (25 nM), **15**, or **22** was adopted. Reference assays were also carried out with AM630 alone or with the cannabinoidergic compounds to be tested without AM630 pre-treatment. Data are expressed as a mean percentage of vehicle (100%) ± SEM. $^{*}p < 0.05$ vs vehicle; $^{\#}p < 0.05$ vs WIN55,212-2; $^{\S}p < 0.05$ vs **15** or **22**.

61.6, 22.0, 14.5; GC–MS *m*/*z* (% relative intensity, ion): 389 (21, M^+), 388 (100, M − 1), 390 (65, M + 1), 391 (13, M + 2), 393 (2, M + 4).

6.2.4. 1-(2,4-Dichlorophenyl)-6-methyl-1H-benzofuro[3,2-c] pyrazole-3-carboxylic acid (**29**)

A mixture of ester **28** (0.17 g, 0.44 mmol) and KOH (0.32 g, 5.70 mmol) in EtOH/H_2O 1:1 (5.6 mL) was heated at 80 °C for 1 h. The mixture was cooled to 0 °C and acidified with concentrated HCl. The resulting precipitate was filtered under vacuum, washed with cold water and dried to afford the desired acid in quantitative yield as a white solid. R_f 0.30 ($CHCl_3$/MeOH 8:2); mp 228.0–230.0 °C; IR (cm^{-1}) 3417, 2096, 1637, 1484, 1434; ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.06 (d, *J* = 4.0 Hz, 1H), 7.85 (d, *J* = 8.0 Hz, 1H), 7.74 (dd, *J* = 4.0, 12.0 Hz, 1H), 7.64 (s, 1H), 7.37 (d, *J* = 8.0 Hz, 1H), 7.20 (d, *J* = 12.0 Hz, 1H), 2.47 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 162.4, 161.8, 147.2, 138.4, 136.0, 135.9, 135.5, 130.7, 130.3, 130.1, 129.4, 127.3, 125.5, 119.6, 114.0, 113.8, 21.8; GC–MS *m*/*z* (% relative intensity, ion): 360 (1, M − 1), 83 (100, M − 278).

6.3. General procedure I: preparation of carbohydrazides **15**–**17** and carboxamides **18**–**24**

A mixture of the benzofuro[3,2-c]pyrazole-3-carboxylic acid **29** (1.0 eq, 0.25 mmol), 1-hydroxybenzotriazole (HOBt) (1.2 eq, 0.30 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) (1.2 eq, 0.30 mmol) in dichloromethane (2.0 mL) was stirred at room temperature for 1 h. A solution of the appropriate amine or hydrazine (2.0 eq, 0.50 mmol) in dichloromethane (3.0 mL) was added drop-wise. The resulting mixture was stirred at room temperature for 22 h. The solvent was removed under vacuum and the residue was purified by flash-chromatography to afford the desired compound.

When the starting amine/hydrazide was used as hydrochloride salt, triethylamine (Et_3N) (2.0 eq, 0.50 mmol) was added to the mixture.

6.3.1. N-(Piperidin-1-yl)-1-(2,4-dichlorophenyl)-6-methyl-1H-benzofuro[3,2-c]pyrazole-3-carbohydrazide (**15**)

General procedure I was used to convert **29** into the title product. White solid; yield: 81%; R_f 0.10 (petroleum ether/Et_2O 6:4); mp 155.0–157.0 °C; IR (cm^{-1}) 3434, 2938, 2856, 1648; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.66 (d, *J* = 4.0 Hz, 1H), 7.61–7.58 (m, 2H), 7.49–7.45 (m, 2H) 7.29 (d, *J* = 8.0 Hz, 1H), 7.09 (d, *J* = 8.0 Hz, 1H), 2.92 (t, *J* = 4.8 Hz, 4H), 2.49 (s, 3H), 1.81–1.75 (m, 4H), 1.49–1.43 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 163.0, 157.6, 146.9, 137.9, 136.3, 136.1, 135.7, 130.6, 130.5, 129.2, 129.0, 128.3, 124.5, 119.1, 114.1, 114.0, 57.1, 25.3, 23.3, 21.9; GC–MS *m*/*z* (% relative intensity, ion): 443 (5, M^+), 445 (1, M + 2), 98 (100, M − 345).

6.3.2. N-(Pyrrolidin-1-yl)-1-(2,4-dichlorophenyl)-6-methyl-1H-benzofuro[3,2-c]pyrazole-3-carbohydrazide (**16**)

General procedure I was used to convert **29** into the title product. White solid; yield: 94%; R_f 0.30 (petroleum ether/EtOAc 1:1); mp 90.0–92.0 °C; IR (cm^{-1}): 3405, 2977, 1677; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.66 (d, *J* = 4.0 Hz, 1H), 7.59 (d, *J* = 8.0 Hz, 1H), 7.47 (dd, *J* = 4.0, 8.0 Hz, 1H), 7.45 (s, 1H), 7.29 (d, *J* = 8.0 Hz, 1H), 7.09 (d, *J* = 8.0 Hz, 1H), 3.08–3.05 (m, 4H), 2.49 (s, 3H), 1.95–1.92 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 163.0, 158.5, 146.9, 137.9, 136.3, 136.1, 135.7, 130.6, 130.5, 129.2, 128.8, 128.3, 124.5, 119.1, 114.0, 113.9,

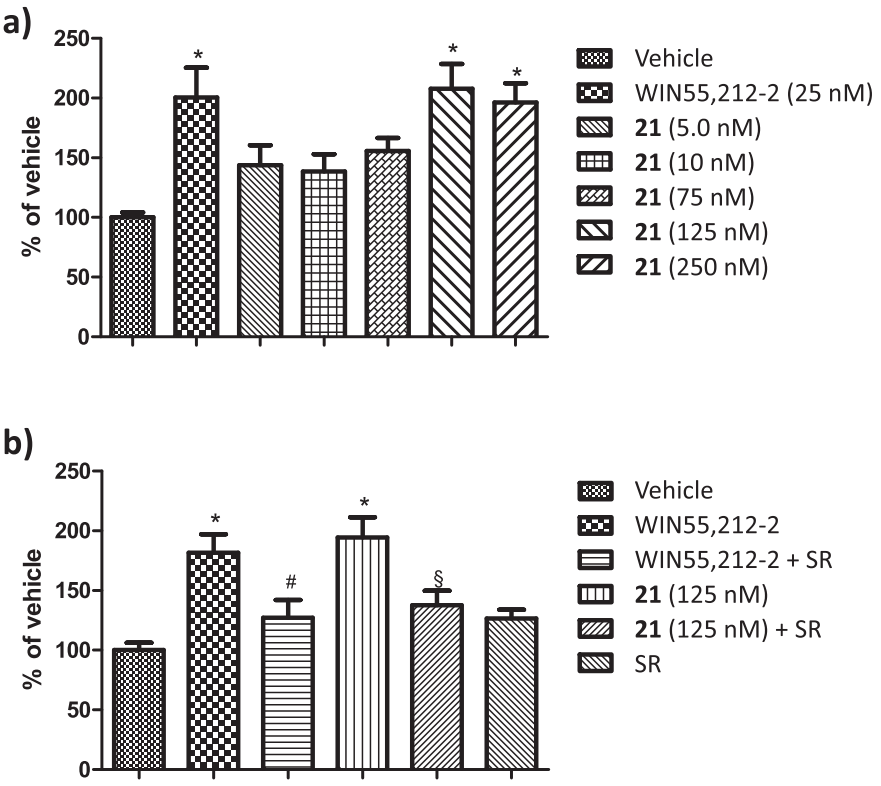


Fig. 6. a) Effect of different doses of the novel CB_1 receptor ligand **21** on P-ERK 1/2 expression in N1E-115 cells after 10 min of treatment. Data are expressed as mean percentage vs vehicle (100%) ± SEM and are the results of five independent experiments. $^{*}p < 0.05$. b) Effect of the CB_1 receptor antagonist/inverse agonist rimonabant (SR) at the dose of 1.0 μM on P-ERK 1/2 expression induced by the reference cannabinoidergic compound WIN55,212-2 (25 nM) or **21** (125 nM). Cells were pre-treated with SR for 5 min, then a 10 min of exposure with WIN55,212-2 (25 nM) or **21** was adopted. Reference assays were also carried out with SR alone or with the cannabinoidergic compounds to be tested without SR pre-treatment. Data are expressed as a mean percentage of vehicle (100%) ± SEM. $^{*}p < 0.05$ vs vehicle; $^{\#}p < 0.05$ vs WIN55,212-2; $^{\S}p < 0.05$ vs **21**.

55.6, 22.3, 21.9; GC–MS *m/z* (% relative intensity, ion): 429 (1, M^+), 430 (1, M + 1), 85 (100, M − 344).

6.3.3. *N-(Morpholin-1-yl)-1-(2,4-dichlorophenyl)-6-methyl-1H-benzofuro[3,2-c]pyrazole-3-carbohydrazide (**17**)*

General procedure I was used to convert **29** into the title product. White solid; yield: quant.; R_f 0.14 (petroleum ether/EtOAc 1:1); mp 178.0–180.0 °C; IR (cm^{-1}) 3430, 2925, 2857, 1673; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.66 (d, *J* = 4.0 Hz, 1H), 7.65 (s, 1H), 7.60 (d, *J* = 8.0 Hz, 1H), 7.47 (dd, *J* = 4.0, 8.0 Hz, 1H), 7.45 (s, 1H), 7.30 (d, *J* = 8.0 Hz, 1H), 7.10 (d, *J* = 8.0 Hz, 1H), 3.89 (t, *J* = 4.4 Hz, 4H), 3.02 (t, *J* = 4.4 Hz, 4H), 2.49 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 163.0, 157.9, 146.9, 138.1, 136.4, 136.0, 135.8, 130.7, 130.5, 129.2, 128.6, 128.4, 124.6, 119.2, 114.1, 113.9, 66.5, 56.0, 22.0; GC–MS *m/z* (% relative intensity, ion): 445 (2, M^+), 446 (3, M + 1), 447 (1, M + 2), 83 (100, M − 362).

6.3.4. *N-Cyclohexyl-1-(2,4-dichlorophenyl)-6-methyl-1H-benzofuro[3,2-c]pyrazole-3-carboxamide (**18**)*

General procedure I was used to convert **29** into the title product. Off-white solid; yield: quant.; R_f 0.16 (petroleum ether/Et_2O 8:2); mp 140.0–142.0 °C; IR (cm^{-1}) 3409, 3092, 3009, 2932, 2855, 1665; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.65–7.59 (m, 2H), 7.47–7.45 (m, 2H), 7.29 (d, *J* = 8.0 Hz, 1H) 7.08 (d, *J* = 8.0 Hz, 1H), 6.73 (d, *J* = 8.0 Hz, 1H), 4.09–3.99 (m, 1H), 2.49 (s, 3H), 2.08–2.05 (m, 2H), 1.78–1.75 (m, 2H), 1.69–1.59 (m, 1H), 1.49–1.39 (m, 2H), 1.34–1.16 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 162.9, 159.3, 146.8, 137.8, 136.4, 136.2, 135.6, 130.6, 130.5, 129.9, 129.3, 128.3, 124.5, 119.1, 114.1, 113.9, 48.0, 33.1, 25.6, 24.9, 21.9; GC–MS *m/z* (% relative intensity, ion): 442 (3, M^+), 443 (6, M + 1), 444 (1, M + 2), 98 (100, M − 344).

6.3.5. *N-Adamant-2-yl-1-(2,4-dichlorophenyl)-6-methyl-1H-benzofuro[3,2-c]pyrazole-3-carboxamide (**19**)*

General procedure I was used to convert **29** into the title product. White solid; yield: 76%; R_f 0.20 (petroleum ether/Et_2O 8:2); mp 209.0–211.0 °C; IR (cm^{-1}) 3417, 1664; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.66 (d, *J* = 4.0 Hz, 1H), 7.62 (d, *J* = 8.0 Hz, 1H), 7.49–7.45 (m, 2H), 7.22 (d, *J* = 8.0 Hz, 1H), 7.09 (d, *J* = 8.0 Hz, 1H), 4.33 (d, *J* = 8.0 Hz, 1H), 2.49 (s, 3H), 2.10 (s, 2H), 1.95–1.91 (m, 8H), 1.78 (s, 2H), 1.69 (d, *J* = 12.0 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 163.0, 159.4, 146.8, 137.8, 136.3, 136.2, 135.5, 130.6, 130.4, 130.0, 129.3, 128.3, 124.5, 119.1, 114.1, 114.0, 53.1, 37.6, 37.1, 32.0, 27.3, 27.2, 21.9; GC–MS *m/z* (% relative intensity, ion):495 (15, M + 1), 496 (4, M + 2), 498 (3, M + 4), 83 (100, M − 411).

6.3.6. *N-Adamant-1-yl-1-(2,4-dichlorophenyl)-6-methyl-1H-benzofuro[3,2-c]pyrazole-3-carboxamide (**20**)*

General procedure I was used to convert **29** into the title product. White solid; yield: 81%; R_f 0.36 (petroleum ether/Et_2O 8:2); mp 210.0–212.0 °C; IR (cm^{-1}) 3399, 1670; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.65 (d, *J* = 4.0 Hz, 1H), 7.60 (d, *J* = 8.0 Hz, 1H), 7.46 (dd, *J* = 4.0 Hz, 8.0 Hz, 1H), 7.42 (s, 1H) 7.29 (d, *J* = 8.0 Hz, 1H), 7.08 (d, *J* = 8.0 Hz, 1H), 6.58 (s, 1H), 2.49 (s, 3H), 2.18 (s, 6H), 2.13 (s, 3H), 1.77–1.70 (m, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 163.0, 159.4, 147.0, 137.8, 136.4, 136.2, 135.5, 130.7, 130.6, 130.4, 129.3, 128.3, 124.4,

119.1, 114.1, 114.0, 52.3, 41.7, 36.4, 29.5, 21.9; GC–MS *m/z* (% relative intensity, ion): 494 (3, M^+), 495 (6, M + 1), 496 (2, M + 2), 83 (100, M − 411).

6.3.7. N-Menthyl-1-(2,4-dichlorophenyl)-6-methyl-1H-benzofuro [3,2-c]pyrazole-3-carboxamide (**21**)

General procedure I was used to convert **29** into the title product. Off-white solid; yield: 96%; R_f 0.42 (petroleum ether/Et_2O 8:2); mp 70.0–72.0 °C; IR (cm^{-1}) 3403, 3010, 2954, 2867, 1666; ^1H NMR (400 MHz, $CDCl_3$): δ 7.67 (d, *J* = 4.0 Hz, 1H), 7.62 (d, *J* = 8.0 Hz, 1H), 7.47 (dd, *J* = 4.0, 8.0 Hz, 1H), 7.44 (s, 1H), 7.30 (d, *J* = 8.0 Hz, 1H), 7.09 (d, *J* = 8.0 Hz, 1H), 6.55 (d, *J* = 8.0 Hz, 1H), 4.10–4.01 (m, 1H), 2.49 (s, 3H), 2.14–1.99 (m, 2H), 1.77–1.70 (m, 2H), 1.27–1.12 (m, 2H), 1.01–0.87 (m, 12H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 163.0, 159.5, 146.8, 137.8, 136.4, 136.2, 135.6, 130.6, 130.5, 129.9, 129.3, 128.3, 124.5, 119.1, 114.1, 114.0, 49.7, 48.2, 43.1, 34.6, 31.9, 26.9, 23.9, 22.2, 21.9, 21.2, 16.3; GC–MS *m/z* (% relative intensity, ion): 498 (2, M^+), 499 (4, M + 1), 83 (100, M − 415); $[\alpha]_D^{20}$ −36.0 (c 10^{-3}, $CHCl_3$).

6.3.8. N-Isopinocampheyl-1-(2,4-Dichlorophenyl)-6-methyl-1H-benzofuro[3,2-c]pyrazole-3-carboxamide (**22**)

General procedure I was used to convert **29** into the title product. Off-white solid; yield: 89%; R_f 0.32 (petroleum ether/Et_2O 8:2); mp 85.0–87.0 °C; IR (cm^{-1}) 3407, 2935, 1666; ^1H NMR (400 MHz, $CDCl_3$): δ 7.66 (d, *J* = 4.0 Hz, 1H), 7.62 (d, *J* = 8.0 Hz, 1H), 7.47 (dd, *J* = 4.0, 8.0 Hz, 1H), 7.45 (s, 1H), 7.30 (d, *J* = 8.0 Hz, 1H), 7.09 (d, *J* = 8.0 Hz, 1H), 6.75 (d, *J* = 8.0 Hz, 1H), 4.59–4.51 (m, 1H), 2.75–2.68 (m, 1H), 2.49 (s, 3H), 2.47–2.41 (m, 1H), 2.02–1.94 (m, 2H), 1.89–1.86 (m, 1H), 1.72 (ddd, *J* = 4.0 Hz, 8.0 Hz, 16.0 Hz, 1H), 1.25 (s, 3H), 1.20 (d, *J* = 7.2 Hz, 3H), 1.11 (s, 3H), 0.97 (d, *J* = 10.0 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 163.0, 159.7, 146.9, 137.8, 136.4, 136.2, 135.6, 130.6, 130.5, 129.9, 129.3, 128.3, 124.5, 119.1, 114.1, 114.0, 47.9, 47.6, 46.2, 41.6, 38.5, 37.1, 35.1, 28.1, 23.5, 21.9, 20.9; GC–MS *m/z* (% relative intensity, ion): 496 (2, M^+), 497 (4, M + 1), 498 (1, M + 2), 343 (100, M − 153); $[\alpha]_D^{20}$ −12.0 (c 10^{-3}, $CHCl_3$).

6.3.9. N-Bornyl-1-(2,4-dichlorophenyl)-6-methyl-1H-benzofuro [3,2-c]pyrazole-3-carboxamide (**23**)

General procedure I was used to convert **29** into the title product. Off-white solid; yield: 89%; R_f 0.26 (petroleum ether/Et_2O 8:2); mp 78.0–80.0 °C; IR (cm^{-1}) 3414, 2956, 1669; ^1H NMR (400 MHz, $CDCl_3$): δ 7.66–7.62 (m, 2H), 7.49–7.44 (m, 2H), 7.30 (d, *J* = 8.0 Hz, 1H), 7.08 (d, *J* = 8.0 Hz, 1H), 6.90 (d, *J* = 8.8 Hz, 1H), 4.55–4.49 (m, 1H), 2.49 (s, 3H), 1.86–1.77 (m, 1H), 1.72–1.63 (m, 2H), 1.46–1.40 (m, 1H), 1.30–1.13 (m, 3H), 1.02 (s, 3H), 0.92 (d, *J* = 8.0 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 163.0, 160.3, 146.8, 137.8, 136.3, 136.2, 135.6, 130.6, 130.4, 130.0, 129.3, 128.3, 124.4, 119.1, 114.1, 114.0, 53.6, 49.9, 48.3, 45.0, 37.5, 28.4, 28.1, 21.9, 19.9, 18.8, 13.8; GC–MS *m/z* (% relative intensity, ion): 496 (7,$M^{\cdot+}$), 497 (16, M + 1), 498 (4, M + 2), 83 (100, M − 414); $[\alpha]_D^{20}$ +3.0 (c 10^{-3}, $CHCl_3$).

6.3.10. N-Fenchyl-1-(2,4-dichlorophenyl)-6-methyl-1H-benzofuro [3,2-c]pyrazole-3-carboxamide (**24**)

General procedure I was used to convert **29** into the title product. White solid; yield: 96%; R_f 0.30 (petroleum ether/Et_2O 8:2); mp 84.0–86.0 °C; IR (cm^{-1}) 3416, 2853, 2360, 1671, 1458, 1376; ^1H NMR (400 MHz, $CDCl_3$): δ 7.67 (d, *J* = 2.4 Hz, 1H), 7.63 (d, *J* = 8.4 Hz, 1H), 7.47 (dd, *J* = 8.4, 2.4 Hz, 1H), 7.44 (s, 1H), 7.31 (d, *J* = 8.0 Hz, 1H), 7.09 (d, *J* = 8.0 Hz, 1H), 6.92 (d, *J* = 9.6 Hz, 1H), 3.89 (d, *J* = 9.6 Hz, 1H), 2.50 (s, 3H) 1.81–1.80 (m, 1H), 1.75–1.70 (m, 2H), 1.54–1.39 (m, 2H), 1.28–1.21 (m, 5H), 1.14 (s, 3H), 0.89 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 163.0, 160.8, 146.8, 137.8, 136.3, 136.2, 135.5, 130.6, 130.4, 129.8, 129.2, 128.3, 124.5, 119.1, 114.2, 114.0, 63.1, 48.6, 48.2, 42.7, 39.6, 30.9, 27.4, 26.0, 21.9, 21.3, 19.7; GC–MS *m/z* (% relative intensity, ion): 496 (8, M^+), 495 (30, M − 1), 497 (19, M + 1), 498 (4, M + 2), 343 (100, M − 153); $[\alpha]_D^{20}$ −11.0 (c 10^{-3}, $CHCl_3$).

6.4. Pharmacology

6.4.1. Chemicals, drugs and cells for in vitro assays

[^{3}H]-CP-55,940 (180 Ci/mmol) was obtained from PerkinElmer Italia. *N*-piperidinyl-5-(4-chlorophenyl)-1-(2,4-dicholorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide (rimonabant) was purchased from Kemprotech Limited (Middlesbrough, UK) while WIN55,212-2 and AM630 from Tocris Bioscience.

HL-60 and N1E-115 cell lines from the European Collection of Cell Cultures (ECACC), Foetal Bovine Serum (FBS), Penicillin-Streptomycin, L-Glutamine, Amphotericin B, Gentamicin, and dimethyl-sulfoxide (DMSO) were purchased from Sigma–Aldrich (Milan, Italy). RPMI 1640 Medium and Phosphate Buffered Saline (PBS) were obtained from Gibco-BRL (Gaithensburg, MA).

For radioreceptor binding experiments, compounds to be tested were dissolved in DMSO. The solvent concentration in the different assays never exceeded 0.1% (v/v) and was without effects.

Regarding cannabinoid intrinsic activity evaluation, tested and reference compounds were dissolved in culture medium with 1% DMSO.

6.4.2. Animals

Male CD1 mice (Charles River, Calco, LC, Italy) were housed in the animal care quarters at the following conditions: temperature 22 ± 2 °C, humidity 55 ± 5%, 12 h light/dark cycles, food and water available ad libitum. Animal management was performed according to the UE guidelines for the care and use of experimental animals (CEE N° 86/609) and the protocol authorized by Italian Health Ministry. Experiments were carried out with animals weighing 30–35 g.

6.4.3. Radioreceptor binding assays

Assays were performed according to previously reported procedure [31]. Spleen and brain (minus cerebellum) tissues were employed as starting matrixes for CB_2 and CB_1 binding assays, respectively. Both the tissues were removed from male CD1 mice killed by cervical dislocation. Tissues were homogenated in 20 vol. (wt/v) of ice-cold TME buffer (50 mM Tris–HCl, 1 mM EDTA and 3.0 mM $MgCl_2$, pH 7.4). The homogenates were centrifuged at 1086 × *g* for 10 min at 4 °C, and the resulting supernatants were centrifuged at 45,000 × *g* for 30 min.

To reduce non-specific binding, all binding studies were performed in Sigma-Cote pre-treated glass tubes (Sigma Chemical Co. Ltd., Poole, UK). The obtained membranes (30–80 μg of protein as determined by Bradford protein assay according to the supplier protocol by Bio-Rad, Milan, Italy) were incubated with 0.5–1.0 nM of [^{3}H]-CP-55,940 for 1 h at 30 °C. A final volume of 0.5 mL of TME buffer containing 5 mg/mL of fatty acid-free bovine serum albumin was adopted. Non-specific binding was estimated in the presence of 1.0 μM of CP-55,940. The reaction was blocked by rapid filtration through Whatman GF/C filters presoaked in 0.5% polyethyleneimine (PEI). A Brandell 36-sample harvester (Gaithersburg, MD, USA) was used. Filters were washed five times with 4 mL aliquots of ice cold Tris HCl buffer (pH 7.4) containing 1.0 mg/mL BSA. The filter bound radioactivity was measured in a liquid scintillation counter (Trisarb 2900, Packard, Meridien, USA) with 4 mL of scintillation fluid (Ultima Gold MV, Packard).

All experiments were performed in triplicate and the results were confirmed in five independent assays. Non-linear regression analysis of a Sigmoid Curve using Graph Pad Prism program was adopted to analyse radioligand inhibition experiments and to determine IC_{50} values. Affinities to CB_1 and CB_2 receptors were

expressed as K_i (mean ± standard error) obtained from IC_{50} values according to previously described procedures [35].

6.4.4. Intrinsic activity by in vitro assays

CB_2 and CB_1 intrinsic activity was evaluated by analysing P-ERK 1/2 expression induced by the compounds on HL-60 and N1E-115 cell lines, respectively [31,32,37]. In first experiment sets, P-ERK 1/2 was determined after 15 min treatment of HL-60 cells, or 10 min treatment of N1E-115 cells, with the compounds to be assayed. Successively, to confirm the capability of the novel derivatives to act as CB_2 agonists, a 5 min pre-treatment with the selective CB_2 receptor antagonist AM630 (75 nM) was carried out before the exposure of HL-60 cells to the cannabinoid compounds. Analogous assays were performed to ascertain CB_1 intrinsic activity. In these cases, a 5 min pre-treatment with the selective CB_1 antagonist/inverse agonist rimonabant (1.0 μM) was adopted before the administration of the novel compounds to N1E-115 cells.

Cells were grown at 37 °C in humidified 5% CO_2 in RPMI 1640 Medium supplemented with 10% FBS, 1.0 μg/ml Penicillin-Streptomycin, 2.0 mM L-Glutamine, 2.5 μg/mL Amphotericin B, and 50 μg/mL Gentamicin. Treatments with cannabinoid agents were performed in a volume of 10 μl/ml of cell suspension. After appropriate time of exposure, cells were collected by centrifugation at 1000 × *g*. The resulting pellets were washed in ice-cold PBS buffer by centrifugation at 1000 × *g*. The pellet was lysed at 4 °C in HEPES based buffer containing other components (for details see Ref. [31]). The extracts were centrifuged at 10,000 × g for 15 min at 4 °C. The resulting supernatant was collected as total cell extracts. Extracted total proteins were quantified using a Quant-iT™Protein Assay Kit (Invitrogen™) by a Qubit Quantitation platform system (Invitrogen™). Each western blot assay was carried out at fixed total protein amount of 40 μg. Separation was performed by 10% Bis-Tris Gel NuPAGE® Novex (Invitrogen) and nitrocellulose membranes (Bio-Rad). Blots were blocked through Chemi BLOCKER™ (Millipore) in TBST (0.1% Tween 20 in Tris borate saline, Bio-Rad) and probed with Anti-phospho-ERK 1/2 recombinant clone AW39R (Millipore) with a 1:1000 dilution in 1% BSA (Bovine Serum Albumin) in TBST (10 min). After three cycles of washing in TBST, membranes were probed with the secondary antibody Goat anti-Rabbit IgC – HRP (Invitrogen) with a 1:2000 dilution in Chemi BLOCKER™-TBST 1:4. After three cycles of washing in TBST, blots were treated for 5 min with Immobilon Western Chemiluminescent HRP substrate (Millipore). Immunoreactive bands were visualized by a Fujifilm Las-1000 analyzer (Raytest Isotopenmessgeräte GmbH) and immunoreactive band optical density was measured using AIDA 2.11 software (Raytest Isotopenmessgeräte GmbH).

Data were expressed as mean percentage of vehicle ± SEM (results of five independent experiments). Statistical analysis was performed by One-way ANOVA followed by Dunnet's multiple comparison using Graph Pad Prism 5 program (San Diego).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.05.055.

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