Identification of the first synthetic allosteric modulator of the CB_2 receptors and evidence of its efficacy for neuropathic pain relief

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Abstract

The direct activation of cannabinoid receptors (CBRs) results in several beneficial effects, therefore several CBRs ligands have been synthesized and tested *in vitro* and *in vivo*. However none of them reached an advanced phase of clinical development due mainly to side effects on the CNS. Medicinal chemistry approaches are now engaged to develop allosteric modulators that might offer a novel therapeutic approach to achieve potential therapeutic benefits avoiding inherent side effects of orthosteric ligands. Here we identify the first ever synthetized positive allosteric modulator (PAM) that targets CB_2Rs. The evidence for this was obtained using [^{3}H]CP55940 and [^{35}S]GTPγS binding assays. This finding will be useful for the characterization of allosteric binding site(s) on CB_2Rs which will be essential for the further development of CB_2R allosteric modulators. Moreover, the new CB_2R PAM displayed antinociceptive activity *in vivo* in an experimental mouse model of neuropathic pain, raising the possibility that it might be a good candidate for clinical development.

Introduction

The endocannabinoid system (ECS) is a ubiquitous neuromodulatory system with wide-ranging actions.[1] Cannabinoid receptors (CB_1Rs, CB_2Rs) are members of G-protein-coupled receptors family. Several findings indicate that CB_2R ligands, which do not share the ability of CB_1R agonists to produce psychotropic effects or of selective CB_1R antagonists to produce other serious adverse effects,[2-4] appear to be promising drugs for treating several diseases.[5-7] Medicinal chemistry approaches are now being adopted to develop more efficacious agonists, and in particular, to investigate the possibility of developing allosteric modulators (Figure 1) that might offer a novel therapeutic approach with minimal side-effects. It has been reported that CBRs have spatially distinct allosteric sites that can be targeted by exogenous/endogenous substances modulating the receptors' functional state.[8-15] Allosteric modulators offer numerous advantages respect to orthosteric ligands, such as a greater subtype selectivity for the higher sequence divergence at allosteric sites, than the orthosteric domains; furthermore, allosteric modulators possess tissue selectivity because they exert effects only where endocannabinoids are present and eventually they might provide synergizing effects with other co-administered ECS modulators.[16-19] Moreover, they could have the advantage of activating or inhibiting the biased signaling properties of these GPCRs. For these reasons, allosteric modulators might represent a valid alternative method to modulate the ECS and they could be useful for the development of future drugs. They are classified in: positive allosteric modulators (PAMs), negative allosteric modulators (NAMs) which activate or inhibit the receptor response, respectively, neutral or silent allosteric modulators, which do bind to the allosteric site without affecting the response of the endogenous agonist and bitopic ligands that bind to both the orthosteric and allosteric sites.

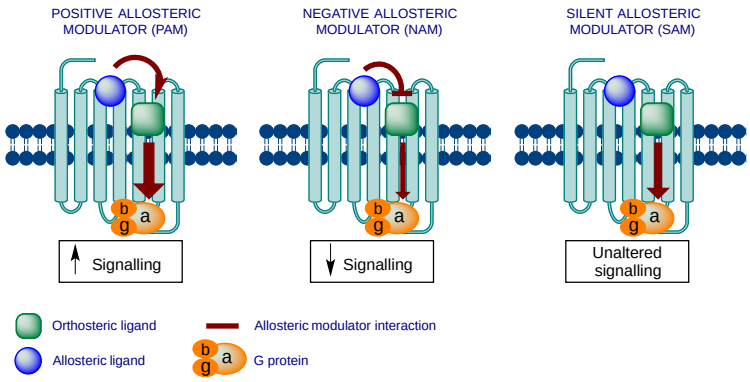


Figure 1. General classification of allosteric modulators: PAMs, NAMs and SAMs.

To date, few allosteric modulators for CB_1Rs have been developed. Among them, indole derivatives,[9,20] urea derivatives[21] and certain other molecules have a PAM profile (Figure 2).[22] Up to now, the only CB_2R allosteric modulator reported in the literature is the endogenous Pepcan-12 (Figure 2) which was reported also to be a CB_1R NAM.[23]

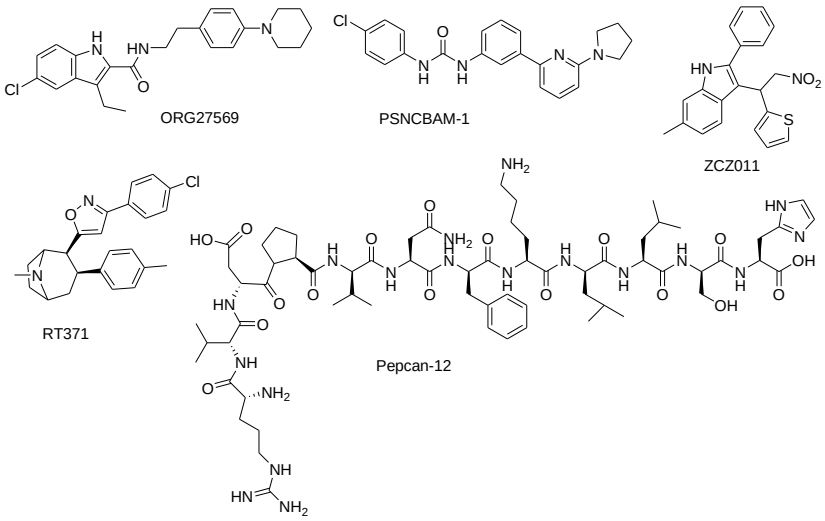


Figure 2. Representative CBRs allosteric modulators.

In this work, we present evidence that the novel synthetic compound **C2** (Figure 3), is a positive allosteric modulator (PAM) of the CB_2Rs. This evidence was obtained by carrying out [^{3}H]CP55940 and [^{35}S]GTPγS binding assays. **C2** is a member of a group of compounds that were obtained by modifying the structure of 2-oxopyridine-3-carboxamides derivatives of general structure **A** and **B**

(Figure 3) that had been previously shown to be orthosteric CBR ligands.[24] With the aim of deeply exploring the structure-activity relationships of derivatives **A** and **B**, we synthesized derivatives **C1-C5**, **D1-D6** and **E1** in which the amide group in position C-3 of the 2-oxopyridine-3-carboxamides **A** and **B** was substituted with the corresponding retro-amide (Figure 3). The substituents of derivatives **C** and **D** have been selected on the basis of the best results achieved with the series **A** and **B**. Moreover, compound **E1**, characterized by an OH group in position 4, has been synthesized, to understand the role of the methyl group and, at the same time, to define the effect on the affinity of binding to CBRs of a polar group in the same position (Figure 3). Finally, derivative **C2** displayed antinociceptive activity *in vivo* in an experimental mouse model of neuropathic pain.

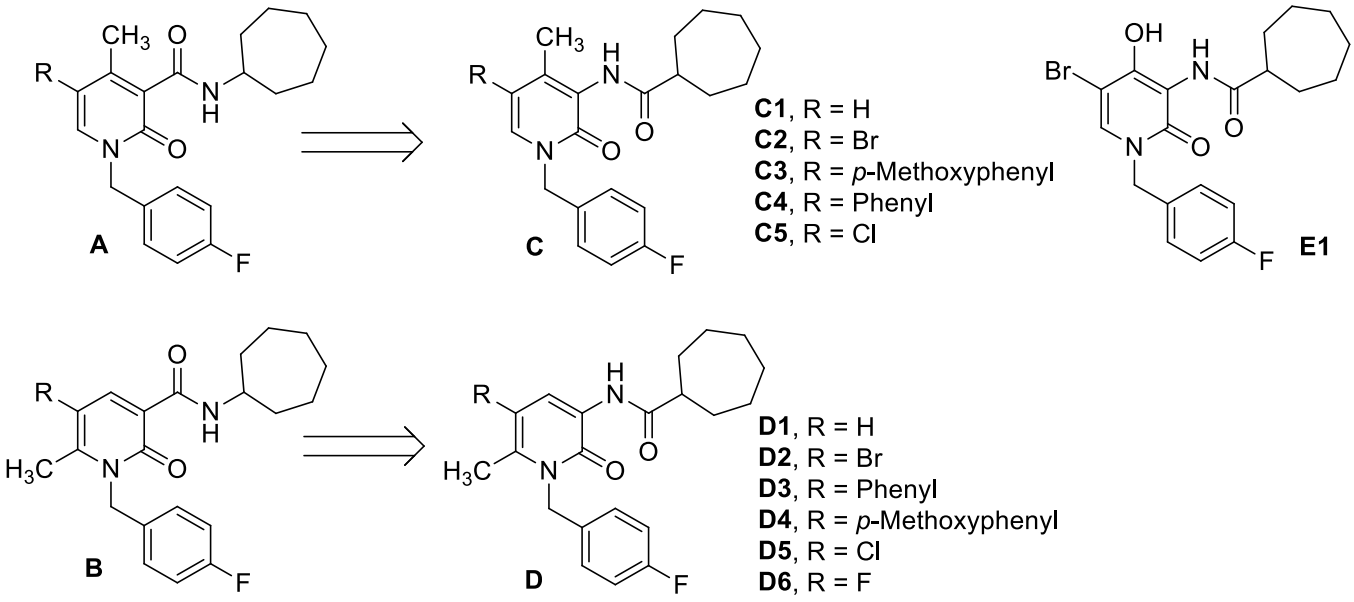


Figure 3. Design of retro-amides **C1-C5**, **D1-D6** and **E1**.

Results and Discussion

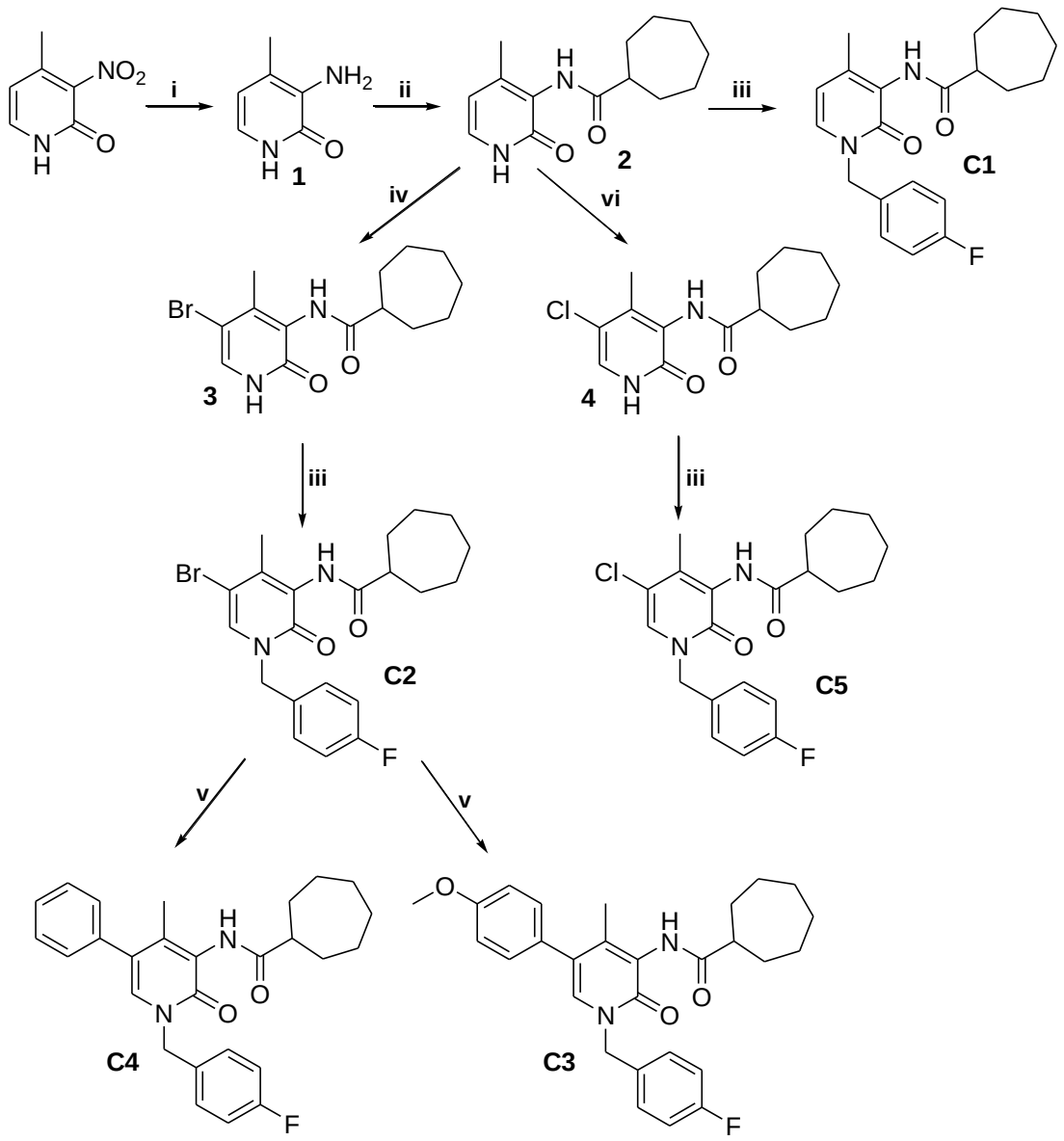
Chemistry. The synthesis of 2-oxopyridines **C**, **D** and **E1** was obtained as reported in Schemes 1-3.

The cycloheptanecarboxamide derivatives **C1-C5** were synthesized (Scheme 1) starting from the 2-hydroxy-4-methyl-3-nitropyridine which underwent a catalytic hydrogenation to afford derivative **1**. The reaction between the amine **1** and the cycloheptanecarbonyl chloride in toluene, DMF and

triethylamine, carried out initially at 0 °C and then at 30 °C for 48 hours, afforded the amide **2**. The acyl chloride was prepared from reaction between cycloheptanecarboxylic acid with $SOCl_2$ at 76 °C for 3 hours. The derivative **2** was reacted with CsF and then with *p*-fluorobenzyl chloride, yielding the desired *N*-alkylated compound **C1**. The derivatives **C2-C5** were synthesized starting from the amide **2** which was treated with a solution of bromine in $CHCl_3$, affording the corresponding 5-bromo derivative **3**. The treatment of **3** with *p*-fluorobenzyl chloride *via* a similar method to that used for the preparation of **C1**, afforded the desired *N*-alkylated compound **C2** (Scheme 1). The 5-aryl derivatives **C3** and **C4** were yielded starting from the 5-bromo derivative **C2** *via* a Suzuki-Miyaura cross-coupling reaction with the suitable boronic acids in dry toluene and $Pd(PPh_3)_4$ as catalyst (Scheme 1). The 5-chloro derivative **C5** was obtained from amide **2** which was treated with *N*-chlorosuccinimide to give compound **4**. The *N*-alkylation of **4**, carried out following the same method used to yield **C1**-**C4**, afforded the desired compound **C5** (Scheme 1).

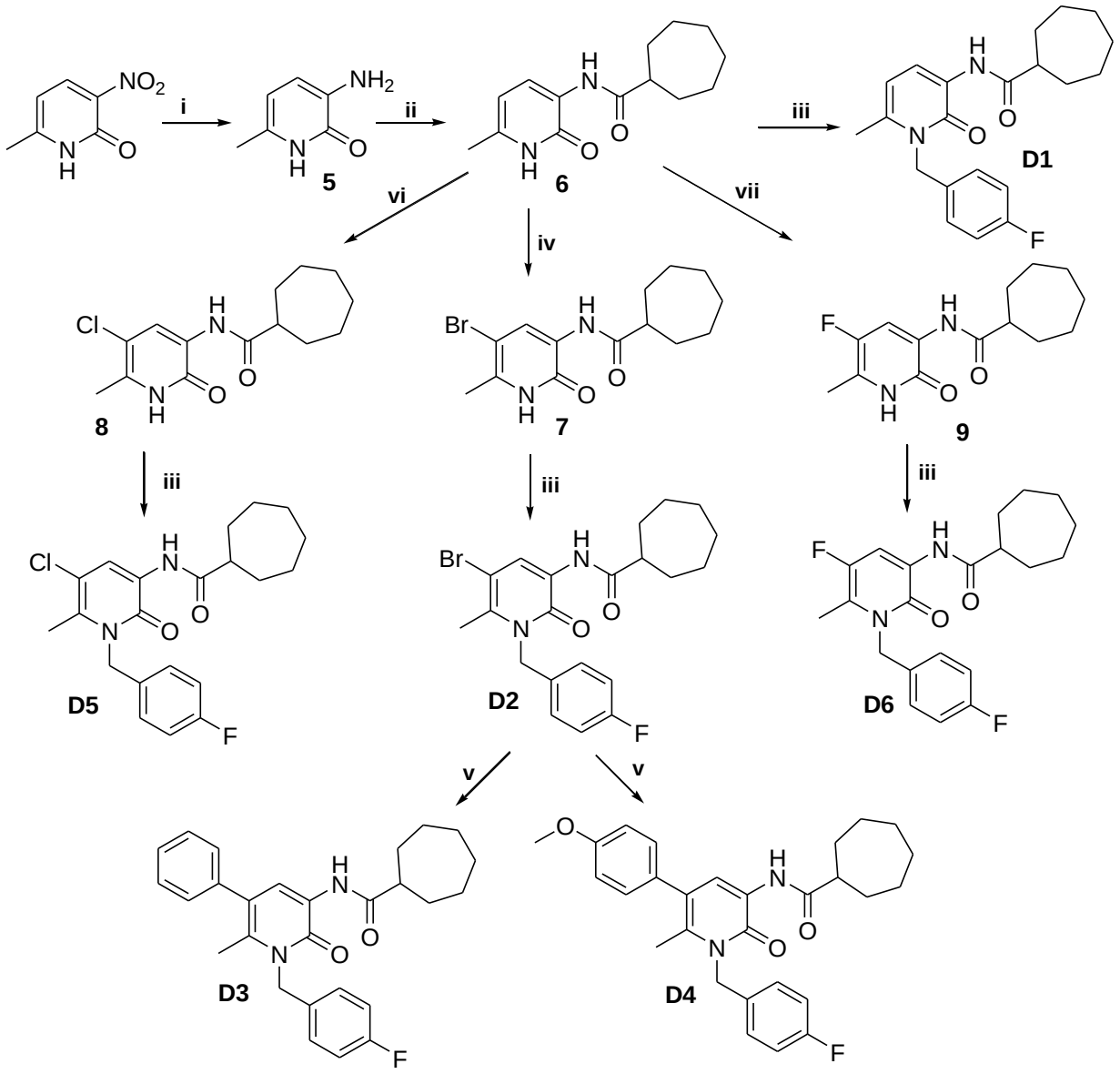
As reported in Scheme 2, the compounds **D1**-**D6** were obtained starting from 2-hydroxy-6-methyl-3-nitropyridine which underwent a catalytic hydrogenation, using Pd/C as catalyst, to afford compound **5**. Subsequently, the condensation of **5** with cycloheptanecarboxylic acid using TBTU as condensing reagent in the presence of triethlylamine, yielded the carboxamide **6** which, for *N*-alkylation with NaH, LiBr and *p*-fluoro-benzylchloride, gave **D1**. The reaction of **6** with a solution of bromine in $CHCl_3$ produced the corresponding 5-bromo derivative **7** which was *N*-alkylated by the same procedure previously described, to yield the desired compound **D2**. The derivatives **D3** and **D4** were obtained from **D2** by Suzuki cross-coupling reaction with the suitable arylboronic acid (Scheme 2). The reaction of compound **6** with *N*-chlorosuccinimide in acetonitrile at reflux gave the 5-chloro derivative **8** which was *N*-alkylated using the procedure already described, to afford compound **D5** (Scheme 2). The carboxamide **6** was also treated with 1-chloromethyl-4-fluoro-1,4-diazoniabicyclo[2.2.2] octanebis(tetrafluoroborate) in acetonitrile at reflux for 12 hours, yielding the 5-fluorinated derivative **9** which was *N*-alkylated to afford the desired compound **D6** (Scheme 2).

Scheme 1. Synthesis of compounds **C1-C5**



Reagents and conditions: (i) H_2, Pd/C, MeOH, rt, 12 h; (ii) 1) cycloheptancarboxylic acid, $SOCl_2$, 76 °C, 3 h; 2) acyl chloride, toluene, DMF, NEt_3, 0-32 °C, 48 h; (iii) 1) DMF, CsF, rt, 1 h; 2) *p*-fluorobenzyl chloride, 50 °C, 12 h; (iv) Br_2, $CHCl_3$, 30 °C, 12 h; (v) triphenylphosphine, $Pd(OAc)_2$, K_2CO_3, suitable arylboronic acid, 110 °C, 12 h; (vi) *N*-chlorosuccinimide, ACN, reflux, 12 h.

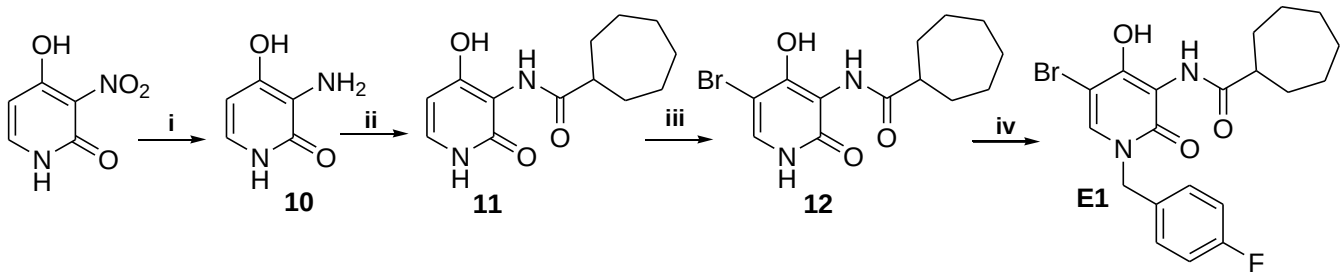
Scheme 2. Synthetic pathway for compounds **D1**-**D6**



Reagents and conditions: (i) H_2, Pd/C, MeOH, rt, 12 h; (ii) cycloheptancarboxylic acid, TBTU, NEt_3, DMF, 0 °C rt, overnight; (iii) NaH 60%, LiBr, *p*-fluorobenzyl chloride, DMF, DME, 65 °C, 12 h; (iv) Br_2, $CHCl_3$, rt, 12 h; (v) triphenylphosphine, $Pd(OAc)_2$, K_2CO_3, suitable arylboronic acid, 110 °C, overnight; (vi) *N*-chlorosuccinimide, ACN, reflux, 12 h; (vii) 1-chloromethyl-4-fluoro-1,4-diazoniabicyclo[2.2.2] octanebis(tetrafluoroborate), ACN, reflux, 12 h.

Finally, compound **E1** was synthesized as reported in Scheme 3, starting from 4-hydroxy-3-nitro-2-oxo-1,2-dihydropyridine which underwent a catalytic hydrogenation to yield derivative **10**. Reaction of **10** with cycloheptanecarbonyl chloride prepared as reported above, gave compound **11** which, by treatment with a solution of bromine in $CHCl_3$, yielded compound **12**. The *N*-alkylation of **12** carried out with CsF and *p*-fluorobenzyl chloride, afforded the derivative **E1**.

Scheme 3. Synthetic pathway for compound **E1**



Reagents and conditions: (i) H_2, Pd/C, MeOH, rt, 12 h; (ii) 1) cycloheptancarboxilic acid, $SOCl_2$, 76 °C, 3 h; 2) Toluene, DMF, NEt_3, 0-32 °C, 48 h; (iii) Br_2, $CHCl_3$, 30 °C, 12 h; (iv) 1) DMF, CsF, rt, 1 h; 2) *p*-fluorobenzylchloride, 50 °C, 12 h.

[^{3}H]CP55940 Binding Assays. Compounds **C1-C5**, **D1-D6** and **E1** were evaluated for CBRs binding by incubating these compounds with membrane preparations obtained from Chinese hamster ovary (CHO) cells overexpressing *h*CB_1Rs or *h*CB_2Rs in the presence of 0.7 nM [^{3}H]CP55940, a high-affinity orthosteric CB_1R and CB_2R radioligand. Also, the *N*1-unsubsituted analogue of **C2**, derivative **3**, was assayed with the aim of exploring the influence of the substituent in position 1.

Regarding the CB_1Rs, initially, all compounds were screened at 100 nM. The results (Figure S1 in Supporting Information) showed that only compounds **C1**, **D1**, **D5** and derivative **3** displaced [^{3}H]CP55940. Rather unexpectedly, all the other compounds enhanced the binding of the radioligand to CB_1Rs. In particular, compounds **C2**, **C3** and **D3** were found to be effective as enhancers at 100 nM (Figure S1 in Supporting Information) and their concentration-dependent (1 nM to 1 μM) CB_1R binding curves (**C3** and **D3** Figure S2 in Supporting Information) indicated compound **C2** (Figure 4) to be the best enhancer, inducing approximately a 40% increase in the binding of [^{3}H]CP55940 up to a concentration of 1 μM. However, no increase in such binding was induced by C2 at 10 μM. These data suggest that **C2** may be a CB_1R PAM (Figure 4).

In the case of the CB_2Rs, concentration-dependent binding curves (Figure S3 in Supporting Information) indicate that **D1-D6**, **C1**, **C3-C5** and **3** were either weak orthosteric ligands or completely inactive. Derivative **E1** was found to enhance weakly the binding of [^{3}H]CP55940 (Figure S3 in Supporting information), whereas compound **C2** strongly increased the binding of

[^{3}H]CP55940 at very low concentrations (Figure 4) raising the possibility that it may be a CB_2R PAM.

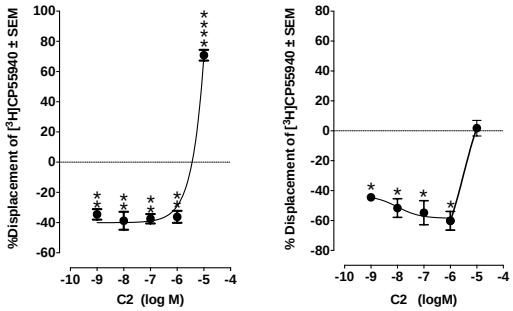


Figure 4. Effects of compounds **C2** (at 1 nM to 10 µM) on [^{3}H]CP55940 binding to CB_1R (left) and CB_2R (right). Asterisks indicate mean values significantly different from zero (*P < 0.05; **P < 0.01; ****P < 0.0001) (one sample t test). Data are expressed as means ± SEM of six independent experiments, each performed in duplicate.

[^{35}S]GTPγS assays. To evaluate the functional activity of **C2** on CBRs, [^{35}S]GTPγS assays were carried out in the presence of [^{35}S]GTPγS (0.1 nM), GDP (30 µM), GTPγS (30 µM), with CHO cell membranes (1 mg/ml) overexpressing *h*CB_1Rs or *h*CB_2Rs and in the presence of CP55940, a full agonist of both CBRs. The choice of a **C2** concentration of 100 nM for these experiments was influenced by CB_1R and CB_2R binding data. The effects of **C2** on the modulation of CBR-mediated signaling was studied by measuring [^{35}S]GTPγS receptor binding (Figure 5).

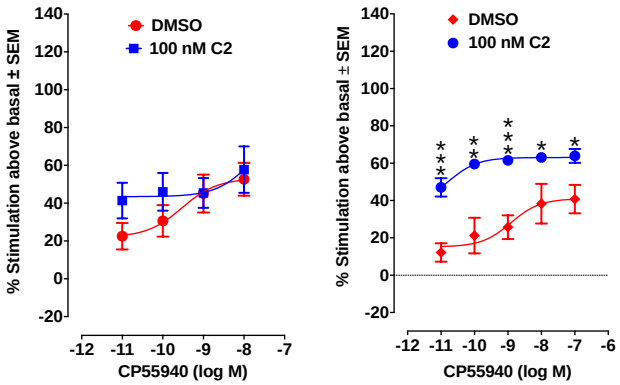


Figure 5. CB_1R [^{35}S]GTPγS binding assays (left) and CB_2R [^{35}S]GTPγS binding assays (right) performed with CP55940 and compound **C2**. . Asterisks indicate mean values significantly different from zero (*P < 0.05; **P < 0.01; ***P < 0.001) (Student's unpaired t test). Data are expressed as means ± SEM of six and twelve independent experiments for CB_1R and CB_2R respectively, each performed in duplicate.

The CP55940-induced stimulation of [^{35}S]GTPγS CB_1R binding was not significantly altered by 100 nM **C2** (Figure 5). This result suggests that although this concentration of compound **C2** increases the binding of the orthosteric ligand [^{3}H]CP55940 to CB_1Rs, this enhancement does not significantly affect the ability of CP55940 to activate CB_1Rs. However, the current data is not sufficient to indicate **C2** as SAM for CB_1R.

Conversely **C2** appeared to target CB_2Rs in a very promising and interesting manner (Figure 5). Thus, the ability of CP55940 to stimulate [^{35}S]GTPγS binding to CB_2Rs was significantly enhanced by 100 nM **C2**. Furthermore as shown in Figure S4 (Supporting Information), in the absence of the orthosteric ligand, compound **C2** did not produce any stimulation or inhibition of [^{35}S]GTPγS binding to CB_2Rs. Collectively, these data suggest that **C2** acts as a PAM of CB_2Rs.

Further studies should be carried out with the aim to investigate the allosteric effects of **C2** more fully (e.g. more concentrations and additional functional in vitro assays) and to highlight different behaviors at other CB_1R- and CB_2R-mediated signaling pathways (e.g. cAMP production, β-arrestin, ion channels).

Consequently, the study of this compound was extended by performing [^{35}S]GTPγS receptor binding assays using one or the other of the two major endocannaboinoids, anandamide (AEA) and 2-arachidonoylglycerol (2-AG), as CB_2R agonists, rather than CP55940. As shown in Figure 6, 100 nM of **C2** significantly enhanced the stimulation of [^{35}S]GTPγS binding to CB_2R induced by 2-AG, but not the enhancement induced by AEA. Thus, it is likely that **C2** would be able to facilitate the

activation of CB_2Rs by low concentrations of endogenously-released 2-AG, although not by endogenously-released AEA.

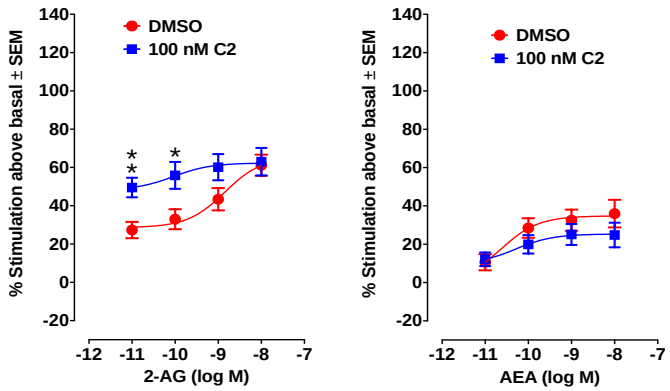


Figure 6. CB_2R [^{35}S]GTPγS binding assays performed in the presence of **C2** with 2-arachidonoylglycerol (2-AG) (left) or anandamide (AEA) (right). Asterisks indicate mean values significantly different from zero (*P < 0.05; **P < 0.01) (Student's unpaired t test). Data are expressed as means ± SEM of six independent experiments, each performed in duplicate.

Dissociation Kinetic Assays. In order to better characterize the postulated allosteric action of derivative **C2**, some dissociation kinetic assays in the absence or in the presence of **C2** (Figure 7) were performed. These assays were carried out with the CB_1/CB_2R ligand [^{3}H]CP55940 (1 μM), **C2** (100 nM) and CHO cell membranes (1 mg/ml) overexpressing *h*CB_2Rs. Dissociation was monitored from +0.5 to +120 min at 25 °C. As shown in Figure 7, [^{3}H]CP55940 dissociation was completely prevented by 100 nM of **C2** for at least 2 hours. The absence of [^{3}H]CP55940 dissociation from CB_2Rs was in line with the increased binding of [^{3}H]CP55940 induced by **C2** in binding experiments. These findings support our hypothesis that **C2** targets an allosteric binding site of the CB_2Rs. Each point on the graph represents a mean of 4 replicates.

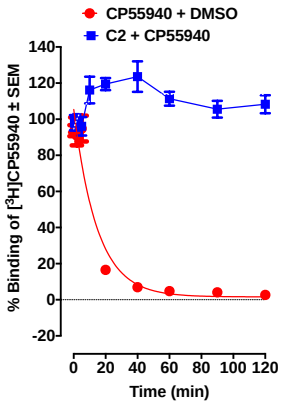


Figure 7. Dissociation of [^{3}H]CP55940 from CB_2Rs in the presence or absence of **C2**. Data are expressed as means ± SEM of four independent experiments, each performed in duplicate.

Inhibitory activity on MAGL. Finally, in line with the hypothesis that **C2** enhanced 2-AG-induced stimulation of [^{35}S]GTPγS binding to the CB_2Rs by acting as a CB_2R PAM, evidence was obtained that **C2** did not induce this enhancement by inhibiting monoacylglycerol lipase (MAGL), the main 2-AG degrading enzyme. This evidence was obtained from experiments in which the enzymatic activity of human isoforms of MAGL was measured by using a colorimetric assay that exploits 4-nitrophenylacetate (4-NPA) as a non-specific chromogenic substrate for MAGL and by measuring the absorbance values at 405 nm due to 4-nitrophenol (PNP) released from 4-NPA, upon enzymatic hydrolysis.[25] The obtained results showed that **C2** produced no significant inhibition of MAGL at < 10 μM.

Antinociceptive Effects of C2 in Animal Models of Neuropathic Pain. Neuropathic pain may be induced by traumatic injury, metabolic challenges and chemotherapeutic agents. Pharmacotherapies used to treat neuropathic pain produce inadequate pain relief and/or unwanted side effects. Thus, the identification of novel therapeutic approaches with limited side effect profiles remains an urgent medical need. Cannabinoids suppress behavioural responses to noxious stimulation

and suppress nociceptive transmission through activation of CB_1Rs and CB_2Rs. Moreover, the CB_2Rs are upregulated in the CNS and dorsal root ganglia by pathological pain states and they were identified as a therapeutic target for treating pathological pain states.[26] These observations prompted us to examine the ability of **C2** to alleviate signs of neuropathic pain in a mouse model of nociceptive behavior caused by the chemotherapeutic agent, oxaliplatin (OXP).

A daily treatment with the neurotoxic compound oxaliplatin (2.4 mg kg^{-1} intraperitoneally - i.p.) progressively decreased the pain threshold of mice evaluated as hypersensitivity to a cold non-noxious stimulus (allodynia-like measurements; cold plate test).[27] The licking latency decreased to 9.7 ± 0.9 s in comparison to control mice (18.2 ±0.6 s) treated with vehicle (Figure 8). The effect of single oral (p.o.) administration of four different doses of derivative **C2** (1, 5, 10, 20 mg kg^{-1}) on oxaliplatin-treated mice is shown in Figure 8. **C2** significantly increased licking latency in the animals starting from 5 mg kg^{-1}. The doses of 5 and 10 mg kg^{-1} of **C2** were equally effective between 30 and 60 min after administration (Figure 8). The effect of the higher 20 mg kg^{-1} dose was detectable within 15 min and lasted 60 min, reducing animal pain threshold back to the value of controls between +30 and +45 min.

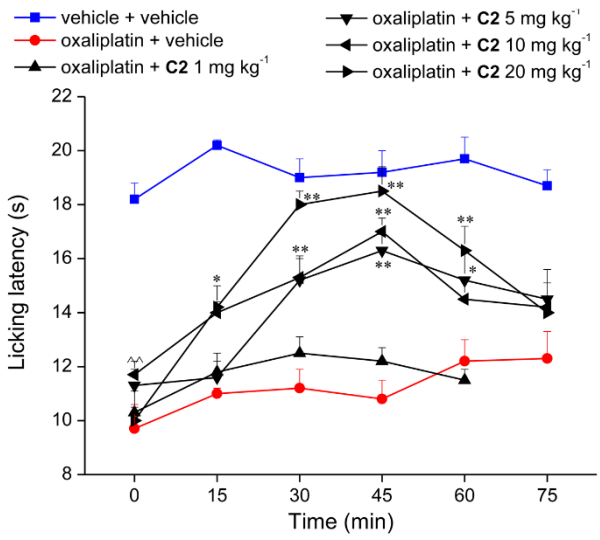


Figure 8. Neuropathic pain modulation by **C2**. Hypersensitivity was induced by repeated treatment with oxaliplatin, the response to a thermal stimulus was evaluated by the cold plate test measuring the latency (s) to pain-related behaviors (lifting or licking of the paw). Tests were performed on day 15. **C2** or vehicle were administered p.o., measurements were performed 15, 30, 45, 60 and 75 min. after treatment. Each point on the graph represents a mean of 16 individual values obtained from 16 mice analyzed in 2 different experimental sets. ^^$P < 0.01$ vs vehicle + vehicle treated mice. *$P < 0.05$ and **$P < 0.01$ vs oxaliplatin + vehicle treated mice. ANOVA followed by Bonferroni test was performed.

Additional experiments were performed to establish if an increase in endocannabinoid levels induced by a monoacylglycerol lipase (MAGL) inhibitor would potentiate the effect of **C2**. The used MAGL inhibitor **F** (compound 4a in the reference[28]) was previously reported in literature and it showed to be efficacious in the same model of neuropathic pain.[28] It was administered p.o. at a minimum active dose of 1 mg kg^{-1} 15 min before compound **C2** (used at the completely inactive dose 1 mg kg^{-1}, and the partially effective dose 10 mg kg^{-1}). The effect observed after the co-administration of **C2** with the MAGL inhibitor, was significantly increased in comparison to that produced by each compound administered alone (Figure 9). The lowest dose of **C2** (1 mg kg^{-1}) became able to fully revert oxaliplatin-induced hypersensitivity when administered after the MAGL inhibitor **F** (1 mg kg^{-1}). The analysis of synergism performed by the Chou-Talalay method[29] revealed a combination index (CI; CompuSyn software) 0.20123 considered as “synergism”. MAGL inhibitor, administered alone at dose 1 mg kg^{-1} had a partial pain relieving efficacy (Figure 9).

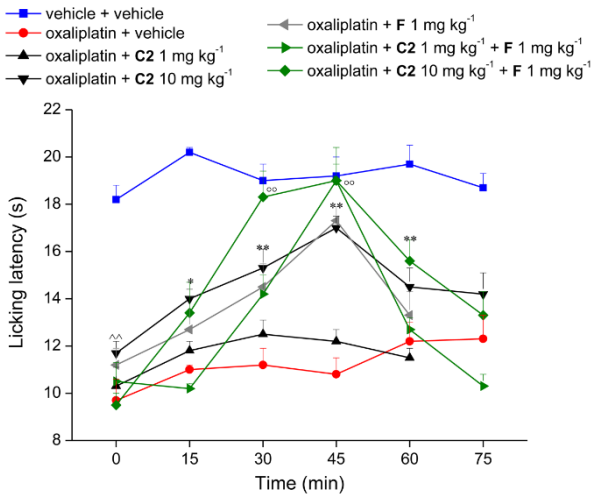


Figure 9. Neuropathic pain. Effect of **C2** administered 15 min after the MAGL inhibitor **F**. Measurements were performed 15, 30, 45, 60 and 75 min after the administration (cold plate test). Control mice were treated with vehicle. Each point on the graph represents a mean of 16 individual values obtained from 16 mice analyzed in 2 different experimental sets. ^^P < 0.01 vs vehicle + vehicle treated mice. *P < 0.05 and **P < 0.01 vs oxaliplatin + vehicle treated mice. °°P < 0.01 vs oxaliplatin + **C2** treated mice. ANOVA followed by Bonferroni test was performed.

Finally, the possible predominance of a CBR subtype in the anti-neuropathic effect of **C2** was studied. Selective CB_1R (SR141716A) and CB_2R (SR144528) antagonists were administered i.p. 15 min before **C2** (20 mg kg^{-1} p.o.; Figure 10). The efficacy of **C2** was strongly decreased by pre-treating with SR144528 (10 mg kg^{-1} i.p.), whereas SR141716A only reduced the duration of **C2** effect without affecting its anti-hyperalgesic efficacy. A main involvement of CB_2Rs is indicated as pharmacodynamic mechanism of **C2** efficacy against chemotherapy-induced neuropathic pain.

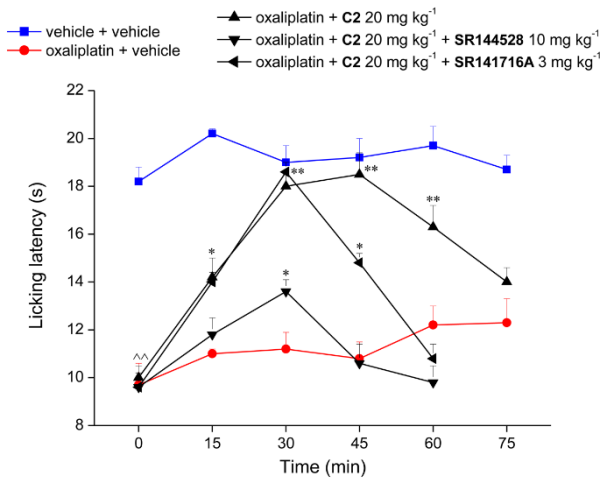


Figure 10. Neuropathic pain. Effects of CB_1R and CB_2R antagonism on **C2** pain relieving efficacy. Measurements were performed 15, 30, 45, 60 and 75 min after the administration of **C2**. Control mice were treated with vehicle. Each point on the graph represents a mean of 16 individual values obtained from 16 mice analyzed in 2 different experimental sets. ^^P < 0.01 vs vehicle + vehicle treated mice. *P < 0.05 and **P < 0.01 vs oxaliplatin + vehicle treated mice. ANOVA followed by Bonferroni test was performed.

Conclusion

In conclusion, we have presented evidence that compound **C2** is the first ever synthesized CB_2R PAM. This compound was obtained from the CBR orthosteric ligands, 2-oxopyridine-3-carboxamide, simply by changing its amide side chain into a retro-amide side chain.

Derivative **C2** behaved as a potent CB_2R PAM as indicated by its ability, at 1 nM to 1 µM, to produce marked increases in the binding of [^{3}H]CP55940 to CB_2Rs. At 100 nM, **C2** also significantly enhanced the ability of CP55940 and 2-AG, but not of AEA, to stimulate [^{35}S]GTPγS binding to CB_2Rs. As expected for an allosteric modulator, **C2** did not affect signs of CB_2R receptor signalling in the [^{35}S]GTPγS assay, in the absence of a CB_2R agonist. Interestingly, **C2** significantly improved the ability of 2-AG, but not of AEA, to stimulate [^{35}S]GTPγS binding to CB_2Rs. Thus, derivative **C2**

would be able to activate CB2Rs by low concentrations of endogenously-released 2-AG and also it could potentiate the pharmacological activity of MAGL inhibitors.

Although we studied only a small compound library, the obtained results suggest that the **C** and **D** series of compounds display interesting SARs. Undoubtedly, it was the introduction of a retro-amide sidechain into compounds **A** and **B** that converted them into CB_2R allosteric modulators (**C** and **D**). Also the bromo substituent in position 5 seems to be important, because its replacement with an H or with other groups abolished all signs of allosteric activity. Furthermore, the substitution of the methyl group in position 4 with an OH group (compound **E1**) reduced signs of allosteric activity. The display of such activity also seems to require the presence of a substituent at position 1 of the pyridine ring, as suggested by the finding that compound **3** lacks any detectable allosteric activity.

The new CB_2R PAM, **C2**, was tested in cold allodynia assays to investigate its effect on neuropathic pain. Results showed that derivative **C2**, after oral administration, dose-dependently reversed the lowering of the threshold to cold stimuli (cold plate test) induced by oxaliplatin. As demonstrated by pharmacological antagonism, this effect is predominantly mediated by the CB_2Rs.

Finally, **C2** potentiated the anti-allodynic effect of the MAGL inhibitor **F**, which previously showed to be efficacious in the same model of neuropathic pain.[28]

Our findings support our hypothesis that compound **C2** acts as a CB_2R PAM both *in vitro* and *in vivo*. Moreover, **C2** could beneficially influence the CNS damages induced by neuropathies. Several cell types (including microglia, astrocytes and oligodendrocytes) are involved in the maladaptive plasticity of the nervous tissue that is thought to be the basis of chronic pain states.[30] The widespread expression (for anatomic areas and cell types)[31] of the CB_2Rs makes its regulation a powerful therapeutic approach. The antinociceptive effects of **C2** need to be further explored as this compound might be a good candidate for clinical development. Recently, based on a homology model, an allosteric binding pocket was postulated to exist adjacent to the CB_2R orthosteric binding site.[8,14] The CB_2R PAM **C2** could be useful for the characterization of such putative binding site(s), a crucial step

for the future development of CB_2R allosteric modulators. In addition, a better understanding of the physiological consequences of CB_2R allosteric modulation might lead to novel pharmacotherapies that rely on this pharmacological action.

EXPERIMENTAL SECTION

Chemistry. Commercially available reagents were purchased from Sigma Aldrich or Alfa Aesar and used without purification. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AVANCE III™ 400 spectrometer (operating at 400 MHz). Chemical shift (δ) are reported in parts per million related to the residual solvent signal, while coupling constants (*J*) are expressed in Hertz (Hz) Catalitic hydrogenation was performed with CLAIND H_2 generator, type HG2200 (flow meter: 200 ml/min; outlet pressure: 1-6 bar; operating temperature: 5-40°C). A Bechman HPLC instrument equipped with a System Gold Solvent Delivery module (Pumps) 125, System Gold UV/VIS Detector 166, Detector set to 280 nm was employed. Analyses were performed on a reverse phase C18 column (Phenomenex 250 x 4.6 mm, 5 mm particle size, Gemini). The mobile phase was constituted by a H_2O/AcOH (0.1% v/v) (eluent A) and ACN (eluent B). A linear gradient starting from 50% of B, changing to 100% of B over 20 min., and returning to the initial conditions over 10 min., was used for all the compounds. For the HPLC analysis, all target compounds (i.e., assessed in biological assays) were ≥95% pure. Evaporation was carried out *in vacuo* using a rotating evaporator. Silica gel flash chromatography was performed using silica gel 60 Å (0.040-0.063 mm; MERK). Reactions was monitored by TLC on Merck aluminum silica gel (60 F254) plates that were visualized under a UV lamp (λ = 254 nm). Melting points were determined on a Kofler hot-stage apparatus and are uncorrected.

3-Amino-2-hydroxy-4-methylpyridine (1). Pd/C (100.0 mg) was added to a solution of the commercially available 2-hydroxy-4-methyl-3-nitropyridine (1.00 g, 6.49 mmol) in methanol. The reaction mixture was stirred under hydrogen at room temperature for 12 h and then was filtered *under*

vacuum using celite. The filtrate was evaporated under reduced pressure to afford the desired compound **1** as solid (0.80 g, 99%). ^{1}H-NMR ($CDCl_3$ δ): 12.36 (bs,1H), 6.57 (d, 1H, *J*=7.2 Hz), 5.88 (d, 1H, *J*=7.2 Hz), 3.97 (bs, 2H), 2.25 (s, 3H).

***N*-(1,2-dihydro-4-methyl-2-oxopyridin-3-yl)cycloheptanecarboxamide (2).** Cycloheptanecarboxylic acid (0.700 ml, 4.99 mmol) was dissolved in $SOCl_2$ (2.60 ml, 36.5 mmol) in a vial under nitrogen flux. The solution was stirred at 76 °C for 3 hours and then the excess of $SOCl_2$ was removed by evaporation under nitrogen flux. The obtained acyl chloride was added dropwise to a solution of the amino derivative **1** (310 mg, 2.50 mmol) and triethylamine (0.700 ml, 4.99 mmol) in anhydrous toluene (46.5 ml) and anhydrous DMF (6.20 ml) in a round bottom flask at 0 °C. The reaction mixture was stirred at room temperature for 48 hours. Then the solvent was removed under reduced pressure to give a residue which was dissolved in $CHCl_3$ and washed three times with water. Subsequently, the organic phase was dried over Na_2SO_4, filtered and evaporated under reduced pressure. The brown solid obtained was purified by flash chromatography on silica gel (ethyl acetate, 2% acetic acid) to give a solid which was triturated in hexane to afford **2** as white solid (372 mg, 60%). Mp: dec. >150 °C (crystallized from hexane). ^{1}H-NMR ($CDCl_3$ δ): 7.62 (bs, 1H), 7.10 (d, 1H, *J*=6.4 Hz), 6.26 (d, 1H, *J*=6.8 Hz), 2.53-2.48 (m, 1H), 2.16 (s, 3H), 2.02-1.98 (m, 2H), 1.81-1.71 (m, 4H), 1.59-1.53 (m, 6H).

***N*-(5-bromo-1,2-dihydro-4-methyl-2-oxo-pyridin-3-yl)cycloheptanecarboxamide (3)**. A solution of Br_2 (0.11 ml, 2.08 mmol) in $CHCl_3$ (2.1 ml) was added dropwise to a solution of the compound **2** (518 mg, 2.08 mmol) in $CHCl_3$ (3.5 ml) at room temperature. Reaction mixture was stirred at room temperature overnight and then was washed four times with a saturated aqueous solution of $Na_2S_2O_3$. The organic phase was dried over Na_2SO_4, filtered and evaporated under reduced pressure. The solid residue was crystallized from hexane to give **3** as a white solid (578 mg, 85%). Mp: 217-219 °C. ^{1}H-NMR ($CDCl_3$ δ): 7.40 (bs, 1H), 7.39 (s, 1H), 2.56-2.49 (m, 1H), 2.21 (s, 3H), 2.06-2.00 (m, 2H), 1.85-1.73 (m, 4H), 1.61-1.55 (m, 6H). ^{13}C-NMR ($CDCl_3$ δ): 176.32, 158.04,

134.75, 128.17, 127.41, 100.81, 48.21, 31.57, 28.40, 26.47, 19.68. HPLC analysis: retention time = 5.1 min; peak area, 95.5% (280 nm).

***N*-(5-chloro-1,2-dihydro-4-methyl-2-oxo-pyridin-3-yl)cycloheptanecarboxamide (4)**. A solution of *N*-chlorosuccinimide (270 mg, 2.02 mmol) in acetonitrile (2.0 mL) was added to a suspension of the derivative **2** (500 mg, 2.02 mmol) in acetonitrile (3.0 mL). The reaction mixture was refluxed overnight. Then, the solvent was removed under reduced pressure and the residue was treated with water and extracted with ethyl acetate. The organic phase was dried over Na_2SO_4, filtered and concentrated under reduced pressure to give a yellow solid purified by flash chromatography on silica gel using ethyl acetate/petroleum ether 7:3 and 1% acetic acid as eluent, to give a yellow solid which was triturated in hexane to afford compound **4** as white solid (274 mg, 48%). Mp: 200 °C (dec), (crystallized from hexane). ^{1}H-NMR ($CDCl_3$ δ): 8.73 (bs, 1H), 7.52 (s, 1H), 7.29 (bs, 1H), 2.54 (m, 1H), 2.18 (s, 3H), 2.04-2.01 (m, 2H), 1.83-1.73 (m, 4H), 1.66-1.49 (m, 6H).

3-amino-2-hydroxy-6-methylpyridine (5). Compound **5** was prepared as described for compound **1**, starting from 2-hydroxy-6-methyl-3-nitropyridine. Yield: 99%. ^{1}H-NMR ($CDCl_3$ δ): 12.20 (bs, 1H), 6.78 (d, 1H, J=6.8 Hz), 6.09 (d, 1H, J=6.8 Hz), 4.03 (bs, 2H), 2.10 (s, 3H).

***N*-(1,2-dihydro-6-methyl-2-oxopyridin-3-yl)cycloheptanecarboxamide (6)**. A solution of cycloheptanecarboxylic acid (0.870 ml, 6.32 mmol) in anhydrous DMF (15.70 ml) was stirred in an ice-bath. TBTU (2440 mg, 7.59 mmol) and triethylamine (2.40 ml, 17.70 mmol) were added at 0 °C. After 30 minutes, compound **5** (785 mg, 6.32 mmol) was added and the reaction mixture was stirred at 0 °C for 30 minutes and then at room temperature overnight. The solvent was removed under reduced pressure. The solid residue was dissolved in $CHCl_3$ and washed with water. The organic phase was dried over Na_2SO_4, filtered and evaporated under reduced pressure. The brown solid thus obtained was purified by flash chromatography on silica gel using ethyl acetate/petroleum ether 8:2 as eluent to give a yellow solid which was crystallized from hexane to afford compound **6** as white solid (1.290 g). Yield: 82 %. Mp: 182-185 °C. ^{1}H-NMR ($CDCl_3$ δ): 11.77 (bs, 1H), 8.39 (d, 1H, *J*=7.6

Hz), 8.16 (bs, 1H), 6.09 (d, 1H, *J*=7.6 Hz), 2.47-2.44 (m, 1H), 2.32 (s, 3H), 2.01-1.95 (m, 2H), 1.83-1.71 (m, 4H), 1.62-1.49 (m, 6H).

***N*-(5-bromo-1,2-dihydro-6-methyl-2-oxo-pyridin-3-yl)cycloheptanecarboxamide (7)**. Compound **7** was prepared starting from derivative **6**, as described for compound **3** and it was purified by flash chromatography on silica gel using ethyl acetate/petroleum ether 7:3. Yield: 89%. ^{1}H-NMR ($CDCl_3$ δ): 12.25 (bs, 1H), 8.64 (s, 1H), 8.10 (s, 1H), 2.50-2.41 (m, 1H), 2.01 (s, 3H), 1.75-1.61 (m, 6H), 1.84-1.76 (m, 4H), 1.99-1.94 (m, 2H).

***N*-(5-chloro-1,2-dihydro-6-methyl-2-oxo-pyridin-3-yl)cycloheptanecarboxamide (8)**. Compound **8** was prepared starting from derivative **6**, as described for compound **4** and it was purified by flash chromatography on silica gel using ethyl acetate/petroleum ether 1:1, Yield: 19%. Mp: 240-246 °C (crystallized from hexane). ^{1}H-NMR ($CDCl_3$ δ): 11.99 (bs, 1H), 8.55 (s, 1H), 8.11 (bs, 1H), 2.50-2.48 (m, 1H), 2.39 (s, 3H), 2.01-1.96 (m, 2H), 1.85-1.81 (m, 4H), 1.79-1.54 (m, 6H).

***N*-(1,2-dihydro-5-fluoro-6-methyl-2-oxo-pyridin-3-yl)cycloheptanecarboxamide (9)**. 1-chloromethyl-4-fluoro-1,4-diazoniabicyclo[2.2.2] octanebis(tetrafluoroborate) (Selectfluor®)(1.200 g, 3.39 mmol) was added at room temperature to a suspension of compound **6** (700 mg, 2.82 mmol) in acetonitrile. Reaction mixture was refluxed for 12 hours. Then, the solvent was removed under reduced pressure and the residue was treated with ethyl acetate and washed three times with water. The organic phase was dried over Na_2SO_4, filtered and evaporated under reduced pressure. The black solid thus obtained was purified by flash chromatography on silica gel using petroleum ether/ethyl acetate 1:1 as eluent, to give compound **9** (52.0 mg, 0.196 mmol) as a black solid. Yield: 7% Mp: 95-98 °C (crystallized from hexane). ^{1}H-NMR ($CDCl_3$ δ): 8.48 (d, 1H, *J*=10.4 Hz), 8.16 (bs, 1H), 2.51-2.46 (m, 1H), 2.30 (d, 3H, *J*=2.8 Hz), 2.01-1.95 (m, 2H), 1.81-1.75 (m, 4H), 1.62-1.53 (m, 6H).

3-amino-2,4-dihydroxypyridine (10). Compound **10** was prepared starting from 2,4-dihydroxy-3-nitropyridine, as described for compound **1**. Yield: 33%. ^{1}H-NMR (MeOD δ): 12.26 (bs,1H), 6.84 (d, 1H, *J*= 7.2 Hz); 6.13 (d, 1H, *J*= 7.2 Hz), 4.03 (bs, 2H).

***N*-(1,2-dihydro-4-hydroxy-2-oxopyridin-3-yl)cycloheptanecarboxamide (11)**. Compound **11** was prepared starting from derivative **10**, as described for compound **2** and it was purified by trituration with MeOH and then with Et_2O. Yield: 25%. Mp: dec. >130 °C (crystallized from hexane). ^{1}H-NMR ($CDCl_3$ δ): 12.71 (bs, 1H); 11.28 (bs, 1H); 8.39 (s, 1H), 7.03 (d, 1H, *J*= 7.2 Hz), 6.08 (d, 1H, *J*= 7.2 Hz) 2.58-2.57 (m, 1H), 2.02-1.98 (m, 2H), 1.83-1.72 (m, 4H), 1.65-1.50 (m, 6H).

***N*-(5-bromo-1,2-dihydro-4-hydroxy-2-oxo-pyridin-3-yl)cycloheptanecarboxamide (12)**. Compound **12** was prepared starting from derivative **11**, as described for compound **3** and it was purified by flash chromatography on silica gel using ethyl acetate/petroleum ether 7:3. Yield: 68 %. ^{1}H-NMR ($CDCl_3$ δ): 8.34 (bs, 1H), 7.35 (s, 1H), 2.60-2.55 (m, 1H), 2.02-1.98 (m, 2H), 1.83-1,71 (m, 4H), 1.61-1.51 (m, 6H).

***N*-[1,2-dihydro-1-(4'-fluorobenzyl)-4-methyl-2-oxo-pyridin-3yl]cycloheptanecarboxamide (C1)**. An amount of 214.0 mg of CsF (1.41 mmol) was added to a solution of of 1,2-dihydro-pyridin-3-cycloheptanecarboxamide **2** (117.0 mg, 0.470 mmol) in anhydrous DMF (1.40 ml). After 1 hour at room temperature, 4-fluorobenzyl chloride (0.17 ml, 1.41 mmol) was added dropwise and the mixture was stirred at 50 °C overnight. After cooling, the reaction mixture was concentrated under reduced pressure, treated with ice-water and then extracted with CH_2Cl_2. The organic phase was dried over Na_2SO_4, filtered and evaporated under reduced pressure. The yellow oil obtained was purified by flash chromatography on silica gel using petroleum ether/ethyl acetate 1:1), to give **C1** as white solid (67.0 mg, 40%). Mp: 160-165 °C (crystallized from hexane). ^{1}H-NMR: ($CDCl_3$ δ): 7.43 (bs, 1H), 7.28-7.25 (m, 2H), 7.04-7.00 (m, 3H), 6.10 (d, 1H, *J*=7.2 Hz), 5.07 (s, 2H), 2.53-2.46 (m, 1H), 2.12 (s, 3H), 2.07-2.00 (m, 2H), 1.83-1.72 (m, 4H), 1.59-1.52 (m, 6H). ^{13}C-NMR: ($CDCl_3$ δ): 176.06, 162.65 (d, *J*=246.0 Hz), 159.56, 142.40, 132.04 (d, *J*=3.2 Hz), 131.67, 130.02 (d, *J*=9.1 Hz), 125.85, 115.95 (d, *J*=22.0 Hz), 110.21, 51.99, 48.04, 31.92, 28.27, 26.77, 19.83. HPLC analysis: retention time = 8.6 min; peak area 99.7% (280 nm).

***N*-[5-bromo-1,2-dihydro-1-(4'-fluorobenzyl)-4-methyl-2-oxo-pyridin-3yl]cycloheptanecarboxamide (C2)**. Compound **C2** was prepared starting from derivative **3**, as described for compound **C1** and it was purified by flash chromatography on silica gel using petroleum ether/ethyl acetate 7:3 as eluent. Yield: 58% Mp: 167-170 °C (crystallized from hexane). ^{1}H-NMR ($CDCl_3$ δ): 7.48 (bs, 1H), 7.33 (s, 1H), 7.30-7.28 (m, 2H), 7.06-7.02 (m, 2H), 5.05 (s, 2H), 2.53-2.47 (m, 1H), 2.15 (s, 3H), 2.06-1.99 (m, 2H), 1.81-1.71 (m, 4H), 1.59-1.52 (m, 6H). ^{13}C-NMR ($CDCl_3$ δ): 176.08, 162.77 (d, *J*=246.0 Hz), 158.42, 142.07, 132.10, 131.34 (d, *J*=4.1 Hz), 130.21 (d, *J*=9.1 Hz), 126.60, 116.12 (d, *J*=22.0 Hz), 104.08, 52.06, 47.94, 31.81, 28.22, 26.70, 20.67. HPLC analysis: retention time = 9.5 min; peak area 95.5 % (280 nm).

***N*-[1,2-dihydro-1-(4'-fluorobenzyl)-5-(4'-methoxyphenyl)-4-methyl-2-oxo-pyridin-3-yl]cycloheptanecarboxamide (C3)**. A mixture of $Pd(OAc)_2$ (2.20 mg, 0.009 mmol) and PPh_3 (11.80 mg, 0.045 mmol) in 2.50 mL of toluene was stirred under nitrogen at room temperature. After 15 minutes, the suitable boronic acid (37.9 mg, 0.600 mmol), K_2CO_3 (62,2 mg, 0.450 mmol) and compound **C2** (130.6 mg, 0.300 mmol) were added. Reaction mixture was stirred at 110 °C overnight. After cooling, the reaction was filtered *under vacuum* using celite, and the filtrate was evaporated under reduced pressure. The residue was treated with water and extracted with $CHCl_3$. The combined organic layers were washed with brine, dried over anhydrous sodium sulfate, and evaporated to give a residue which was purified by flash chromatography on silica gel using petroleum ether/ethyl acetate 8:2 as eluent affording **C3** as oil (100 mg, 0.216 mmol). Yield: 72%. ^{1}H-NMR ($CDCl_3$ δ): 7.57 (bs, 1H), 7.32-7.29 (m, 2H), 7.17-7.15 (m, 2H), 7.05-7.00 (m, 3H), 6.92-6.90 (m, 2H), 5.12 (s, 2H), 3.83 (s, 3H), 2.56-2.51 (m, 1H), 2.07-2.02 (m, 2H), 1.98 (s, 3H), 1.81-1.76 (m, 4H), 1.60-1.58 (m, 6H). ^{13}C-NMR ($CDCl_3$ δ): 176.15, 162.18 (d, *J*=245.0 Hz), 159.26, 158.70, 142.25, 135.89, 131.97 (d, *J*=32 Hz), 130.92, 130.82, 130.07 (d, *J*=8.6 Hz), 129.05, 125.85, 123.46, 115.92 (d, *J*=22.0 Hz), 113.95, 55.42, 52.00, 47.89, 31.88, 28.26, 26.70, 18.50. HPLC analysis: retention time = 13.2 min; peak area 98.2 % (280 nm).

***N*-[1,2-dihydro-1-(4'-fluorobenzyl)-4-methyl-2-oxo-5-phenyl-pyridin-3-yl]cycloheptanecarboxamide (C4)**. Compound **C4** was prepared from compound **C2**, as described for compound **C3** and it was purified by crystallization from hexane. Yield: 45%. Mp: 143-146 °C (crystallized from hexane). ^{1}H-NMR: ($CDCl_3$ δ): 7.54 (bs, 1H), 7.41-7.29 (m, 5H), 7.25-7.24 (m, 2H), 7.05-7.01 (m, 3H), 5.12 (s, 2H), 2.56-2.51 (m, 1H), 2.07-2.02 (m, 2H), 1.99 (s, 3H), 1.83-1.77 (m, 4H), 1.60-1.53 (m, 6H). ^{13}C-NMR ($CDCl_3$ δ): 176.05, 162.58 (d, *J*=243.8 Hz), 158.72, 141.85, 136.78, 131.93 (d, *J*=3.2 Hz), 131.00, 130.04 (d, *J*=8.6 Hz), 129.67, 128.52, 127.72, 126.02, 123.77, 115.89 (d, *J*=21.8 Hz), 51.98, 47.81, 31.85, 28.25, 26.67, 18.41. HPLC analysis: retention time = 13.5 min; peak area 99.6 % (280 nm).

***N*-[5-chloro-1,2-dihydro-1-(4'-fluorobenzyl)-4-methyl-2-oxo-pyridin-3-yl]cycloheptanecarboxamide (C5)**. Compound **C5** was prepared from compound **4**, as described for compound **C1** and it was purified by flash chromatography on silica gel using petroleum ether/ethyl acetate 7:3 as eluent to give a light yellow solid which was triturated in hexane to afford **C5** as white solid. Yield: 39%. Mp: 197-199 °C (crystallized from hexane). ^{1}H-NMR ($CDCl_3$ δ): 7.49 (bs, 1H), 7.30-7.27 (m, 2H), 7.21 (s, 1H), 7.06-7.02 (m, 2H), 5.06 (s, 2H), 2.53-2.48 (m, 1H), 2.14 (s, 3H), 2.06-2.00 (m, 2H), 1.81-1.74 (m, 4H), 1.58-1.51 (m, 6H). ^{13}C-NMR ($CDCl_3$ δ): 176.06, 162.72 (d, *J*=246.0 Hz), 158.24, 141.17, 131.33 (d, *J*=3.2 Hz), 130.17 (d, *J*=8.6 Hz), 129.62, 126.64, 116.05 (d, *J*=22.0 Hz), 115.75, 52.01, 47.83, 31.77, 28.19, 26.66, 17.86. HPLC analysis: retention time = 11.6 min; peak area 96.8 % (280 nM).

***N*-[1,2-dihydro-1-(4'-fluorobenzyl)-6-methyl-2-oxo-pyridin-3yl]cycloheptanecarboxamide (D1)**. An amount of 35.2 mg (0.879 mmol) of NaH (60% mineral oil dispersion) was added portionwise to a solution of 1,2-dihydropyridin-3-cycloheptanecarboxamide **6** (212 mg, 0.854 mmol) in DME (1.700 ml) and anhydrous DMF (0.400 ml). After 10 minutes at 0 °C, LiBr (147.6 mg, 1.700 mmol) was added and the reaction mixture was stirred at room temperature for 15 minutes. After that, 4-fluorobenzylchloride (0.200 ml, 1.650 mmol) was added dropwise and the mixture was stirred at

65 °C overnight. After cooling, the solvent was removed under reduced pressure and the obtained residue was dissolved in $CHCl_3$ and washed with brine. The organic phase was dried over Na_2SO_4, filtered and evaporated under reduced pressure to obtain a residue which was purified by flash chromatography on silica gel using petroleum ether/ethyl acetate 8:2 as eluent giving **D1** as oil (79.1 mg, 0.220 mmol). Yield: 26% ^{1}H-NMR ($CDCl_3$ δ): 8.31 (bs, 1H), 8.30 (d, 1H, *J*=7.6 Hz), 7.11-7.08 (m, 2H), 6.99-6.96 (m, 2H), 6.09 (d, 1H, *J*=7.6 Hz), 5.31 (s, 2H), 2.44-2.43 (m, 1H), 2.26 (s, 3H), 1.99-1.95 (m, 2H), 1.79-1.70 (m, 4H), 1.58-1.48 (m, 6H). ^{13}C-NMR ($CDCl_3$ δ): 176.44, 162.27 (d, *J*=245.0 Hz), 158.75, 137.97, 131.86, 128.25 (d, *J*=8.6 Hz), 127.36, 122.34, 115.93 (d, J=22.1 Hz), 107.32, 48.58, 47.62, 31.69, 28.31, 26.64, 20.14. HPLC analysis: retention time = 13.2 min; peak area 97.1 % (280 nm).

***N*-[5-bromo-1,2-dihydro-1-(4'-fluorobenzyl)-6-methyl-2-oxo-pyridin-3-y]cycloheptanecarboxamide (D2)**. Compound **D2** was prepared from derivative **7**, as described for compound **D1** and it was purified by flash column chromatography on silica gel using petroleum ether/ethyl acetate 8:2 as eluent. Yield: 40%. Mp: 124-127 °C (crystallized from hexane). ^{1}H-NMR ($CDCl_3$ δ): 8.61 (s, 1H), 8.31 (bs, 1H), 7.12-7.09 (m, 2H), 7.04-7.00 (m, 2H), 5.39 (s, 2H), 2.48-2.43 (m, 1H), 2.42 (s, 3H), 2.02-1.94 (m, 2H), 1.82-1.71 (m, 4H), 1.63-1.54 (m, 6H). ^{13}C-NMR ($CDCl_3$ δ): 176.33, 162.19 (d, *J*=246.0 Hz), 157.76, 135.64, 131.20 (d, *J*=3.2 Hz), 128.05 (d, *J*=8.1 Hz), 127.46, 125.78, 115.91 (d, *J*=22.0 Hz), 101.53, 48.77, 48.23, 31.42, 28.18, 26.39, 19.49. HPLC analysis: retention time = 17.6 min; peak area 98.1 % (280 nM).

***N*-[1,2-dihydro-1-(4'-fluorobenzyl)-5-phenyl-6-methyl-2-oxo-pyridin-3-yl]cycloheptane carboxamide (D3)**. Compound **D3** was prepared starting from derivative **D2**, as described for compound **C3** and it was purified by flash column chromatography on silica gel using petroleum ether/ethyl acetate 8:2 as eluent. Yield: 13%. ^{1}H-NMR ($CDCl_3$ δ): 8.45 (s, 1H), 8.38 (bs, 1H), 7.38−7.28 (m, 4H), 7.25-7.23 (m, 3H), 7.17-7.01 (m, 2H), 5.44 (s, 2H), 2.46−2.43 (m, 1H), 2.24 (s, 3H), 2.01−1.95 (m, 2H), 1.79−1.71 (m, 4H), 1.59−1.49 (m, 6H). ^{13}C-NMR ($CDCl_3$ δ): 176.47, 162.29

(d, *J*=244.0 Hz), 158.22, 139.27, 135.05, 132.00, 129.84, 128.53, 128.25 (d, *J*=8.6 Hz), 127.43, 126.75, 125.23, 121.35, 115.99 (d, *J*=22.0 Hz), 48.46, 48.16, 31.68, 28.42, 26.60, 17.56. HPLC analysis: retention time = 18.0 min; peak area 95.8 % (280 nm).

***N*-[1,2-dihydro-1-(4'-fluorobenzyl)-5-(4'-methoxyphenyl)-6-methyl-2-oxo-pyridin-3-yl]cycloheptanecarboxamide (D4)**. Compound **D4** was prepared starting from compound **D2**, as described for compound **C3** and it was purified by flash column chromatography on silica gel using petroleum ether/ethyl acetate 8:2 as eluent. Yield: 19%. ^{1}H-NMR ($CDCl_3$ δ): 8.43 (s, 1H), 8.38 (bs, 1H), 7.17-7.13 (m, 4H), 7.05-7.01 (m, 2H), 6.90-6.88 (m, 2H), 5.43 (s, 2H), 3.83 (s, 3H), 2.48-2.43 (m, 1H), 2.23 (s, 3H), 2.00-1.97 (m, 2H), 1.80-1.74 (m, 4H), 1.52-1.50 (m, 6H). ^{13}C-NMR ($CDCl_3$ δ): 176.43, 162.24 (d, *J*=244.0 Hz), 158.93, 158.14, 135.03, 131.99 (d, *J*=3.2 Hz), 130.89, 128.22 (d, *J*=8.6 Hz), 126.61, 125.47, 120.95, 115.94 (d, *J*=21.8 Hz), 113.90, 55.42, 48.44, 48.16, 31.65, 28.38, 26.58, 17.53. HPLC analysis: retention time = 17.3 min; peak area, 99.0 % (280 nm).

***N*-[5-chloro-1,2-dihydro-1-(4'-fluorobenzyl)-6-methyl-2-oxo-pyridin-3-yl]cycloheptanecarboxamide (D5)**. Compound **D5** was prepared starting from compound **8**, as described for compound **D1** and it was purified by crystallization from hexane. Yield: 36%. Mp: 116-119 °C (crystallized from hexane). ^{1}H-NMR ($CDCl_3$ δ): 8.49 (s, 1H), 8.33 (bs, 1H), 7.12-7.09 (m, 2H), 7.04-7.00 (m, 2H), 5.37 (s, 2H), 2.46-2.43 (m, 1H), 2.38 (s, 3H), 2.04-1.94 (m, 2H), 1.81-1.71 (m, 4H), 1.61-1.48 (m, 6H). ^{13}C-NMR ($CDCl_3$ δ): 176.39, 162.24 (d, *J*=245.0 Hz), 157.56, 134.35, 131.26 (d, *J*=3.2 Hz), 128.11 (d, *J*=8.1 Hz), 127.37, 123.48, 115.95 (d, *J*=22.0 Hz), 113.35, 48.50, 48.30, 31.47, 28.21, 26.44, 16.68. HPLC analysis: retention time = 16.9 min; peak area 98.2 % (280 nm).

***N*-[1,2-dihydro-5-fluoro-1-(4'-fluorobenzyl)-6-methyl-2-oxo-pyridin-3-yl]cycloheptanecarboxamide (D6)**. Compound **D6** was prepared starting from compound **9**, as described for compound **D1,** and it was purified by flash column chromatography on silica gel using petroleum ether/ethyl acetate 8:2. Yield: 24%. ^{1}H-NMR ($CDCl_3$ δ): 8.43 (d, 1H, *J*=10.4 Hz), 8.40

(bs, 1H), 7.14-7.11 (m, 2H), 7.04-7.00 (m, 2H), 5.32 (s, 2H), 2.48-2.43 (m, 1H), 2.25 (d, 3H, *J*=3.2 Hz), 2.05-1.95 (m, 2H), 1.80-1.74 (m, 4H), 1.55-1.52 (m, 6H). ^{13}C-NMR ($CDCl_3$ δ): 176.54, 162.33 (d, *J*=245.0 Hz), 156.74, 145.52 (d, *J*=224.2 Hz), 131.48 (d, *J*=3.2 Hz), 128.30 (d, *J*=8.6 Hz), 127.23 (d, *J*=13.0 Hz), 123.12 (d, *J*=29.1 Hz), 116.02 (d, *J*=21.0 Hz), 114.34 (d, *J*=32.4 Hz), 48.45, 47.78, 31.56, 28.28, 26.56, 11.78. HPLC analysis: retention time = 15.1 min; peak area 96.3 % (280 nm).

***N*-(5-bromo-1,2-dihydro-1-(4-fluorobenzyl)-4-hydroxy-2-oxo-pyridin-3-yl)cycloheptanecarboxamide (E1)**. Compound **E1** was prepared from compound **12**, as described for compound **C1** and it was purified by flash column chromatography on silica gel using petroleum ether/ethyl acetate 8:2 as eluent. Yield: 8%. Mp: 73-76 °C (crystallized from hexane). ^{1}H-NMR ($CDCl_3$ δ): 13.46 (bs, 1H), 8.51 (bs, 1H), 7.30 (s, 1H), 7.28-7.24 (m, 2H), 7.08-7.03 (m, 2H), 5.06 (s, 2H), 2.61-2.52 (m, 1H), 2.05-1.97 (m, 2H), 1.85-1.68 (m, 4H), 1.62-1.44 (m, 6H). ^{13}C-NMR ($CDCl_3$ δ): 178.70, 161.56, 132.50, 131.40, 129.94 (d, *J*=8.6 Hz), 116.19 (d, *J*=21.8 Hz), 112.40, 97.30, 51.88, 47.72, 31.85, 28.09, 26.50. HPLC analysis: retention time = 15.6 min; peak area 95.8% (280 nM).

Biological Evaluations

CHO cells. CHO cells stably transfected with cDNA encoding human cannabinoid CB_1Rs or CB_2Rs were maintained at 37°C and 5% CO_2 in Gibco™ Ham's F-12 Nutrient Mix supplied by Fisher Scientific UK Ltd that was supplemented both with 2 mM L-glutamine, 10% FBS and 0.6% penicillin-streptomycin, all also supplied by Fisher Scientific UK Ltd, and with the disulphate salt of G418 [(2R,3S,4R,5R,6S)-5-amino-6-{[(1R,2S,3S,4R,6S)-4,6-diamino-3-{[(2R,3R,4R,5R)-3,5-dihydroxy- 5-methyl-4-(methylamino)oxan-2-yl]oxy}-2-hydroxy cyclohexyl]oxy}-2-[(1R)-1-hydroxyethyl]oxane-3,4-diol; 600 mg·mL^{-1}] supplied by Sigma-Aldrich UK. All cells were exposed to 5% CO_2 in their respective media, and were passaged twice a week using non-enzymatic cell dissociation solution. For membrane preparation, cells were removed from flasks by scraping,

centrifuged, and then frozen as a pellet at -20 °C until required. Before use in a radioligand binding assay, cells were defrosted, diluted in Tris buffer (50 mM Tris–HCl and 50 mM Tris–base).

Radioligand displacement assay. The assays were carried out with [^{3}H]CP55940 and Tris binding buffer (50 mM Tris–HCl, 50 mM Tris–base, 0.1% BSA, pH 7.4), total assay volume 500 µl. Binding was initiated by the addition of transfected human CB_1 or CB_2 CHO cell membranes (50 µg protein per well). All assays were performed at 37 °C for 60 min before termination by the addition of ice-cold Tris binding buffer, followed by vacuum filtration using a 24-well sampling manifold (Brandel Cell Harvester; Brandel Inc, Gaithersburg, MD, USA) and Brandel GF/B filters that had been soaked in wash buffer at 4°C for at least 24 h. Each reaction well was washed six times with a 1.2 mL aliquot of Tris-binding buffer. The filters were oven-dried for 60 min and then placed in 3 mL of scintillation fluid (Ultima Gold XR, PerkinElmer, Seer Green, Buckinghamshire, UK). Radioactivity was quantified by liquid scintillation spectrometry. Specific binding was defined as the difference between the binding that occurred in the presence and absence of 1 µM unlabelled CP55940. The concentration of [^{3}H]CP55940 used in our displacement assays was 0.7 nM. The compounds used in this investigation were stored as stock solutions of 10 mM in DMSO, the vehicle concentration in all assay wells being 0.1% DMSO. Each point on each graph represents the mean of data obtained from six independent experiments. Each experiment was performed in duplicate for each concentration.

[^{35}S]GTPγS assay. The assay was carried out in the presence of [^{35}S]GTPγS (0.1 nM), GDP (30 µM), GTPγS (30 µM) and CHO cell membranes (1 mg/ml) overexpressing *h*CB_1Rs or *h*CB_2Rs. The assay buffer contained 50 mM Tris, 10 mM $MgCl_2$, 100 mM NaCl, 0.2 mM EDTA and 1 mM DTT (dithiothreitol) at pH 7.4. Incubations were carried out at 30 °C for 90 min in a total volume of 500 ml. The reaction was terminated by the addition of ice-cold wash buffer (50 mM Tris and 1 mg/ml BSA, pH 7.4) followed by rapid filtration under vacuum through Whatman GF/B glass-fibre filters (pre-soaked in wash buffer) 24-well sampling manifold (Brandel Cell Harvester; Brandel Inc,

Gaithersburg, MD, USA) and Brandel GF/B filters that had been soaked in wash buffer at 4°C for at least 24 h. Each reaction well was washed six times with a 1.2 mL aliquot of Tris-binding buffer. The filters were oven-dried for 60 min and then placed in 3 mL of scintillation fluid (Ultima Gold XR, PerkinElmer, Seer Green, Buckinghamshire, UK). Bound radioactivity was determined by liquid scintillation counting. Basal binding of [^{35}S]GTPγS was determined in the presence of 20 mM GDP and absence of cannabinoid. Non-specific binding was determined in the presence of 10 mM GTPgS. The compounds used in this investigation were stored as stock solutions of 10 mM in DMSO, the vehicle concentration in all assay wells being 0.1% DMSO. Each point on CB1R graph represents the mean of data obtained from six independent experiments. Each experiment was performed in duplicate for each concentration. This protocol was also used to obtain the data shown in the 2-AG and AEA CB2R graphs. Each point on the CP55940 CB2R graph represents the mean of data obtained from twelve independent experiments. Each experiment was performed in duplicate for each concentration.

Dissociation kinetic assay. Dissociation kinetic assays were performed with [^{3}H]CP55940 and Tris binding buffer (50 mM Tris–HCl, 50 mM Tris–base, 0.1% BSA, pH 7.4), total assay volume 500 µl. Dissociation was initiated by addition of transfected human CB_1 or CB_2 CHO cell membranes (50 µg protein per well). Dissociation times of 0.5 to 120 min at 25°C were used. To determine the nonspecific binding, experiments were also performed in the presence of a 1 µM concentration of the unlabeled ligand. All assays were performed at 37 °C for 60 min before termination by the addition of ice-cold Tris binding buffer, followed by vacuum filtration using a 24-well sampling manifold (Brandel Cell Harvester; Brandel Inc, Gaithersburg, MD, USA) and Brandel GF/B filters that had been soaked in wash buffer at 4°C for at least 24 h. Each reaction well was washed six times with a 1.2 mL aliquot of Tris-binding buffer. The filters were oven-dried for 60 min and then placed in 3 mL of scintillation fluid (Ultima Gold XR, PerkinElmer, Seer Green, Buckinghamshire, UK). Radioactivity was quantified by liquid scintillation spectrometry. Specific binding was defined as the difference between the binding that occurred in the presence and absence of 1 µM unlabelled

CP55940. The concentration of [^{3}H]CP55940 used in our displacement assays was 0.7 nM. The compounds used in this investigation were stored as stock solutions of 10 mM in DMSO, the vehicle concentration in all assay wells being 0.1% DMSO. Each point on the graph represents the mean of data obtained from four independent experiments. Each experiment was performed in duplicate for each concentration.

Data analysis. Results were calculated as percentage changes from a basal level (zero) of [^{3}H]CP55940 binding (in the presence of vehicle). GraphPad Prism 5.0 (GraphPad, San Diego, CA) was used to construct sigmoidal log concentration-response curves, and to calculate values of EC50, Emax, S.E.M and 95% confidence intervals. P values < 0.05 were considered to be significant.

MAGL inhibition assay. Human recombinant MAGL and 4-NPA substrate were from Cayman Chemical. The enzymatic reaction was carried out at room temperature (final volume of 200 µL in 10 mM Tris buffer, pH 7.2, containing 1 mM EDTA and 0.1 mg/ml BSA). A total of 150 µL of 4-NPA 133.3 µM (final concentration = 100 µM) was added to 10 µL of DMSO containing the appropriate amount of compound and the reaction was initiated by the addition of 40 µL of MAGL (11 ng/well). After the reaction had proceeded for 30 min, absorbance values were then measured by using a Victor X3 Microplates Reader (PerkinElmer®) at 405 nm.[1] The assay was generated in 96-well microtiter plates. Two reactions were also run: one reaction containing no compounds and the second one containing neither inhibitor nor enzyme.

Pharmacological *in vivo* study. Male CD-1 albino mice (Envigo, Varese, Italy) weighing approximately 22–25 g at the beginning of the experimental procedure, were used. Animals were housed in CeSAL (Centro Stabulazione Animali da Laboratorio, University of Florence) and used at least 1 week after their arrival. Ten mice were housed per cage (size 26 × 41 cm); animals were fed a standard laboratory diet and tap water *ad libitum*, and kept at 23 ± 1 °C with a 12 h light/dark cycle, light at 7 a.m. All animal manipulations were carried out according to the Directive 2010/63/EU of the European parliament and of the European Union council (22 September 2010) on the protection

of animals used for scientific purposes. The ethical policy of the University of Florence complies with the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health (NIH Publication No. 85-23, revised 1996; University of Florence assurance number: A5278-01). Formal approval to conduct the experiments described was obtained from the Animal Subjects Review Board of the University of Florence. Experiments involving animals have been reported according to ARRIVE guidelines.[32] All efforts were made to minimize animal suffering and to reduce the number of animals used.

Oxaliplatin-induced neuropathic pain model. Mice treated with oxaliplatin (2.4 mg kg^{-1}) were administered i.p. on days 1-2, 5-9, 12-14 (10 i.p. injections).[27,33] Oxaliplatin was dissolved in 5% glucose solution. Control animals received an equivalent volume of vehicle. Behavioral tests were performed on day 15.

Cold plate test. The animals were placed in a stainless steel box (12 cm × 20 cm × 10 cm) with a cold plate as floor. The temperature of the cold plate was kept constant at 4 °C ± 1 °C. Pain-related behavior (licking of the hind paw) was observed and the time (seconds) of the first sign was recorded. The cut-off time of the latency of paw lifting or licking was set at 60 seconds.

Compounds administration. C2 (1, 5, 10, 20 mg kg^{-1}) was dissolved in 1% carboxymethylcellulose and orally administered. Measurements were performed 15, 30, 45, 60 and 75 min after injection. Control mice were treated with vehicle. The MAGL inhibitor **F** (1 mg kg^{-1} p.o.; Cayman Chemicals, USA) was dissolved in 1% carboxymethylcellulose and administered 15 min after **C2** (1-10 mg kg^{-1} p.o.). Measurements were performed 15, 30, 45, 60 and 75 min after **C2** injection. The selective CB_1R (SR141716A; Tocris Bioscience, UK) and CB_2R (SR144528; Tocris Bioscience, UK) antagonists were dissolved in saline solution with 5% DMSO and 5% Tween 20. Antagonists were administered i.p. 15 minutes before **C2** (20 mg kg^{-1} p.o.). The dosages of SR141716A and SR144528 were according to previously published articles.[34,35]

Statistical analysis. Behavioral measurements were performed on 16 mice for each treatment carried out in 2 different experimental sets (8 animals for single experimental session). Results were expressed as mean ± S.E.M. The analysis of variance of behavioral data was performed by one way ANOVA, a Bonferroni's significant difference procedure was used as post-hoc comparison. *P* values of less than 0.05 or 0.01 were considered significant. Investigators were blind to all experimental procedures. Data were analysed using the "Origin 9" software (OriginLab, Northampton, USA).

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI :

Molecular formula strings for target compounds and some data (CSV)

Screening on CB_1R of compounds **C1-C5**, **D1-D6**, **E1** and **3**; effects of compounds **C3** and **D3** on [^{3}H]CP55940 binding to CB_1R; effects of compounds **C1**, **C3-C5**, **D1-D6**, **E1** and **3** on [^{3}H]CP55940 binding to CB_2R; CB_2R [^{35}S]GTPγS binding assays performed in the presence of **C2** only**;** compound **C2**-MAGL inhibition analysis; ^{1}H-,^{13}C-NMR Spectra and HPLC chromatograms of the final compounds.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

ECS, endocannabinoid system; CB_1R, type-1 cannabinoid receptor; CB_2R, type-2 cannabinoid receptor; ECs, endocannabinoids; AEA, anadamide; 2-AG, 2-arachidonoylglycerol; MAGL, monoacylglycerol lipase; MeOH, methanol; $SOCl_2$, thionyl chloride; DMF, *N*, *N*-dimethylformamide; DMSO, Dimethyl sulfoxide; NEt_3, triethylamine; $CHCl_3$, chloroform; CH_2Cl_2, dichloromethane; ACN, acetonitrile; TBTU, 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate; DME, dimethoxy ethane; TLC, thin layer chromatography, 4-NPA, 4-nitrophenylacetate; EDTA, Ethylenediaminetetraacetic acid; BSA, Bovine serum albumin.

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REFERENCES

1. Pacher P.; Kunos G. Modulating the endocannabinoid system in human health and disease--successes and failures. *FEBS J.* **2013**, *280*, 1918−1943.
2. Jourdan T.; Szanda G.; Rosenberg A. Z.; Tam J.; Earley B. J.; Godlewski G.; Cinar R.; Liu Z.; Liu J.; Ju C.; Pacher P.; Kunos G. Overactive cannabinoid 1 receptor in podocytes drives type 2 diabetic nephropathy. *Proc. Natl. Acad. Sci. USA,* **2014**, *111*, E5420–8.

3. Gatta-Cherifi B.; Cota D. New insights on the role of the endocannabinoid system in the regulation of energy balance. *Int. J. Obes. (Lond)*, **2016**, *40*, 210–219.
4. Mazier W.; Saucisse N.; Gatta-Cherifi B.; Cota D. The endocannabinoid system: pivotal orchestrator of obesity and metabolic disease. *Trends Endocrinol. Metab.* **2015**, *26*, 524–537.
5. Cassano T.; Calcagnini S.; Pace L.; De Marco F.; Romano A.; Gaetani S. Cannabinoid receptor 2 signaling in neurodegenerative disorders: from pathogenesis to a promising therapeutic target. *Front Neurosci.* **2017**, *11*, 30.
6. Niu J.; Huang D.; Zhou R.; Yue M.; Xu T.; Yang J.; He L.; Tian H.; Liu X.; Zeng J. Activation of dorsal horn cannabinoid CB2 receptor suppresses the expression of P2Y12 and P2Y13 receptors in neuropathic pain rats. *J. Neuroinflammation.* **2017**,*14*, 185.
7. Fonseca B. M.; Teixeira N. A.; Correia-da-Silva G. Cannabinoids as modulators of cell death: clinical applications and future directions. *Rev. Physiol. Biochem. Pharmacol.* **2017**, *173*, 63-88.
8. Martínez-Pinilla E.; Varani K.; Reyes-Resina I.; Angelats E.; Vincenzi F.; Ferreiro-Vera C.; Oyarzabal J.; Canela E. I.; Lanciego J. L.; Nadal X.; Navarro G.; Borea P. A.; Franco R. Binding and signaling studies disclose a potential allosteric site for cannabidiol in cannabinoid CB2 receptors. *Front. Pharmacol.* **2017**, *8*, 744.
9. Price M. R.; Baillie G. L.; Thomas A.; Stevenson L. A; Easson M.; Goodwin R.; McLean A.; McIntosh L.; Goodwin G.; Walker G.; Westwood P.; Marrs J.; Thomson F.; Cowley P.; Christopoulos A.; Pertwee R. G.; Ross R. A. Allosteric modulation of the cannabinoid CB1 receptor. *Mol. Pharmacol.* **2005**; *68*, 1484–1495
10. Sabatucci A.; Tortolani D.; Dainese E.; Maccarrone M. In silico mapping of allosteric ligand binding sites in type-1 cannabinoid receptor. *Biotechnol. Appl. Biochem.* **2018** Jan;65(1):21-28. doi: 10.1002/bab.1589
11. Kulkarni R.; Garai S.; Janero D. R.; Thakur G. A. Design and synthesis of cannabinoid 1 receptor (CB1R) allosteric modulators: drug discovery applications. *Methods Enzymol.* **2017**, *593*, 281-315.

12. Khurana L.; Mackie K.; Piomelli D.; Kendall D. A. Modulation of CB1 cannabinoid receptor by allosteric ligands: pharmacology and therapeutic opportunities. *Neuropharmacology*. **2017**, *124*, 3-12.
13. Morales, P.; Goya P.; Jagerovic N.; Hernandez-Folgado L. Allosteric modulators of the CB1 cannabinoid receptor: a structural update review. *Cannabis Cannabinoid Res.* **2016**, *1*, 22-30.
14. Feng Z.; Alqarni M. H.; Yang P.; Tong Q.; Chowdhury A.; Wang L.; Xie X. Q. Modeling, molecular dynamics simulation and mutation validation for structure of cannabinoid receptor 2 based on known crystal structures of GPCRs. *J. Chem. Inf. Model.* **2014**, *54*, 2483-2499.
15. Shore D. M.; Baillie G. L.; Hurst D. H.; Navas F.; Seltzman H. H.; Marcu J. P.; Abood M. E.; Ross R. A.; Reggio P H. Allosteric modulation of a cannabinoid g protein- coupled receptor: binding site elucidation and relationship to g protein signaling. *J. Biol. Chem.* **2014**, *289*, 5828-5845.
16. Christopoulos A.; Kenakin T. G protein-coupled receptor allosterism and complexing. *Pharmacol. Rev.* **2002**, *54*, 323-374.
17. Bridges T. M.; Lindsley C. W. G-protein-coupled receptors: from classical modes of modulation to allosteric mechanisms. *ACS Chem. Biol.* **2008**, *3*, 530–541.
18. Conn P. J.; Christopoulos A.; Lindsley C W. Allosteric modulators of GPCRs: a novel approach for the treatment of CNS disorders. *Nat. Rev. Drug. Disc.* **2009**, *8*, 41–54.
19. Slivicki R. A.; Xu Z.; Kulkarni P. M.; Pertwee R. G.; Mackie K.; Thakur G. A.; Hohmann A. G. Positive allosteric modulation of cannabinoid receptor type 1 suppresses pathological pain without producing tolerance or dependence. *Biol. Psychiatry* **2017** In press and published on-line. doi: 10.1016/j.biopsych.2017.06.032.
20. Ignatowska-Jankowska M.; Baillie G. L.; Kinsey S.; Crowe M.; Ghosh S.; Owens R. A.; Damaj I. M.; Poklis J.; Wiley J. L.; Zanda M.; Zanato C.; Greig I. R.; Lichtman A. H.; Ross R. A. A Cannabinoid CB1 receptor-positive allosteric modulator reduces neuropathic pain in the mouse with no psychoactive effects. *Neuropsychopharmacology.* **2015**, *40*, 2948-2959.

21. Horswill J. C.; Bali U.; Shaaban S.; Keily J. F.; Jeevaratnam P.; Babbs A. J.; Reynet C.; Wong Kai In P. PSNCBAM-1, a novel allosteric antagonist at cannabinoid CB_1 receptors with hypophagic effects in rats. *Br. J. Pharmacol.* **2007**, *152*, 805-814.
22. Navarro H. A.; Howard J. L.; Pollard G. T.; Carroll F. T. Positive allosteric modulation of the human cannabinoid (CB_1) receptor by RTI-371, a selective inhibitor of the dopamine transporter. *Br. J. Pharmacol*. **2009**, *156*, 1178–1184.
23. Petrucci V.; Chicca A.; Glasmacher S.; Paloczi J.; Cao Z.; Pacher P.; Gertsch J. Pepcan-12 (RVD-hemopressin) is a CB2 receptor positive allosteric modulator constitutively secreted by adrenals and in liver upon tissue damage. *Sci. Rep.* **2017**, *7*, 9560.
24. Chicca A.; Arena C.; Bertini S.; Gado F.; Ciaglia E.; Abate M.; Digiacomo M.; Lapillo M.; Poli G.; Bifulco M.; Macchia M.; Tuccinardi T.; Gertsch J.; Manera C. Polypharmacological profile of 1,2-dihydro-2-oxo-pyridine-3-carboxamides in the endocannabinoid system. *Eur. J. Med. Chem.* **2018**, *154*, 155-171.
25. Muccioli G. G.; Labar G.; Lambert D. M. CAY10499, a novel monoglyceride lipase inhibitor evidenced by an expeditious MGL assay. *Chembiochem* **2008**, *9*, 2704-2710.
26. Guindon J.; Hohmann A. G. Cannabinoid CB_2 receptors: a therapeutic target for the treatment of inflammatory and neuropathic pain. *Br. J. Pharmacol.* **2008**, *153*, 319–334.
27. Di Cesare Mannelli L.; Lucarini, E.; Micheli L.; Mosca I.; Ambrosino P.; Soldovieri M. V.; Martelli A.; Testai L.; Taglialatela M.; Calderone V.; Ghelardini C. Effects of natural and synthetic isothiocyanate-based H_2S-releasers against chemotherapy-induced neuropathic pain: role of Kv7 potassium channels. *Neuropharm.* **2017**, *121*, 49-59.
28. Brindisi M.; Maramai S.; Gemma S.; Brogi S.; Grillo A.; Di Cesare Mannelli L.; Gabellieri E.; Lamponi S.; Saponara S.; Gorelli B.; Tedesco D.; Bonfiglio T.; Landry C.; Jung K. M.; Armirotti A.; Luongo L.; Ligresti A.; Piscitelli F.; Bertucci C.; Dehouck M. P.; Campiani G.; Maione S.; Ghelardini C.; Pittaluga A.; Piomelli D.; Di Marzo V.; Butini S. Development and pharmacological

characterization of selective blockers of 2-arachidonoyl glycerol degradation with efficacy in rodent models of multiple sclerosis and pain. *J. Med. Chem*. **2016**, *59*, 2612-2632.

29. Chou T. C.; Drug combination studies and their synergy quantification using the Chou-Talalay method. *Cancer Res.* **2010**, *70*, 440-446.
30. Di Cesare Mannelli L.; Pacini A.; Bonaccini L.; Zanardelli M.; Mello T.; Ghelardini C. Morphologic features and glial activation in rat oxaliplatin-dependent neuropathic pain. *J. Pain.* **2013**, *14*, 1585-1600.
31. Luongo L.; Maione S.; Di Marzo V. Endocannabinoids and neuropathic pain: focus on neuron–glia and endocannabinoid–neurotrophin interactions, *Eur. J. Neurosci*. **2014**, *39*, 401-408.
32. McGrath J. C.; Lilley E. Implementing guidelines on reporting research using animals (ARRIVE etc.): new requirements for publication in BJP. *Br. J. Pharmacol.* **2015**, *172*, 3189-3193.
33. Cavaletti G.; Tredici G.; Petruccioli M. G.; Donde E.; Tredici P.; Marmiroli P.; Minoia C.; Ronchi A.; Bayssas M.; Etienne G. G. Effects of different schedules of oxaliplatin treatment on the peripheral nervous system of the rat. *Eur. J. Cancer*. **2001**, *37*, 2457-2463.
34. Lake K. D.; Compton D. R.; Varga K.; Martin B. R.; Kunos G. Cannabinoid-induced hypotension and bradycardia in rats mediated by CB1-like cannabinoidreceptors. *J. Pharmacol. Exp. Ther.* **1997**, *281*, 1030-1037.
35. Li A. L.; Carey L. M., Mackie K. M.; Hohmann A. G. Cannabinoid CB_2 Agonist GW405833 Suppresses Inflammatory and Neuropathic Pain through a CB_1 Mechanism that is Independent of CB_2 Receptors in Mice. *J. Pharmacol. Exp. Ther.* **2017**, *362*, 296-305.

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