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Investigations on the 4-Quinolone-3-carboxylic Acid Motif. 3. Synthesis, Structure−Affinity Relationships, and Pharmacological Characterization of 6-Substituted 4-Quinolone-3-carboxamides as Highly Selective Cannabinoid-2 Receptor Ligands

Serena Pasquini,[†] Alessia Ligresti,[‡] Claudia Mugnaini,[†] Teresa Semeraro,[†] Lavinia Cicione,[†,#] Maria De Rosa,[†] Francesca Guida,[§] Livio Luongo,[§] Maria De Chiaro,[§] Maria Grazia Cascio,[⊥] Daniele Bolognini,[∥,⊥] Pietro Marini,[⊥] Roger Pertwee,[⊥] Sabatino Maione,[§] Vincenzo Di Marzo,*[,‡] and Federico Corelli*[,†]

[†]*Dipartimento Farmaco Chimico Tecnologico, Università degli Studi di Siena, Via A. Moro, 53100 Siena, Italy,* [‡]*Endocannabinoid Research Group, Institute of Biomolecular Chemistry, Consiglio Nazionale delle Ricerche, Via dei Campi Flegrei 34, 80078 Pozzuoli (Naples), Italy,* [§]*Department of Experimental Medicine—Section of Pharmacology "L. Donatelli", Second University of Naples, Via S. Maria di Costantinopoli 16, 80138 Naples, Italy,* [∥]*DBSF, Pharmacology Section and Neuroscience Centre, University of Insubria, Busto Arsizio (Va), Italy, and* [⊥]*Institute of Medical Sciences University of Aberdeen, Aberdeen AB25 2ZD, Scotland, U.K.* [#] *Present address: Dipartimento di Scienze Chimiche, Alimentari, Farmaceutiche e Farmacologiche, Università del Piemonte Orientale "A. Avogadro", Vercelli, Italy.*

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A set of quinolone-3-carboxamides **2** bearing diverse substituents at position 1, 3, and 6 of the bicyclic nucleus was prepared. Except for six compounds exhibiting $K_i > 100$ nM, all the quinolone-3-carboxamides **2** proved to be high affinity CB2 ligands, with K_i values ranging from 73.2 to 0.7 nM and selectivity [SI = K_i(CB1)/K_i(CB2)] varying from > 14285 to 1.9, with only **2ah** exhibiting a reverse selectivity (SI < 1). In the formalin test of peripheral acute and inflammatory pain in mice, **2ae** showed analgesic activity that was antagonized by a selective CB2 antagonist. By contrast, **2e** was inactive per se and antagonized the effect of a selective CB2 agonist. Finally, **2g** and **2p** exhibited CB2 inverse agonist-like behavior in this in vivo test. However, two different functional assays carried out in vitro on **2e** and **2g** indicated for both compounds an overall inverse agonist activity at CB2 receptors.

Introduction

Cannabinoid receptors, their endogenous ligands, and proteins responsible for endocannabinoid cellular uptake and inactivation, i.e. the putative anandamide membrane transporter (AMT[a]),[1] and the fatty acid amide hydrolase (FAAH),[2] or the monoacylglycerol lipase (MAGL)[3] in the case of 2-AG, constitute the "endocannabinoid (EC) system" and represent potentially interesting targets[4] for the development of new therapeutic agents to be employed in many pathologies, such as, for example, pain, loss of appetite in patients with AIDS, obesity, chemotherapy-induced nausea and vomiting, immune and inflammatory disorders, cardiovascular and gastrointestinal disorders, and neurodegenerative diseases.[5]

So far, two cannabinoid receptors (cannabinoid receptor 1 [CB1] and cannabinoid receptor 2 [CB2]), have been characterized and cloned.[6,7] CB1 receptors are expressed at high levels in the central nervous system (CNS), whereas CB2 receptors are found predominantly, but not exclusively, outside the CNS and are most abundant in the immune system, i.e. in tonsils, spleen, macrophages, and lymphocytes (B-cells and natural killer cells).[8]

Agonists of both cannabinoid receptor subtypes produce strong antinociceptive effects in animal models of chronic, neuropathic, and inflammatory pain and are intensively investigated as potential new analgesic and antiinflammatory agents.[9] Unfortunately, CB1/CB2 agonists are not devoid of unwanted side effects (such as muscle weakness, palpitations, dry mouth, disorientation, altered time perception, impairment of memory, tremor, confusion, paranoia and hallucination, nausea and vomiting), many of which are thought to be due to activation of central CB1 receptors rather than peripheral CB1 or CB2 receptors.[10] The occurrence of these adverse effects limits the therapeutic usefulness of mixed cannabinoid agonists that show high affinity for CB1 receptors.

Differences in receptor distribution and signal transduction mechanisms[11,12] are likely to account for the relative absence of CNS side effects induced by CB2 agonists. These considerations suggest that novel pharmacotherapies selectively targeting CB2 receptors may have considerable therapeutic potential.[13] Significant medicinal chemistry efforts have been directed to the characterization of selective CB2 agonists (Chart 1), leading to the identification of compounds eliciting antinociceptive effects in models of acute pain, persistent inflammatory pain, postoperative pain, cancer pain, and neuropathic pain.[14]

In recent years a number of reports have suggested that also CB2 inverse agonists/antagonists (Chart 1) may possess

*To whom correspondence should be addressed. For F.C.: phone, +39 0577 234308; fax, +39 0577 234333; E-mail, corelli@unisi.it. For V. D.: phone, +39 081 8675093; fax, +39 081 8041770; E-mail, vdimarzo@icmib.na.cnr.it.

[a] Abbreviations: AMT, anandamide membrane transporter; FAAH, fatty acid amide hydrolase; MAGL, monoacylglycerol lipase; EC, endocannabinoid system; CB1, cannabinoid receptor type 1; CB2, cannabinoid receptor type 2; HOBt, 1-hydroxybenzotriazole; HBTU, *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; DIPEA, diisopropylethylamine; TEA, triethylamine; SI, selectivity index.

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Chart 1. Representative CB2-Selective Agonists (HU-308,[40] JWH-133,[27] AM1241,[31] GW405833,[41] and GW842166X[42]) and Inverse Agonists/Antagonists (JTE-907,[15b] SR144528,[15a] AM630,[26] and Sch.336[43])

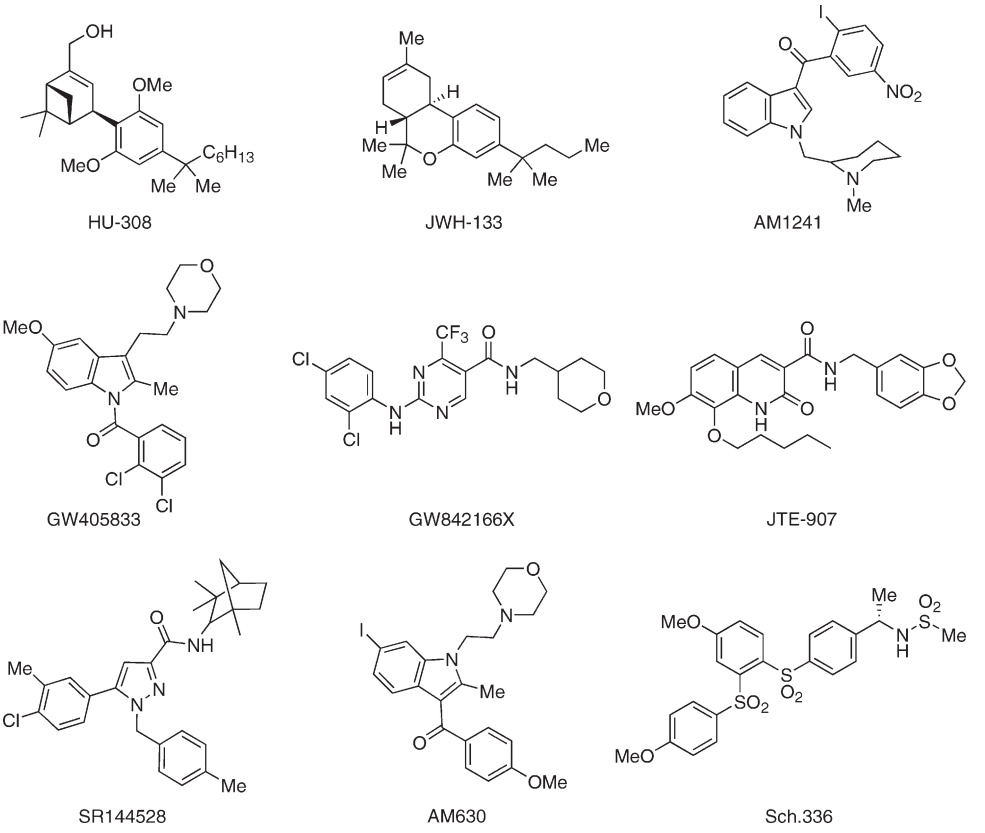
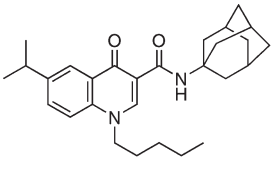


Chart 2. Lead Compound **1** and General Structure of 6-Substituted 4-Quinolone-3-carboxamide Derivatives **2**



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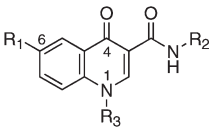
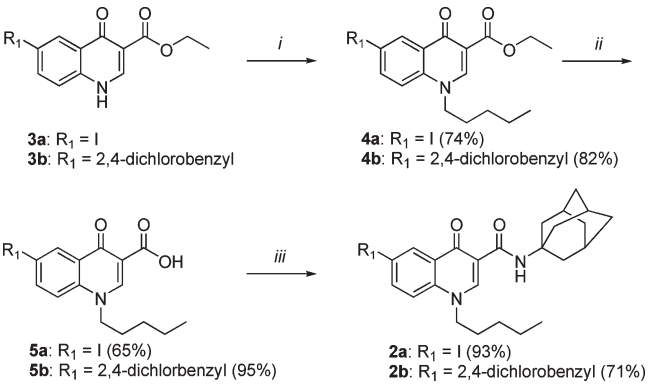


2

R_1 = iodo, substituted phenyl, (un)substituted heteroaryl, $PhSO_n$, (aryl)alkyl, (aryl)alkenyl, (aryl)alkynyl
R_2 = 1-adamantyl, 1-fenchyl, 1-hexyl, 1-piperidinyl
R_3 = 1-pentyl, 1-buten-4-yl, allyl

antiinflammatory activity, being able to inhibit carrageenan-induced paw edema in mice, to reduce leukocytes trafficking and to impair the migration of cells toward cannabinoid agonists.[15] Taken together, these previous results show that selective cannabinoid CB2 inverse agonists may serve as novel immunomodulatory agents in the treatment of a variety of acute and chronic inflammatory disorders. On the other hand, CB2 agonists have also been proposed as useful therapeutic agents against chronic (neuropathic) pain and inflammation.[16] Although it may appear puzzling that both agonists and inverse agonists at a given receptor may cause anti-inflammatory effects, this phenomenon has been widely described for CB2 receptors and was ascribed to the capability of these receptors to be coupled, under different conditions and in a time- and site-selective way, to either recruitment of immune cells or interference with the action of other chemoattractants.[4a,17]

Scheme 1. Synthesis of 6-Substituted 4-Quinolone-3-carboxamide Derivatives **2a**,**b**[a]

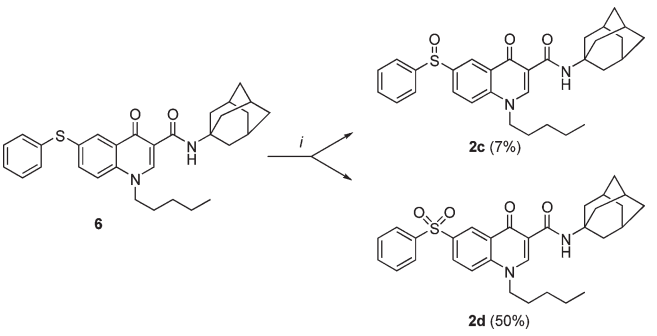


[a] Reagents and conditions: (i) pentyl iodide, K_2CO_3, DMF, 100 °C, 4 h; (ii) 10% aq NaOH, reflux, 2 h; (iii) 1-aminoadamantane, HBTU, HOBt, DIPEA, DMF, rt, 4 h.

Within a research program aimed at characterizing novel cannabinoid ligands,[18] we have recently described a family of quinolone-3-carboxamides bearing diverse substituents at different positions of the aromatic ring as new CB2 ligands. The tested compounds exhibited high CB2 affinity and selectivity over the CB1 receptor subtype; compound **1** (Chart 2), in particular, exhibited >190-fold selectivity over CB1 in the [^{3}H]CP-55,940 binding assay (hCB2/hCB1 K_i=6.3 nM/1220 nM). Moreover, this compound showed analgesic activity in the formalin test of acute peripheral and inflammatory pain in mice as a result of selective CB2 agonistic activity.[18a,19] On the basis

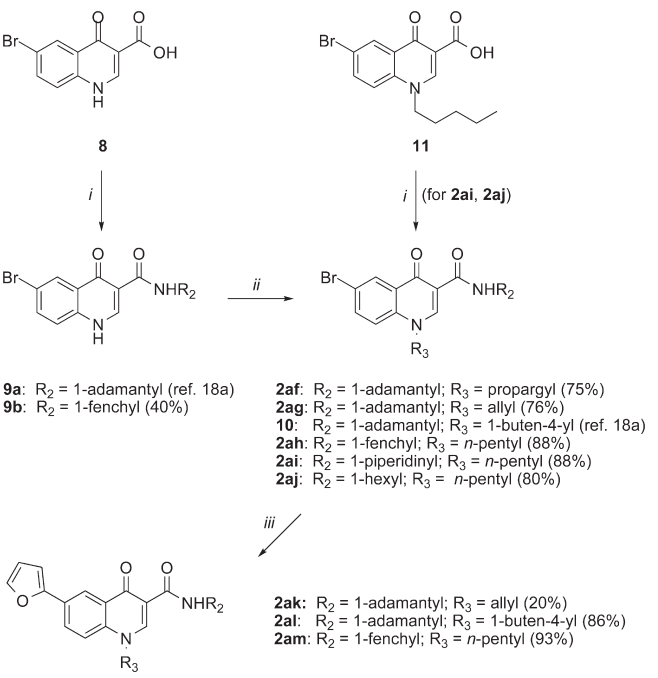
of these promising results, we chose derivative **1** as the prototype for the development of a set of 6-substituted quinolone-3-carboxamides of general structure **2**, where the N-1 pentyl chain and adamantyl group of **1** were either retained or replaced by other lipophilic groups, whereas a number of different substituents in place of the 2-propyl group at C-6 position were extensively investigated.[20] Our main objectives were: (i) to identify new compounds endowed with increased CB2 affinity and/or selectivity, (ii) to highlight the relationship between chemical structure and the in vitro/in vivo pharmacological profile of the new compounds.

Scheme 2. Synthesis of 6-Substituted 4-Quinolone-3-carboxamide Derivatives **2c**,**d**[a]



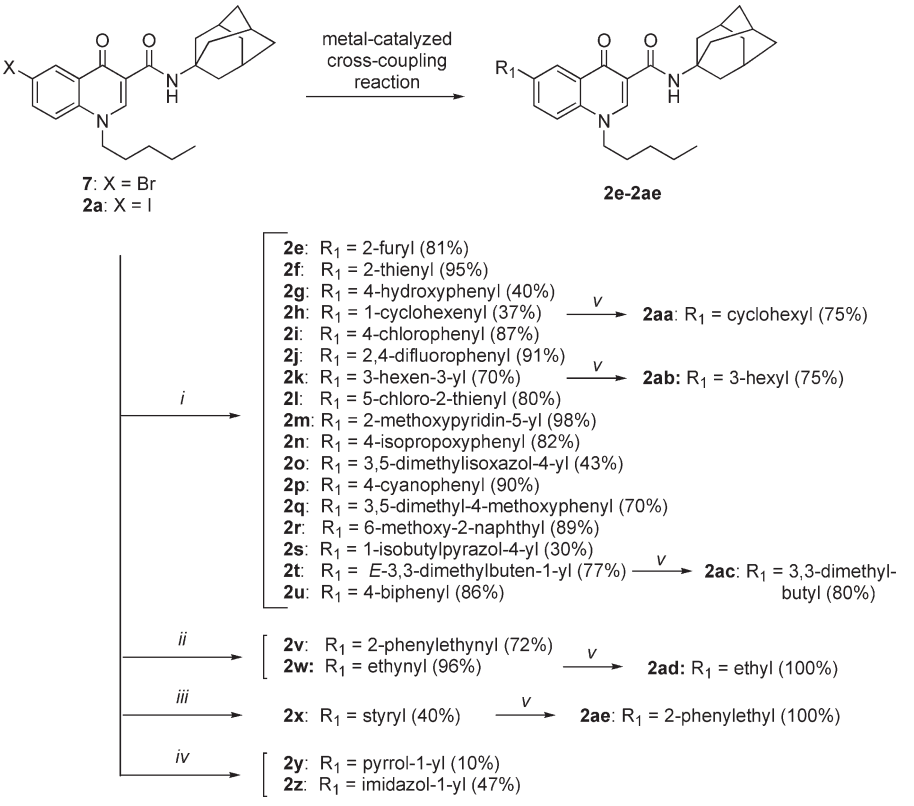
[a] Reagents and conditions: (i) Oxone, 1,4-dioxane, H_2O, rt, 24 h.

Scheme 4. Synthesis of 6-Substituted 4-Quinolone-3-carboxamide Derivatives **2af**−**2am**[a]



[a] Reagents and conditions: (i) amine, HBTU, HOBt, DIPEA, DMF, rt, 8 h; (ii) alkyl halide, K_2CO_3, DMF, 100 °C, 4 h; (iii) 2-furanboronic acid, $Pd(OAc)_2$, PPh_3, 1 N Na_2CO_3, DME, EtOH, MW, 150 °C, 10 min.

Scheme 3. Synthesis of 6-Substituted 4-Quinolone-3-carboxamide Derivatives **2e**−**2ae**[a]



[a] Reagents and conditions: (i) (for **2e**, **2 g**−**k**, **2m**, **2n**, **2q**, **2r**, **2t**, **2u**): **7**, aryl- or alkenylboronic acid, $Pd(OAc)_2$, PPh_3, 1 N Na_2CO_3, DME, EtOH, MW, 150 °C, 10 min; (for **2f**, **2l**, **2o**, **2p**, **2s**): **2a**, aryl- or heteroarylboronic acid, $Pd(OAc)_2$, PPh_3, 1 N Na_2CO_3, DME, EtOH, MW, 150 °C, 10 min. (ii) (for **2v**): **2a**, phenylacetylene, *i*-Pr_2NH, $PdCl_2(PPh_3)_2$, toluene, reflux, 2 h; (for **2w**): (1) **2a**, (trimethylsilyl)acetylene, *i*-Pr_2NH, $PdCl_2(PPh_3)_2$, toluene, reflux, 2 h; (2) K_2CO_3, MeOH, THF, rt, 30 min. (iii) **2a**, styrene, $Pd(OAc)_2$, TEA, PPh_3, DMF, reflux, 3 h. (iv) **2a**, imidazole or pyrrole, $Cu(OAc)_2$, DBU, DMSO, 130 °C, 10 min. (v) H_2, Pd/C, EtOH, 4 h.

Table 1. CB1 and CB2 Receptor Affinity Values for Compounds **2a**−**2am**[a]

compd	R1	R2	R3	CB1[b,d] K_i[f] (nM)	CB2[c,d] K_i (nM)	SI[e]
2a	iodo	1-adamantyl	1-pentyl	2080	20.6	101
2b	2,4-dichlorobenzyl	1-adamantyl	1-pentyl	> 10000	3881	> 3
2c[g]	phenylsulfinyl	1-adamantyl	1-pentyl	460	73.2	6
2d	phenylsulfonyl	1-adamantyl	1-pentyl	> 10000	65.9	> 152
2e	2-furyl	1-adamantyl	1-pentyl	> 10000	0.7	> 14285
2f	2-thienyl	1-adamantyl	1-pentyl	> 10000	2.3	> 4348
2g	4-hydroxyphenyl	1-adamantyl	1-pentyl	> 10000	4.2	> 2381
2h	1-cyclohexenyl	1-adamantyl	1-pentyl	> 10000	8.3	> 1205
2i	4-chlorophenyl	1-adamantyl	1-pentyl	> 10000	11.0	> 909
2j	2,4-difluorophenyl	1-adamantyl	1-pentyl	> 10000	16.0	> 625
2k	3-hexen-3-yl	1-adamantyl	1-pentyl	NT[h]	NT	
2l	5-chloro-2-thienyl	1-adamantyl	1-pentyl	> 10000	44.8	> 223
2m	2-methoxypyridin-5-yl	1-adamantyl	1-pentyl	> 10000	59.3	> 169
2n	4-isopropoxyphenyl	1-adamantyl	1-pentyl	NT	NT	
2o	3,5-dimethylisoxazol-4-yl	1-adamantyl	1-pentyl	> 10000	125.7	> 80
2p	4-cyanophenyl	1-adamantyl	1-pentyl	> 10000	41.9	> 239
2q	3,5-dimethyl-4-methoxyphenyl	1-adamantyl	1-pentyl	510	21.5	24
2r	6-methoxy-2-naphthyl	1-adamantyl	1-pentyl	360	52.6	7
2s	1-isobutylpyrazol-4-yl	1-adamantyl	1-pentyl	245.7	51.0	5
2t	*E*-3,3-dimethylbuten-1-yl	1-adamantyl	1-pentyl	> 10000	71.6	> 140
2u	4-biphenyl	1-adamantyl	1-pentyl	> 10000	181	> 55
2v	2-phenylethynyl	1-adamantyl	1-pentyl	> 10000	343.0	> 29
2w	ethynyl	1-adamantyl	1-pentyl	> 10000	8.8	> 1136
2x	styryl	1-adamantyl	1-pentyl	> 10000	65.4	> 153
2y	pyrrol-1-yl	1-adamantyl	1-pentyl	29.7	0.7	42
2z	imidazol-1-yl	1-adamantyl	1-pentyl	> 10000	8.0	> 1250
2aa	cyclohexyl	1-adamantyl	1-pentyl	> 10000	7.3	> 1370
2ab	3-hexyl	1-adamantyl	1-pentyl	NT	NT	
2ac	3,3-dimethylbutyl	1-adamantyl	1-pentyl	NT	NT	
2ad	ethyl	1-adamantyl	1-pentyl	900	16.9	53
2ae	2-phenylethyl	1-adamantyl	1-pentyl	> 10000	56.6	> 177
2af	bromo	1-adamantyl	propargyl	3210	49.8	64
2ag	bromo	1-adamantyl	allyl	NT	NT	
2ah	bromo	1-fenchyl	1-pentyl	2.8	5.0	0.6
2ai	bromo	1-piperidinyl	1-pentyl	1900	400	5
2aj	bromo	1-hexyl	1-pentyl	1700	900	2
2ak	2-furyl	1-adamantyl	allyl	> 10000	59.0	> 169
2al	2-furyl	1-adamantyl	1-buten-4-yl	> 10000	4.4	2273
2am	2-furyl	1-fenchyl	1-pentyl	480	2.4	200
1[i]				1220	6.3	194
SR144528[i,j]				> 2820	5.4	> 522
Rimonabant[i,k]				12.0	790	0.015

[a] Data represent mean values for at least three separate experiments performed in duplicate and are expressed as K_i (nM). [b] CB1: human cannabinoid type 1 receptor. [c] CB2: human cannabinoid type 2 receptor. [d] For both receptor binding assays, the new compounds were tested using membranes from HEK cells transfected with either the CB1 or CB2 receptor and [^{3}H]-(−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)-phenyl]-*trans*-4-(3-hydroxy-propyl)-cyclohexanol ([^{3}H]CP-55,940). [e] SI: selectivity index for CB2, calculated as K_i(CB1)/K_i(CB2) ratio. [f] K_i: "Equilibrium dissociation constant", that is, the concentration of the competing ligand that will bind to half the binding sites at equilibrium in the absence of radioligand or other competitors. [g] Tested as the racemate. [h] NT, not tested because of solubility problems. [i] The binding affinities of reference compounds were evaluated in parallel with compounds **2** under the same conditions. [j] CB2 reference compound. [k] CB1 reference compound.

Chemistry

The synthesis of the new compounds **2** was accomplished as depicted in Schemes 1−4. 4-Oxo-1,2-dihydroquinolin-3-carboxylic acid ethyl esters **3a**[21] and **3b**[22] (Scheme 1) were alkylated with pentyl iodide in the presence of potassium carbonate to give derivatives **4a** and **4b**, respectively. Alkaline hydrolysis of the ester function to **5a** and **5b** was followed by coupling (HBTU, HOBt, DIPEA) with 1-aminoadamantane to afford the final compounds **2a** and **2b** in an overall yield of 45 and 55%, respectively.

Oxidation of the sulfide **6**[18a] (Scheme 2) by means of oxone in 1,4-dioxane/water gave a mixture of the corresponding sulfoxide **2c** (7% yield) and sulfone **2d** (50% yield), which were separated by column chromatography on silica gel.

Most of the final compounds **2** (namely, **2e**−**2ae**) were obtained through metal-catalyzed cross-coupling reactions starting from either the bromo derivative **7**[18a] or the corresponding iodo derivative **2a** (Scheme 3). In particular, all the Sonogashira, Heck, and Ullman-type reactions (conditions ii, iii, and iv in Scheme 3) were performed on precursor **2a** in order to obtain the expected products in satisfactory yield, whereas in the case of the Suzuki reaction (condition i), the choice between **2a** and **7** as the reaction substrate basically depended upon the nature of the boronic acid to be used; thus, aryl- and alkenylboronic acids gave good results also with the less reactive bromo derivative **7**, while the cross-coupling reaction using heteroarylboronic acids, with the only exception of 2-furylboronic acid, was best carried out on the iodo derivative **2a**. Compounds **2h**, **2k**, **2t**, **2w**, and **2x** were

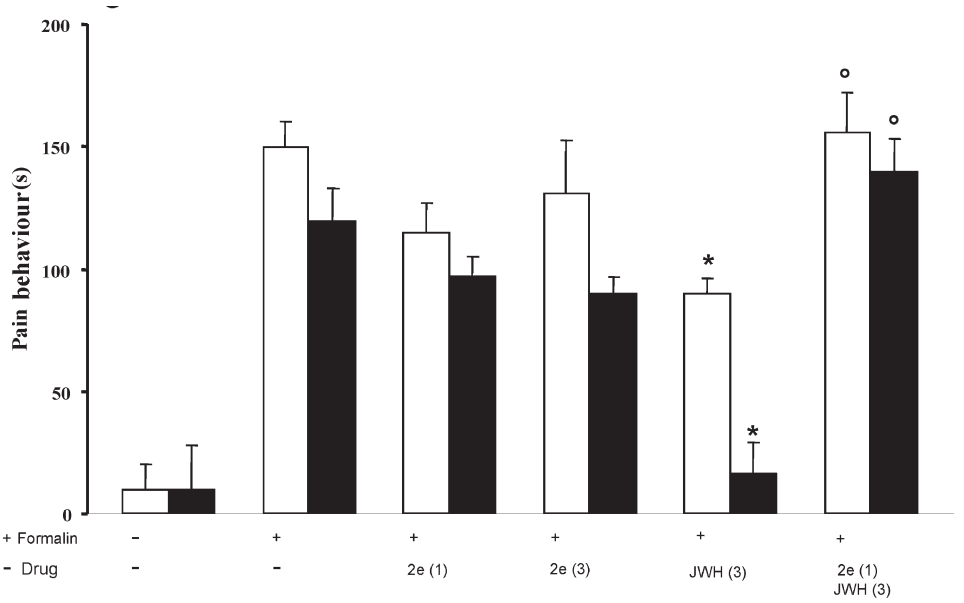


Figure 1. Effect of intraperitoneal (ip) administration of vehicle (20% DMSO in saline), compound **2e** (1 or 3 mg/kg) alone, or compound **2e** (1 mg/kg ip) in combination with JWH-133 (3 mg/kg ip), a selective CB2 receptor agonist, on the nociceptive behavior (seconds) induced by subcutaneous formalin (1.25%) injection into the hind paw of mice ($n = 8-10$). Open columns represent the early phase and filled columns the late phase of formalin-evoked nociception. Formalin was injected 15 min after vehicle or drug treatments. Asterisks indicate significant differences ($P < 0.05$) vs vehicle and circles vs compound **2e** (1 mg/kg ip).

subjected to catalytic hydrogenation to yield the corresponding saturated derivatives **2aa**−**2ae**.

Finally, with the aim of exploring the effects on receptor affinity of enhanced chemical diversity within this family of 4-oxoquinoline-3-carboxamides, diverse substituents were introduced at either the N-1 position or carboxamide group. Accordingly, 6-bromo-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**8**)[18a] (Scheme 4) by reaction with 1-aminoadamantane and *rac*-1-fenchylamine[23] was converted into the *N*-(1-adamantyl)amide **9a** and *N*-(1-fenchyl)amide **9b**, which were in turn alkylated at the N-1 position; in particular, the adamantylamide **9a** was treated with propargyl, allyl, and 1-buten-4-yl halides to afford the corresponding derivatives **2af**, **2ag**, and **10**,[18a] while the fenchylamide **9b** was alkylated with *n*-pentyl iodide to give **2ah**. Conversely, compounds **2ai** and **2aj** were obtained by amidation with 1-aminopiperidine and 1-aminohexane, respectively, of the acid **11**, in turn prepared according to a general procedure for the synthesis of 4-quinolone-3-carboxylic acids (see Supporting Information). Suzuki reaction with 2-furylboronic acid on 6-bromo derivatives **2ag**, **10**, and **2ah** provided the corresponding 6-(2-furanyl) derivatives **2ak**, **2al**, and **2am**.

Pharmacology

The binding affinities (K_i values) of compounds **2** for human recombinant CB1 and CB2 receptors are reported in Table 1. The tested compounds were evaluated in parallel with SR144528[15a] (Chart 1) and rimonabant[24] as reference CB2 and CB1 ligands, respectively, as previously described.[25]

The functional activity of compounds **2e**, **2g**, **2p**, **2aa**, and **2ae** was assessed using the formalin test of acute peripheral and inflammatory pain in mice. Formalin injection induces a biphasic stereotypical nocifensive behavior. Nociceptive responses are divided into an early, short lasting first phase (0−7 min) caused by a primary afferent discharge produced by the stimulus, followed by a quiescent period, and then a second, prolonged phase (15−60 min) of tonic pain. Fifteen minutes before injection of formalin, mice received intraperitoneal (ip) administration of vehicle or compound **2e**, **2g**, **2p**, **2aa**, and **2ae** (1 or 3 mg/kg), alone or in combination with either the selective CB2 antagonist, 6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1*H*-indol-3-yl](4-methoxyphenyl)methanone[26] (AM630, Chart 1) (1 mg/kg, ip) or the selective CB2 agonist 3-(1′,1′-dimethylbutyl)-1-deoxy-Δ8-THC[27] (JWH-133, Chart 1) (3 mg/kg, ip), administered 5 min before the compound. The results are presented in Figures 1−5. Finally, the effect of compounds **2e** and **2g** on forskolin-induced elevation of cAMP levels in CHO cells transfected with human CB2 and on [^{35}S]GTPγS binding to membranes from these cells as well as from the mouse spleen (which expresses selectively CB2 receptors) were also assessed. The effect of **2e** as a potential antagonist against CP-55,940 agonist effects on forskolin-induced elevation of cAMP levels and [^{35}S]-GTPγS binding in these assays was also evaluated.

Results and Discussion

In Vitro Pharmacology and SAR. Out of the 39 synthesized quinolones **2**, compounds **2k**, **2n**, **2ab**, **2ac**, **2ag** could not be tested in the cannabinoid receptor binding assay because of their very low solubility in DMSO/water. With the exception of six compounds (**2b**, **2o**, **2u**, **2v**, **2ai**, **2aj**) exhibiting $K_i >$ 100 nM (Table 1), all the remaining 28 quinolone carboxamides of the **2** series proved to be high affinity CB2 ligands, with K_i values ranging from 73.2 nM (**2c**) to 0.7 nM (**2e** and **2y**). Under the conditions of our assay, the CB2-selective reference ligand SR144528[15a] showed $K_i = 5.4$ nM. With regard to the CB1 affinity, K_i values spanned 3 orders of magnitude in the range from >10000 nM (22 out of the 34 tested compounds) to 245.7 nM (**2s**), with rimonabant (SR141716A)[24] used as the CB1-selective reference ligand, displaying $K_i = 12.0$ nM. Because of the high variability of affinity for both CB1 and CB2 receptors, the CB2 selectivity index (SI), calculated as K_i(CB1)/K_i(CB2) ratio, for tested compounds also varied widely, from >14285 to 1.9. Only in

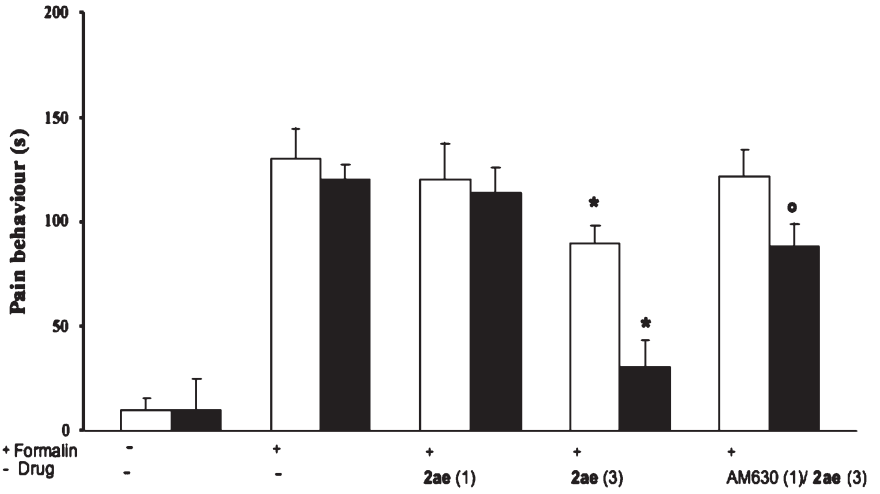


Figure 2. Effect of intraperitoneal (ip) administration of vehicle (20% DMSO in saline), compound **2ae** (1 or 3 mg/kg) alone, or compound **2ae** (3 mg/kg) in combination with AM630 (1 mg/kg), a selective CB2 antagonist, on the nociceptive behavior (seconds) induced by subcutaneous formalin (1.25%) injection into the hind paw of mice ($n = 8-10$). Open columns represent the early phase and filled columns the late phase of formalin-evoked nociception. Formalin was injected 15 min after vehicle or drug treatments. Asterisks indicate significant differences ($P < 0.05$) vs vehicle and circles vs compound **2ae** (3 mg/kg).

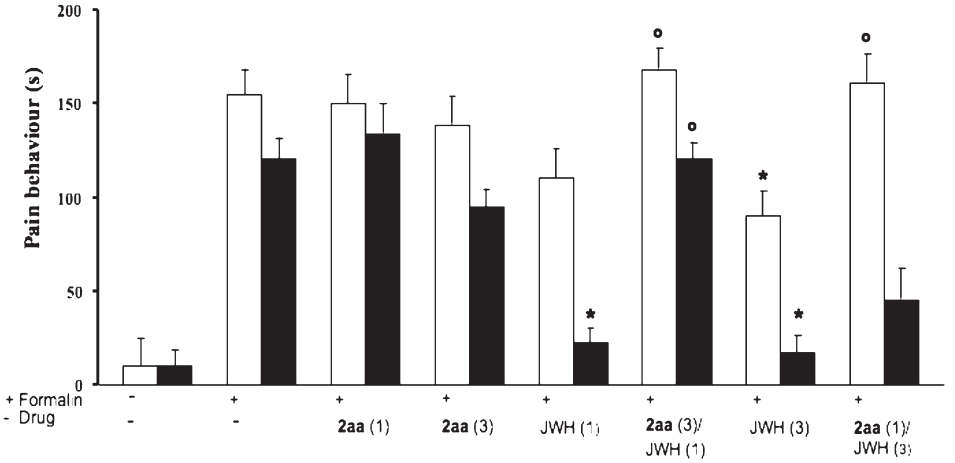


Figure 3. Effect of intraperitoneal (ip) administration of vehicle (20% DMSO in saline), compound **2aa** (1 or 3 mg/kg), JWH-133 (1 or 3 mg/kg), compound **2aa** (3 mg/kg) in combination with JWH-133 (1 mg/kg), or compound **2aa** (1 mg/kg) in combination with JWH-133 (3 mg/kg) on the nociceptive behavior (seconds) induced by subcutaneous formalin (1.25%) injection into the hind paw of mice ($n = 8-10$). Open columns represent the early phase and filled columns the late phase of formalin-evoked nociception. Formalin was injected 15 min after vehicle or drug treatments. Asterisks indicate significant differences ($P < 0.05$) vs vehicle and circles vs JWH-133 (1 and 3 mg/kg).

one case the SI was lower than 1, with compound **2ah** [K_i(CB1) = 2.8, K_i(CB2) = 5.0] exhibiting a reverse selectivity. Derivatives **2e**−**h**, **2w**, **2z**, **2aa**, and **2al** all showed CB1 and CB2 affinities >10000 nM and < 10 nM, respectively and hence a SI > 1000. To our knowledge, compound **2e** is the CB2 ligand endowed with the highest affinity (K_i = 0.7 nM) *and* selectivity (SI > 14285) described so far in the literature and compares favorably with structurally different compounds claimed in recent patent applications, such as for instance a benzimidazole derivative showing SI > 13000[28] and an indole derivative with K_i = 0.24 nM.[29,30]

A chain of appropriate length and lipophilicity at position 1 of the quinolone nucleus is required for best CB2 affinity/selectivity. In fact, replacement of the pentyl group of **2e** with a four-carbon atom chain (1-buten-4-yl in compound **2al**) and then with a three-carbon atom chain (allyl in compound **2ak**) progressively reduced CB2 affinity by approximately 1 order of magnitude. Similarly, the insertion of a propargyl chain at the N-1 position (**2af**) decreased both CB2 and CB1 affinities by more than 3 times with respect to compound **7**[18a] bearing a *n*-pentyl moiety.

The nature of the 3-carboxamido substituent also markedly affects both receptor affinity and selectivity. Thus, compounds **2ah** and **2am**, where the adamantyl group has been replaced with the fenchyl moiety characteristic of SR144528, retained basically the same CB2 affinity as the corresponding adamantyl derivatives **7** [K_i(CB2) = 14.3 nM][18a] and **2e**, respectively, while exhibiting dramatically improved affinity for the CB1 receptor, that increased from 996 nM (for **7**) to 2.8 nM (for **2ah**) and from > 10000 nM (for **2e**) to 480 nM (for **2am**). These changes in CB1 affinity strongly influenced receptor selectivity, leading to the much less CB2-selective ligand **2am** or even to a compound (**2ah**) showing *reversed* selectivity. On the other hand, *N*-1-piperidinyl and *N*-1-hexyl amides **2ai** and **2aj** proved to be substantially devoid of affinity toward both receptor subtypes.

The most striking results were obtained by inserting substituents at position 6 of the quinolone scaffold, particularly

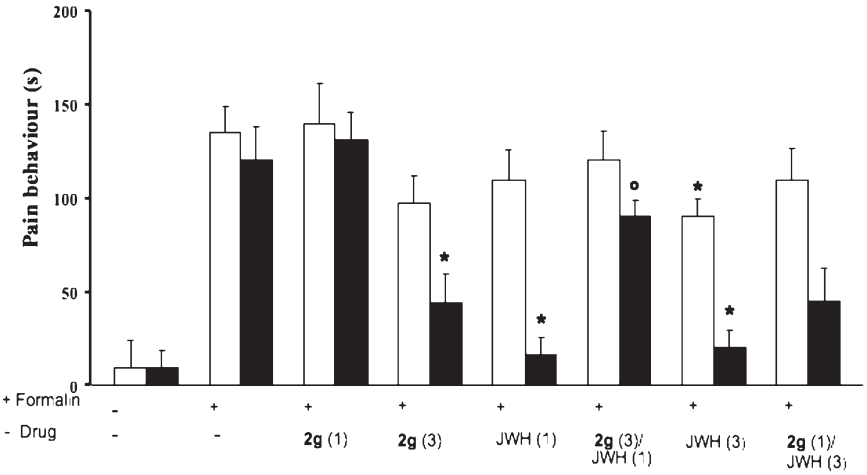


Figure 4. Effect of intraperitoneal (ip) administration of vehicle (20% DMSO in saline), compound **2g** (1 or 3 mg/kg), JWH-133 (1 or 3 mg/kg), compound **2g** (3 mg/kg) in combination with JWH-133 (1 mg/kg), or compound **2g** (1 mg/kg) in combination with JWH-133 (3 mg/kg) on the nociceptive behavior (seconds) induced by subcutaneous formalin (1.25%) injection into the hind paw of mice ($n = 8-10$). Open columns represent the early phase and filled columns the late phase of formalin-evoked nociception. Formalin was injected 15 min after vehicle or drug treatments. Asterisks indicate significant differences ($P < 0.05$) vs vehicle and circles vs JWH-133 (1 and 3 mg/kg).

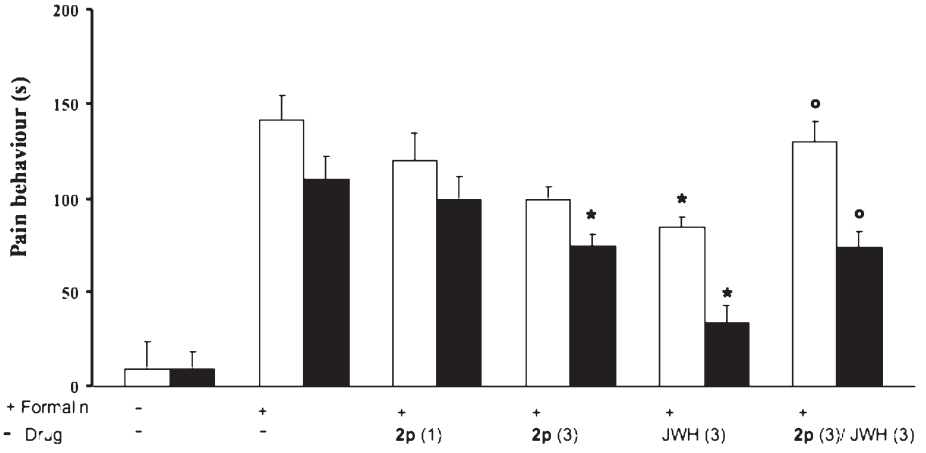


Figure 5. Effect of intraperitoneal (ip) administration of vehicle (20% DMSO in saline), compound **2p** (1 or 3 mg/kg), JWH-133 (3 mg/kg), or compound **2p** (3 mg/kg) in combination with JWH-133 (3 mg/kg), on the nociceptive behavior (seconds) induced by subcutaneous formalin (1.25%) injection into the hind paw of mice ($n = 8-10$). Open columns represent the early phase and filled columns the late phase of formalin-evoked nociception. Formalin was injected 15 min after vehicle or drug treatments. Asterisks indicate significant differences ($P < 0.05$) vs vehicle and circles vs JWH-133 (3 mg/kg).

aryl and heteroaryl groups. Quinolones **2e**−**g**, **2i**, **2j**, **2l**, **2m**, **2p**, and **2z**, all characterized by this type of substitution, displayed high CB2 affinity and selectivity, although more sterically demanding aromatic substituents, as in compounds **2o**, **2q**−**s**, and **2u**, negatively affected either CB2 affinity or selectivity. No definitive explanation can yet be given for the particular biochemical profile of the 6-pyrrolyl derivative **2y**, which seems to be an outlier within this series, showing the same CB2 potency as **2e**, but greatly reduced selectivity. The replacement of the 6-bromo substituent with the 2-furyl group favorably influenced the whole biochemical profile even in compounds, such as **2al** and **2am**, with a diverse substitution pattern at N-1 and carboxamide nitrogen. It is interesting to note how the conversion of **2ah** into **2am** was able not only to maintain very high CB2 affinity, but also to restore the CB2 selectivity, despite the CB1-directing effect of the fenchyl group on the amide.

When the aromatic ring at the position 6 was moved away from the quinolone nucleus by means of different spacers, less active compounds were generally obtained. Thus, the 6-dichlorobenzyl derivative **2b** was actually inactive, and further spacing out of the aromatic rings by either unsaturated or saturated spacers resulted in compounds possessing moderate (**2x**, **2ae**) or low (**2v**) CB2 affinity. It is worth noting that **2ae** showed lower CB2 affinity than the corresponding 6-ethyl derivative (**2ad**) or 6-phenyl derivative,[18a] but the presence of the aromatic ring in the 6-substituent probably prevented a favorable interaction with the CB1 receptor, thus giving rise to a more selective ligand than **2ad**.

Also alkyl, alkenyl, and alkynyl substituents at the 6 position are well tolerated, as demonstrated by the CB2 affinity and selectivity values of compounds **2h**, **2t**, **2w**, **2aa**, and **2ad**. However, the risk exists to get too lipophilic compounds, the biological testing of which becomes problematic (as for **2k**, **2ab**, **2ac**).

Oxidation of the 6-phenylthio analogue **6**[18a] to the sulfoxide **2c** and sulfone **2d** caused a descrease in CB2 affinity by approximately 20 times. Considering the modest affinity/

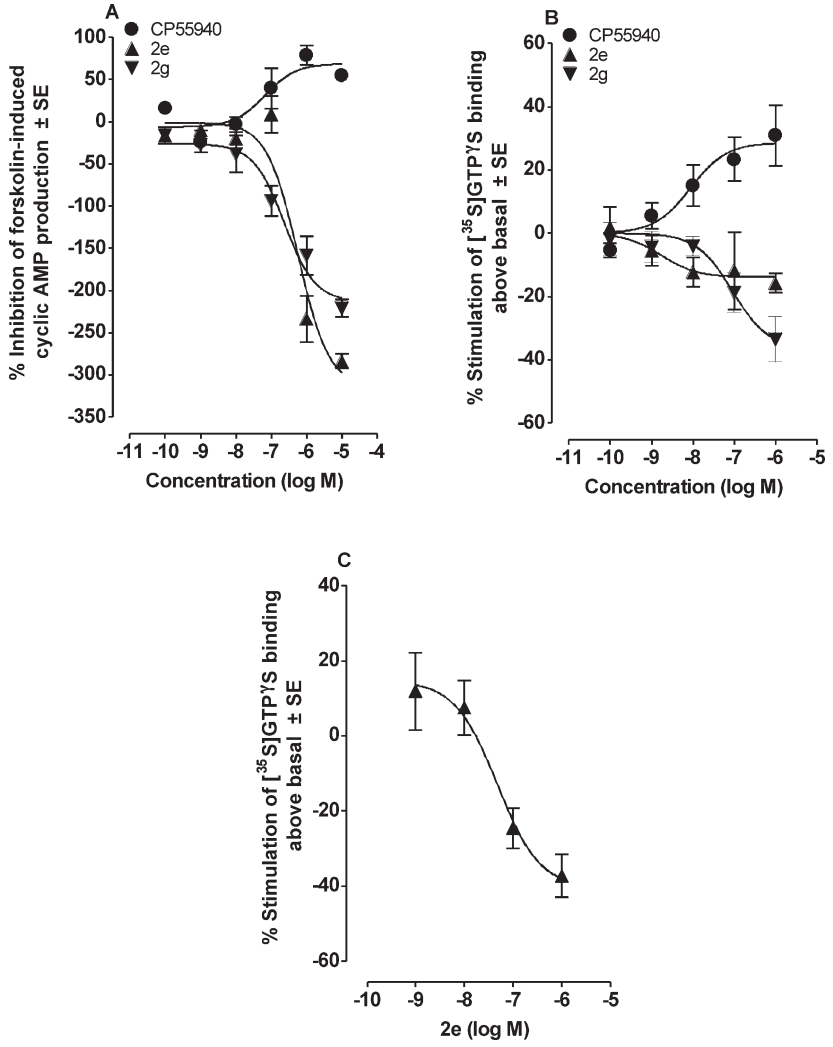


Figure 6. Dose-dependent effects (means ± SE) (A) of CP-55,940 (n = 2) and of **2e** and **2g** (n = 3) on forskolin-induced stimulation of cyclic AMP production by CHO-hCB2 cells, (B) of CP-55,940, **2e** and **2g** on [^{35}S]GTPγS binding to mouse spleen membranes (n = 4−12), and (C) of **2e** on [^{35}S]GTPγS binding to CHO-hCB2 cell membranes (n = 4).

selectivity profile of **2c** as well as the low yield in its preparation, no attempt was made to separate the racemate in order to test the individual enantiomers. Finally, the 6-iodo derivative **2a** elicited receptor affinity and selectivity comparable to the 6-bromo analogue **7**, and in particular it showed 4-fold reduced CB2 affinity but 10-fold enhanced receptor selectivity when compared to its 6-unsubstituted counterpart.[18a]

In Vivo Pharmacology. Compounds with high to moderate affinity (K_i ranging from 0.7 to 56.6 nM) and selectivity (SI ranging from >177 to >14286) for CB2, i.e. **2e**, **2g**, **2p**, **2aa** and **2ae**, were selected to be tested in the formalin test of acute (both phases) and inflammatory (second phase) pain (Figures 1−5). Compound **2e** (Figure 1), possibly the highest affinity and most selective CB2 ligand developed to date (see above), behaved as a potent CB2 neutral antagonist in this test because, unlike CB2 inverse agonists,[31] it did not reduce the second phase of the nocifensive response to formalin but fully antagonized, already at the dose of 1 mg/kg, the effect of even a high dose (3 mg/kg) of the CB2-selective ligand JWH-133 on both phases of the formalin response. Given the lack of CB2-neutral antagonists in the scientific literature, this CB2-selective neutral antagonism-like behavior of **2e** might provide an unprecedented pharmacological tool for in vivo studies of CB2 receptor biological function. By contrast, compound **2ae** (Figure 2) behaved as a CB2 agonist, although weaker than JWH-133 because it counteracted both phases of the nocifensive response to formalin but only at the highest dose tested (3 mg/kg), and this effect was antagonized by the CB2 receptor inverse agonist AM630 at a per se inactive dose (1 mg/kg). Importantly, the CB2 affinity of **2ae** (K_i = 56.6 nM) was significantly lower than that (K_i = 3.4 nM) previously reported for JWH-133,[27] in agreement with its lower efficacy in vivo. Compound **2aa** (Figure 3), like **2e**, was also inactive in the formalin test, but it fully antagonized only the effect of a low dose of JWH-133 (1 mg/kg) at the highest dose tested (3 mg/kg). Therefore, also **2aa** might be considered as a neutral CB2-selective antagonist, albeit less efficacious than **2e**, in agreement with its lower affinity for CB2 receptors as compared to **2e** (K_i = 7.3 nM vs 0.7 nM). Unlike **2e**, **2ae**, and **2aa**, the effects of compounds **2g** and **2p** in the formalin test were more open to interpretation. Compound **2g** (Figure 4) significantly inhibited only the second phase of the nocifensive response to formalin only at the highest dose tested (3 mg/kg). This, in view of the high affinity (K_i = 4.2 nM) and selectivity (SI > 2381) of this compound for CB2, is the behavior previously reported for high affinity CB2 inverse agonists.[32] Accordingly, the effect of **2g** (3 mg/kg) was partly antagonized by a low dose of

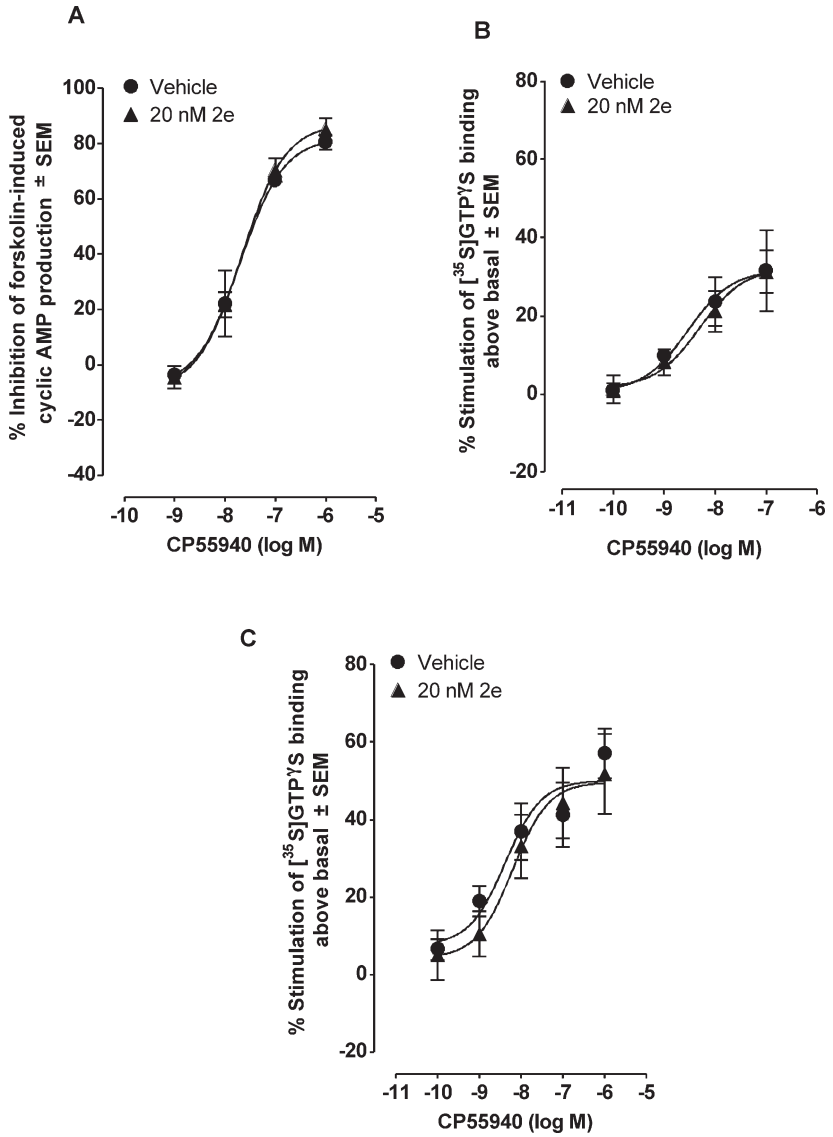


Figure 7. (A) Inhibitory effect of CP-55,940 on forskolin-induced stimulation of cyclic AMP production by CHO-hCB2 cells in the presence of 20 nM **2e** or of its vehicle, DMSO (means ± SE, $n = 4$). Stimulatory effect of CP-55,940 on [^{35}S]GTPγS binding to mouse spleen membranes (B) or CHO-hCB2 cell membranes (C) in the presence of 20 nM **2e** or of its vehicle, DMSO (means ± SE, $n = 4$).

JWH-133 (1 mg/kg). However, a per se inactive dose of **2g** (1 mg/kg) was not capable of fully antagonizing the antinocifensive effect of JWH-133 (3 mg/kg) although it rendered the effect of the latter nonstatistically significant. This observation, given the high affinity of **2g** for CB2, is not completely in agreement with it behaving as an inverse agonist.[32] Indeed, the pharmacological behavior of **2g** is also compatible with that of a potent, albeit partial, CB2 agonist because, as mentioned above, it obstructed the effect of a low, but still active, dose of JWH-133. Finally, compound **2p** (Figure 5), endowed with lower affinity (K_i = 41.9 nM) and selectivity (SI > 177) for CB2 receptor than **2g**, appeared also less efficacious than the latter compound at reducing the second phase of the nocifensive response to formalin at the highest dose tested (3 mg/kg). This behavior might be due to **2p** acting again as an inverse agonist, as suggested by the fact that this compound and JWH-133 obstructed each other, rather than to its activity as a potential dual CB1/CB2 agonist at high doses, which would have resulted in an inhibition of both the first and second phase of the nocifensive response to formalin.

Functional Activity at CB2 Receptors in Vitro. The above hypotheses on the possible agonist, neutral antagonist-, and inverse agonist-like behaviors of the five compounds examined here in the formalin test needed to be confirmed, at least to some extent, by appropriate in vitro assays of CB2 receptor functional activity. Therefore, of the five compounds, we selected the ones with highest affinity on CB2, i.e. **2e** and **2g**, and tested them in two assays of CB2-mediated functional activity. These two compounds were also selected because, for the former, we needed to confirm the unprecedented behavior as a neutral antagonist in the formalin test and, for the latter, we needed to distinguish between the two possibilities (inverse agonism vs partial agonism) suggested by this in vivo assay (see above). Both compounds behaved as inverse agonists in the forskolin-induced cAMP elevation assay carried out in CHO cells overexpressing the human recombinant CB2 receptor (Figure 6A), although the effect of **2e** in this assay was only seen at concentrations higher than 100 nM, i.e. more than 140-fold higher than its K_i. Both compounds also acted as inverse agonists in the [^{35}S]GTPγS binding assay carried out with mouse spleen membranes

(Figure 6B), which preferentially express CB2 receptors over CB1. In this case, compound **2e** was more potent but significantly less efficacious than **2g**, with an E_{max} of only 13.9%. Therefore, given its activity in vivo, we thought that **2e**, at concentrations < 100 nM, could behave as a neutral antagonist, and tested it against CP-55,940-induced inhibition of forskolin-induced cAMP elevation in CHO cells overexpressing the human recombinant CB2 receptors or stimulation of [^{35}S]GTPγS binding in mouse spleen membranes. Surprisingly, in neither of these assays, **2e**, at concentrations up to 20 nM, could antagonize the effect of CP-55,940 (Figure 7), the reported CB2 receptor affinity of which is similar to that observed here for **2e**. Thus, while the in vitro assays performed here confirmed for **2g** its behavior as inverse agonist, observed in the formalin test, they failed to confirm the very promising neutral antagonist activity of **2e** suggested by the in vivo data. A possible explanation for this discrepancy is that this compound might act, in vivo, rather than as a competitive antagonist, as a "functional" antagonist instead, for example by blocking effects that are downstream of CB2 receptors, and hence counteracting only some effects (e.g., analgesia) of certain agonists (e.g., JWH133) rather than other effects (e.g., inhibition of adenylate cyclase) by other agonists (e.g., CP-55,940). Indeed, it is known that different agonists can induce the trafficking of their G-protein-coupled receptors toward different G-proteins and biological effects. It is also possible that **2e** is transformed in vivo to a metabolite that instead does act as a neutral competitive antagonist at CB2 receptors. Indeed, unpublished in vitro data from our laboratories indicate that this compound is rapidly oxidized by cytochrome P450 enzymes (after 1 h incubation with CYP3A4, only 4% of compound **2e** remains unaltered). Finally, a possibly much simpler explanation is that **2e** behaves in vitro predominantly as an inverse agonist at CB2 receptors, and that the putative neutral antagonism observed with **2e** in vivo might be observed also in vitro but only at concentrations that are too low to counteract the agonist effects of a potent agonist such as CP-55,940. Indeed, we did find here that **2e** behaves as an inverse agonist at concentrations > 40 nM also in a [^{35}S]GTPγS binding assay carried out with CHO cells overexpressing the human recombinant CB2 receptor (Figure 6C), in which, again, a per se inactive concentration of the compound could not counteract the stimulatory effect of CP-55,940 (Figure 7C).

Conclusions

Within a research program aimed at characterizing novel cannabinoid ligands, we describe herein new 6-substituted 4-quinolone-3-carboxamides possessing high affinity for the human CB2 receptor at nanomolar or subnanomolar concentration and selectivity index values as high as >14000. Thus, some of these compounds proved to be very potent toward the human CB2 receptor, while no affinity for the human CB1 receptor could be measured at all. It should be considered that the issue of receptor selectivity has a major relevance in the cannabinoid area, since none of the CB2-selective agonists that have been developed to date are completely CB2-specific.[5b] Thus they are all expected to display CB2 selectivity only within a finite dose range and to target CB1 receptors as well when administered at a dose that lies above this range.

As far as the functional activity is concerned, among the new compounds it was possible to identify compounds that, at least in vivo, behaved as agonists, inverse agonists or, most importantly, neutral antagonists. This would have been a quite significant finding, since the CB2 "antagonists" most widely used so far (such as SR144528 and AM630) all behave indeed as inverse agonists,[5b] able to reverse the constitutive activity of the receptor, whereas neutral antagonists have affinity but no efficacy for their receptor. Selected compounds were assayed in the formalin test of acute peripheral and inflammatory pain in mice by ip administration. In previous studies, compounds acting as CB2 agonists exhibited in this test analgesic activity, whereas CB2 antagonists antagonized the antinociceptive responses of agonists and inverse agonists also caused antinocifensive effects at the highest dose tested.[33] In the present study, data obtained in this animal model of inflammatory pain allowed us to conclude that 6-substituted 4-quinolone-3-carboxamides agonists may possess CB2 receptor-mediated analgesic activity in vivo and show favorable drug-safety profile. These data suggest that the compound with highest CB2 affinity obtained in this study, i.e **2e**, might behave as a neutral antagonist in vivo, whereas others, such as **2g**, might act as inverse agonists. However, two different functional assays carried out in vitro could only confirm the latter hypothesis, thus opening the way to further future studies aimed at understanding how the very high affinity of **2e** for CB2, and its weak efficacy and/or potency as an inverse agonist at this receptor in vitro, result in the antagonism of a CB2-mediated effect only in vivo. A different behavior of CB2 ligands in functional assays carried out in vitro or in vivo is not unusual. Indeed, the concept of "protean agonists", defined as those compounds the functional efficacies of which in various assay systems may depend on the local levels of receptor constitutive activities, and for which, therefore, the efficacies observed in vitro may not necessarily predict in vivo activities,[34] was applied to explain the pharmacological activity of certain CB2 receptor-selective ligands as early as 2006.[35] Importantly, a recent study showed that such different behaviors in functional tests do not seem to affect the final outcome in assays of antihyperalgesic activity,[36] exactly as observed here, possibly because, as emphasized by many authors,[4a,17,44] both agonists and inverse agonists at CB2 may inhibit inflammation via different cellular mechanism of actions. Finally, further investigations will be also directed to enhance both water solubility and metabolic stability of the most promising CB2 ligands within this series of compounds.

Experimental Section

Chemistry. Reagents were purchased from commercial suppliers and used without further purification. Anhydrous reactions were run under (a positive pressure) dry N_2. Merck silica gel 60 was used for flash chromatography (23−400 mesh). IR spectra were recorded on a Perkin−Elmer BX FT-IR system using $CHCl_3$ as the solvent or a Nujol dispersion. ^{1}H NMR and ^{13}C NMR were recorded at 200 and 50 MHz respectively on a Bruker AC200F spectrometer and at 400 and 100 MHz on a Bruker Advance DPX400. Chemical shifts are reported relative to tetramethylsilane at 0.00 ppm. Mass spectral (MS) data were obtained using Agilent 1100 LC/MSD VL system (G1946C) with a 0.4 mL/min flow rate using a binary solvent system of 95:5 methanol/water. UV detection was monitored at 254 nm. Mass spectra were acquired either in positive or in negative mode scanning over the mass range of 105−1500. Melting points were determined on a Gallenkamp apparatus and are uncorrected. Microwave irradiations were conducted using a CEM Discover Synthesis Unit (CEM Corp., Matthews, NC). Elemental

analyses were performed on a Perkin-Elmer PE 2004 elemental analyzer and the data for C, H, and N are within 0.4% of the theoretical values. The chemical purity of the target compounds was determined using the following conditions: an Agilent 1100 series LC/MSD with a Lichrocart 125-4 Lichrospher 100 RP-18 (4.6 mm × 100 mm, 5 μm) reversed phase column; method: 86% (v/v) of MeOH in H_2O, isocratic, flow rate of 1 mL/min, UV detector, 254 nm. The purity of each compound was ≥95% in either analysis.

Synthesis of Compounds 4a,b and 2af−2ah by *N*-Alkylation Reaction. General Procedure. To a solution of the appropriate substrate **3a**,**b** or **9a**,**b** (1 mmol) in DMF (1 mL) were added K_2CO_3 (0.39 g, 2.8 mmol) and the alkyl halide (2.8 mmol). The reaction mixture was heated at 100 °C for 4 h, then diluted with water (10 mL) and extracted with CH_2Cl_2 (3 × 3 mL). The organic layer was dried over Na_2SO_4 and evaporated to dryness. The crude residue was recrystallized from EtOH to provide the title compound.

Example. 6-Bromo-4-oxo-1-pentyl-*N*-(1,3,3-trimethylbicyclo-[2.2.1]heptan-2-yl)-1,4-dihydroquinoline-3-carboxamide (2ah). Prepared in 88% yield starting from **9b**; white solid; mp 159−160 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 10.01 (d, J = 9.1 Hz, 1H), 8.71 (s, 1H), 8.63 (d, J = 1.9 Hz, 1H), 7.72 (d, J_1 = 1.9, J_2 = 8.9 Hz, 1H), 7.34 (d, J = 8.9 Hz, 1H), 4.15 (t, J = 6.9 Hz, 2H), 3.80 (d, J = 9.1 Hz, 1H), 1.82−1.58 (m, 6H), 1.51−1.17 (m, 7H), 1.10 (s, 3H), 1.04 (s, 3H), 0.87−0.81 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 175.54, 165.03, 147.78, 137.78, 135.64, 130.20, 129.38, 118.88, 117.66, 112.68, 64.17, 54.39, 48.80, 48.35, 42.70, 39.57, 31.02, 28.70, 27.76, 26.07, 22.20, 21.71, 19.78, 13.80. MS (ESI): m/z: 474 $[M + H]^+$. IR ($CHCl_3$): ν 1607, 1658 cm^{-1}. Anal. ($C_{25}H_{33}BrN_2O_2$) C, H, N.

Synthesis of Compounds 5a and 5b by Basic Hydrolysis. General Procedure. A suspension of the appropriate ester **4a**,**b** (1 mmol) in 10% aq NaOH (7 mL) was refluxed for 3 h. After cooling at room temperature, the reaction mixture was acidified using conc HCl. The solid precipitated was collected by filtration and washed with water and petroleum ether. The solid obtained was recrystallized from EtOH to afford the pure acid.

Example. 6-Iodo-4-oxo-1-pentyl-1,4-dihydroquinoline-3-carboxylic Acid (5a). Prepared from compound **4a** in 65% yield; beige solid; mp 186−187 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 14.64 (s, 1H), 8.82 (s, 1H), 8.72 (s, 1H), 8.06 (d, J = 8.8 Hz, 1H), 7.34 (d, J = 8.8 Hz, 1H), 4.27 (t, J = 7.2 Hz, 2H), 1.98−1.79 (m, 2H), 1.36−1.34 (m, 4H), 1.00−0.80 (m, 3H). MS (ESI): m/z: 386 $[M + H]^+$. IR ($CHCl_3$): ν 1722 cm^{-1}. Anal. ($C_{15}H_{16}INO_3$) C, H, N.

Synthesis of Compounds 2a,b, 9a,b, and 2ai,aj by Amidation Reaction. General Procedure. The appropriate carboxylic acid **5a**,**b**, **8**, or **11** (2 mmol) was dissolved in DMF (5 mL). HOBt (270 mg, 2 mmol), HBTU (1.72 g, 4 mmol), DIPEA (152 μL, 3 mmol), and the amine (2.4 mmol) were added to the solution and the reaction mixture was stirred at room temperature for 30 min. Further DIPEA (152 μL, 3 mmol) was thereafter added, and the reaction mixture was stirred at room temperature for further 4 h. The reaction mixture was poured into ice and the solid precipitated was collected by filtration and washed with water and petroleum ether. Recrystallization from EtOH gave the pure amide.

Example. 6-Bromo-4-oxo-1-pentyl-*N*-(piperidin-1-yl)-1,4-dihydroquinoline-3-carboxamide (2ai). Prepared in 88% yield from **11**; white solid; mp 195−196 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 10.06 (s, 1H), 8.61 (d, J = 1.6 Hz, 1H), 8.43 (s, 1H), 7.64 (dd, J_1 = 1.6, J_2 = 7.0 Hz, 1H), 7.28 (d, J = 7.0 Hz, 1H), 4.11, (t, J = 6.8 Hz, 2H), 2.77−2.65 (m, 4H), 1.72−1.62 (m, 6H), 1.44−1.28 (m, 6H), 0.76 (t, J = 6.8 Hz, 3H). MS (ESI): m/z: 421 $[M + H]^+$. IR ($CHCl_3$): ν 1601, 1655 cm^{-1}. Anal. ($C_{20}H_{26}BrN_3O_2$) C, H, N.

Synthesis of Compounds 2c and 2d. To a solution of oxone (240 mg, 0.39 mmol) in H_2O (3 mL), a solution of **6** (65 mg, 0.13 mmol) in 1,4-dioxane (2 mL) was added. The reaction mixture was stirred at room temperature for 20 h and extracted with CH_2Cl_2. The organic layers were washed with brine, dried over anhydrous Na_2SO_4, and evaporated to dryness. The crude residue was purified by chromatography using light petroleum ether/AcOEt (2:1) as eluent to provide compounds **2c** (5 mg, 7% yield) as a colorless oil and **2d** (32 mg, 50% yield) as a white solid.

***N*-(Adamantan-1-yl)-4-oxo-1-pentyl-6-(phenylsulfinyl)-1,4-dihydroquinoline-3-carboxamide (2c).** Colorless oil. ^{1}H NMR (200 MHz, $CDCl_3$): δ 9.69 (s, 1H), 8.78 (d, J = 1.9 Hz; 1H), 8.50 (s, 1H), 8.10 (dd, J_1 = 1.9, J_2 = 8.6 Hz, 1H), 7.90−7.55 (m, 6H), 4.20 (t, J = 7.2 Hz, 2H), 2.14−2.09 (m, 10H), 1.90−1.81 (m, 2H), 1.80−1.71 (m, 5H), 1.37 (m, 4H), 0.92−0.86 (m, 3H). MS (ESI): m/z 517 $[M + 1]^+$, 539 $[M + Na]^+$. IR ($CHCl_3$): ν 1601, 1653 cm^{-1}. Anal. ($C_{31}H_{36}N_2O_3S$) C, H, N.

***N*-(Adamantan-1-yl)-4-oxo-1-pentyl-6-(phenylsulfonyl)-1,4-dihydroquinoline-3-carboxamide (2d).** White solid; mp 133−135 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 9.60 (s, 1H), 8.89 (d, J = 2.4 Hz, 1H), 8.67 (s, 1H), 8.15 (dd, J_1 = 2.4, J_2 = 8.8 Hz, 1H), 7.91 (d, J = 6.8 Hz, 2H), 7.56 (d, J = 8.8 Hz, 1H), 7.50−7.43 (m, 3H), 4.16 (t, J = 6.8 Hz, 2H), 2.11−2.09 (m, 9H), 1.79−1.65 (m, 8H), 1.35−1.31 (m, 4H), 0.82−0.77 (m, 3H). MS (ESI): m/z: 533 $[M + H]^+$. IR ($CHCl_3$): ν 1601, 1655 cm^{-1}. Anal. ($C_{31}H_{36}N_2O_4S$) C, H, N.

Synthesis of Compounds 2e−2u and 2ak−2am by Suzuki Reaction. General Procedure. To a solution of the appropriate bromoquinolone **7** or iodoquinolone **2a** (1 mmol) in DME (4 mL), $Pd(OAc)_2$ (0.1 mmol), PPh_3 (0.3 mmol), the appropriate boronic acid (0.5 mmol), EtOH (1 mL), and 1 N Na_2CO_3 (2 mL) were added. The mixture was irradiated with microwaves at 150 °C for 10 min, then filtered through a plug of Celite. The filtrate was washed with H_2O, brine, dried over anhydrous Na_2SO_4, and evaporated to dryness. The solid residue was purified by flash chromatography using CH_2Cl_2/MeOH (98:2) as eluent.

Example. *N*-(Adamantan-1-yl)-6-(furan-2-yl)-4-oxo-1-pentyl-1,4-dihydroquinoline-3-carboxamide (2e). Prepared from **7** in 81% yield; light-brown solid; mp 180−181 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 9.88 (s, 1H), 8.65−8.61 (m, 2H), 7.93−7.89 (m, 1H), 7.44−7.40 (m, 2H), 6.73−6.72 (m, 1H), 6.43−6.42 (m, 1H), 4.14 (t, J = 7.6 Hz, 2H), 2.15 (s, 6H), 2.06 (s, 3H), 1.80−1.74 (m, 2H), 1.66−1.61 (m, 6H), 1.31−1.28 (m, 4H), 0.83−0.80 (m, 3H). MS (ESI): m/z: 459 $[M + H]^+$. IR ($CHCl_3$): ν 1601, 1653 cm^{-1}. Anal. ($C_{29}H_{34}N_2O_3$) C, H, N.

Synthesis of Compounds 2v and 2w by Sonogashira Reaction. General Procedure. To a solution of **2a** (518 mg, 1 mmol) in toluene (7 mL) under N_2 were added successively CuI (38 mg, 0.2 mmol), *i*-Pr_2NH (7 mL), the appropriate alkyne (2.2 mmol), and $PdCl_2(PPh_3)_2$ (70.2 mg, 0.1 mmol). The reaction mixture was refluxed for 2 h and volatiles were removed under reduced pressure. The crude residue was purified by flash chromatography using CH_2Cl_2/MeOH (99:1) as eluent to give **2v** or the trimethylsilyl derivative of **2w**.

***N*-(Adamantan-1-yl)-4-oxo-1-pentyl-6-(2-phenylethynyl)-1,4-dihydroquinoline-3-carboxamide (2v).** Yield: 72%; yellow solid; mp 199−200 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 9.86 (s, 1H), 8.68 (s, 1H), 8.63 (d, J = 1.4 Hz, 1H), 7.35−7.31 (m, 3H), 4.17 (t, J = 7.3 Hz, 2H), 2.16 (s, 6H), 2.09 (s, 3H), 1.85−1.78 (m, 2H), 1.75−1.70 (m, 6H), 1.35−1.32 (m, 4H), 0.91−0.85 (m, 3H). MS (ESI): m/z: 493 $[M + H]^+$, 515 $[M + Na]^+$. IR ($CHCl_3$): ν 1601, 1653 cm^{-1}. Anal. ($C_{33}H_{36}N_2O_2$) C, H, N.

***N*-(Adamantan-1-yl)-6-ethynyl-4-oxo-1-pentyl-1,4-dihydroquinoline-3-carboxamide (2w).** The trimethylsilyl derivative obtained by the Sonogashira reaction (40 mg, 43% yield) was dissolved in MeOH/THF (1:1, 10 mL) and K_2CO_3 (110 mg, 0.8 mmol) was added. The reaction mixture was stirred at room temperature for 30 min and evaporated to dryness. The crude residue was taken up in CH_2Cl_2 and washed with water and brine. The organic layer was evaporated to dryness to afford compound **2w** (32 mg, 96% yield) as a colorless oil. ^{1}H NMR (200 MHz, $CDCl_3$): δ 9.81, (s, 1H), 8.69 (s, 1H), 8.61 (d, J = 1.9 Hz, 1H), 7.74 (dd, J_1 = 1.9 Hz, J_2 = 7.0 Hz, 1H), 7.42 (d, J = 7.0 Hz, 1H), 7.42 (d, J = 8.9 Hz, 1H), 4.17 (t, J = 7.4 Hz, 2H), 3.13

(s, 1H), 2.15 (s, 6H), 2.09 (s, 3H), 1.85−1.70 (m, 2H), 1.63−1.61 (m, 6H), 1.36−1.33 (m, 4H), 0.91−0.88 (m, 3H). MS (ESI): *m*/*z*: 417 $[M + H]^+$, 439 $[M + Na]^+$. IR ($CHCl_3$): ν 1599, 1660, 3303 cm^{-1}. Anal. ($C_{27}H_{32}N_2O_2$) C, H, N.

(*E*)-*N*-(Adamantan-1-yl)-4-oxo-1-pentyl-6-styryl-1,4-dihydroquinoline-3-carboxamide (2x). A suspension of compound **2a** (100 mg, 0.19 mmol), styrene (65 μL, 0.57 mmol), $Pd(OAc)_2$ (42 mg, 0.19 mmol), TEA (26 μL, 0.19 mmol),and PPh_3 (20 mg, 0.076 mmol) in DMF (1 mL) was refluxed under N_2 for 3 h. The reaction mixture was poured into ice−water and extracted with CH_2Cl_2. The organic layer was washed with water, then brine, dried over anhydrous Na_2SO_4, and evaporated to dryness. The crude residue was purified by flash chromatography using CH_2Cl_2/MeOH (99:1) as eluent to give the title compound **2x**. Yield: 40%; yellow oil. ^{1}H NMR (200 MHz, $CDCl_3$): δ 9.96 (s, 1H), 8.69 (s, 1H), 8.8.62−8.61 (m, 1H), 7.87−7.83 (m, 1H), 7.54−7.40 (m, 3H), 7.37−7.31 (m, 3H), 7.26 (d, J = 5.4 Hz, 1H), 4.20 (t, J = 7.4 Hz, 2H), 2.17 (s, 6H), 2.10 (s, 3H), 1.88−1.72 (m, 2H), 1.61−1.60 (m, 6H), 1.37−1.35 (m, 4H), 0.90 (t, J = 6.8 Hz, 3H). MS (ESI): *m*/*z*: 495 $[M + H]^1$. IR ($CHCl_3$): ν 1600, 1655 cm^{-1}. Anal. ($C_{33}H_{38}N_2O_2$) C, H, N.

Synthesis of Compounds 2y and 2z by Ullman-Type Reaction. General Procedure. To a solution of imidazole or pyrrole (1.5 mmol) in dry DMSO (0.2 mL), $Cu(OAc)_2$ (182 mg, 1 mmol), **2a** (518 mg, 1 mmol), and DBU (300 μL, 2 mmol) were added under N_2. The reaction mixture was irradiated with microwaves at 130 °C for 10 min and then diluted with MeOH/AcOEt and filtered through a plug of Celite. The filtrate was washed with water, brine, dried over anhydrous Na_2SO_4, and evaporated to dryness. The solid residue was purified by flash chromatography using CH_2Cl_2/MeOH (98:2) as eluent.

Example. *N*-(Adamantan-1-yl)-6-(1*H*-imidazol-1-yl)-4-oxo-1-pentyl-1,4-dihydroquinoline-3-carboxamide (2z). Yield: 47%; light-brown solid; mp 282−283 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 9.76, (s, 1H), 8.74 (s, 1H), 8.52 (s, 2H), 7.75−7.70 (m, 2H), 7.64−7.60 (m, 2H), 4.23 (t, J = 7.2 Hz, 2H), 2.14 (s, 6H), 2.09 (s, 3H), 1.99−1.69 (m, 2H), 1.65−1.63 (m, 6H), 1.37−1.33 (m, 4H), 0.92−0.85 (m, 3H). MS (ESI): *m*/*z*: 459 $[M + H]^+$, 481 $[M+Na]^+$. IR ($CHCl_3$): ν 1601, 1654 cm^{-1}. Anal. ($C_{28}H_{34}N_4O_2$) C, H, N.

Synthesis of Compounds 2aa−2ae by Hydrogenation Reaction. General Procedure. To a previously degassed solution of the appropriate starting material (1 mmol) in EtOH (5−10 mL) was added 10% Pd/C (0.1 mmol). The reaction mixture was stirred under H_2 (1 atm) at room temperature for 4 h and then was filtered through a plug of Celite and evaporated to dryness.

Example. *N*-(Adamantan-1-yl)-6-ethyl-4-oxo-1-pentyl-1,4-dihydroquinoline-3-carboxamide (2ad). Yield: 100%; colorless oil. ^{1}H NMR (200 MHz, $CDCl_3$): δ 10.01 (s, 1H), 8.70 (s, 1H), 8.32 (d, J = 1.7 Hz, 1H), 7.54 (dd, J_1 = 1.7 Hz, J_2 = 7.2 Hz, 1H), 7.41 (d, J = 7.2 Hz, 1H), 4.18 (t, J = 7.4 Hz, 2H), 2.77 (d, J = 7.6 Hz, 2H)q, 2.15 (s, 6H), 2.08 (s, 3H), 1.89−1.82 (m, 2H), 1.76−1.70 (m, 6H), 1.35−1.31 (m, 7H), 0.88 (t, J = 6.9 Hz, 3H). MS (ESI): *m*/*z*: 421 $[M + H]^+$. IR ($CHCl_3$): ν 1601, 1656 cm^{-1}. Anal. ($C_{27}H_{36}N_2O_2$) C, H, N.

Binding Assays. CB1 and CB2 receptor binding assays were performed exactly as described previously,[18] using membranes of cells overexpressing the human recombinant CB1 or CB2 receptors.

Formalin Test. The experimental procedures applied in the formalin test were approved by the Animal Ethics Committee of the Second University of Naples. Animal care was in compliance with the IASP and European Community guidelines on the use and protection of animals in experimental research (EC L358/1 18/12/86). All efforts were made to minimize animal suffering and to reduce the number of animals used. Formalin injection induces a biphasic stereotypical nocifensive behavior.[33] Nociceptive responses are divided into an early, short-lasting first phase (0−7 min) caused by a primary afferent discharge produced by the stimulus, followed by a quiescent period and then a second, prolonged phase (15−60 min) of tonic pain. Mice received formalin (1.25% in saline, 30 μL) in the dorsal surface of one side of the hind-paw. Each mouse was randomly assigned to one of the experimental groups (n = 8−10) and placed in a Plexiglas cage and allowed to move freely for 15−20 min. A mirror was placed at a 45° angle under the cage to allow full view of the hind-paws. Lifting, favoring, licking, shaking, and flinching of the injected paw were recorded as nociceptive responses. The duration of those mentioned noxious behaviors were monitored by an observer blind to the experimental treatment for periods of 0−10 min (early phase) and 20−60 min (late phase) after formalin administration. Results are expressed as means ± SEM. Significant differences between groups were evaluated by using analysis of variance followed by the Dunnett's test. The version of the formalin test we applied is based on the fact that a correlational analysis showed that no single behavioral measure can be a strong predictor of formalin or drug concentrations on spontaneous behaviors.[37] Consistently, we considered that a simple sum of time spent licking plus elevating the paw, or the weighted pain score, is in fact superior to any single (lifting, favoring, licking, shaking, and flinching) measure (r ranging from 0.75 to 0.86).[38] Treatments: groups of 8−10 animals per treatment were used with each animal being used for one treatment only. Mice received intraperitoneal vehicle (20% DMSO in 0.9% NaCl) or different doses of before mentioned compounds.

In Vitro Functional Assays. CHO Cells. Chinese hamster ovary (CHO) cells transfected with human cannabinoid CB2 receptors[39] were maintained in Dulbecco's modified Eagles's medium (DMEM) nutrient mixture F-12 HAM, supplemented with 1 mM L-glutamine, 10% fetal bovine serum, 0.6% penicillin−streptomycin, and G418 (400 μg/mL). Cells were maintained at 37 °C and 5% CO_2 in their media and passaged twice a week using nonenzymatic cell dissociation solution.

Membrane Preparation. Binding assays with [^{35}S]GTPγS were performed with membranes obtained from CHO-hCB_2 cells[39] or mouse spleens.[40] The hCB2 transfected cells were removed from flasks by scraping and then frozen as a pellet at −20 °C until required. Spleen tissue was obtained from adult male C57BL/6J mice weighing 25−40 g and maintained on a 12/12 h light/dark cycle with free access to food and water. Spleens were cut into several pieces and placed in a Choi lysis buffer (Tris-HCl 20 mM, sucrose 0.32 M, EDTA 0.2 mM, EGTA 0.5 mM, pH 7.5) containing Roche protease inhibitor cocktail (1:40 v/v; Roche Diagnostics, Mannheim, Germany) and PMSF (150 μM) and then homogenized. The homogenate was centrifuged at 500*g* for 2 min and the resultant supernatant was recentrifuged at 16000*g* for 20 min. The harvested membranes were resuspended in TME buffer (50 mM Tris-HCl; EDTA 1.0 mM; $MgCl_2$ 3.0 mM; pH 7.4) and stored at −80 °C for no more than 1 month. Protein assays were performed using a Bio-Rad Dc kit (Hercules, CA).

Cyclic AMP Assay. Assays were performed using HitHunter cyclic AMP assay kits according to the vendor's protocol. Briefly, CHO cells expressing hCB2 receptors were detached using cell dissociation buffer, counted, and seeded at 2×10^4 cells per well in 100 μL of complete medium onto white 96-well plates. They were then incubated at 37 °C with 5% CO_2 for approximately 24 h before running the experiment. The assays and the drug dilutions were performed using a 1:1 mixture of DMEM and Ham's F12 medium without phenol red (DMEM/F12 Media), containing 10 μM of rolipram and forskolin. Before running the assay, the medium was discarded and cells were washed once with DMEM/F12 Media. Cells were then treated with the compounds under investigation (30 μL per well) and incubated for 30 min at 37 °C with 5% CO_2. Finally, a cyclic AMP standard curve was constructed, after which the appropriate mixture of kit components was added, as described by the manufacturer (DiscoveRx). Plates were incubated overnight at room temperature in the dark. Chemiluminescent signals were

detected using a Synergy HT Multi-Mode Microplate Reader (BioTek, Winooski, VT).

[^{35}S]GTPγS Binding Assay. This assay was performed as described previously.[40] It was carried out with GTPγS binding buffer (50 mM Tris-HCl, 100 mM NaCl, 0.1% BSA) in the presence of [^{35}S]GTPγS and GDP, in a final volume of 500 μL. The GTPγS binding buffer also contained 50 mM Tris-Base, 5 mM $MgCl_2$, 1 mM dithiothreitol and 1 mM EDTA (CHO cell membrane experiments) or 3 mM $MgCl_2$ and 0.2 mM EGTA (spleen membrane experiments). Binding was initiated by the addition of [^{35}S]GTPγS to the wells. Nonspecific binding was measured in the presence of 30 μM GTPγS. Compound(s) under investigation were incubated in the assay for 60 min at 30 °C. The reaction was terminated by a rapid vacuum filtration method using Tris-binding buffer, and the radioactivity was quantified by liquid scintillation spectrometry. In all the [^{35}S]-GTPγS-binding assays we used 0.1 nM [^{35}S]GTPγS, 30 μM GDP and 40 μg (spleen membranes) or 50 μg (cell membranes) protein per well. Additionally, mouse spleen membranes were preincubated for 30 min at 30 °C with 0.5 U/mL adenosine deaminase (200 U/mL) to remove any endogenous adenosine.

Materials. Rolipram was supplied by Sigma-Aldrich (Poole, Dorset, UK) and forskolin and CP-55,940 {(−)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl)cyclohexan-1-ol} by Tocris (Bristol, UK). The HitHunter cyclic AMP assay kit was purchased from DiscoveRx (Fremont, CA).

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Supporting Information Available: Additional synthetic, spectral, and analytical data. This material is available free of charge via the Internet at http://pubs.acs.org.

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