Rational Design, Synthesis, and Pharmacological Properties of New 1,8-Naphthyridin-2(1*H*)-on-3-Carboxamide Derivatives as Highly Selective Cannabinoid-2 Receptor Agonists

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The CB₂ receptor activation can be exploited for the treatment of diseases such as chronic pain and tumors of immune origin, devoid of psychotropic activity. On the basis of our already reported 1,8-naphthyridin-4(1H)-on-3-carboxamide derivatives, new 1,8-naphthyridin-2(1H)-on-3-carboxamide derivatives were designed, synthesized, and tested for their affinities toward the human CB₁ and CB₂ cannabinoid receptors. Some of the reported compounds showed a subnanomolar CB₂ affinity with a CB₁/CB₂ selectivity ratio greater than 200 (compounds 6, 12, *cis*-12, 13, and *cis*-13). Further studies revealed that compound 12, which presented benzyl and carboxy-4-methylcyclohexylamide substituents bound in the 1 and 3 positions, exerted a CB₂-mediated inhibitory action on immunological human basophil activation. On the human T cell leukemia line Jurkat the same derivative induced a concentration-dependent decrease of cell viability. The obtained results suggest that 1,8-naphthyridin-2(1H)-on-3-carboxamides represent a new scaffold very suitable for the development of new promising CB₂ agonists.

Introduction

Following the discovery of the endocannabinoid system (ECS^{*a*}), it has become apparent that research in the cannabinoid field has promise of becoming therapeutically important. The ECS includes cannabinoid receptors, several endocannabinoids (e.g., 2-arachidonoylglycerol, *N*-arachidonoylethanolamine), and the enzymes responsible for their production and inactivation (fatty acid amide hydrolases I and II, monoacylglycerol lipase, and *N*-acylethanolamine acid amidase).^{1,2} To date, two G-protein-coupled seven-transmembrane receptors, namely, CB₁ (CB₁R) and CB₂ (CB₂R), have been identified. On the basis of pharmacological evidence of cannabinoid action in CB₁R- and CB₂R-deficient mice, recent studies support the presence of yet uncloned cannabinoid receptors.³

The CB₁R appears to be responsible for most of the centrally mediated effects of cannabinoids.⁴ This receptor is concentrated in areas of the brain that control movement, coordination, sensory perception, learning and memory, reward and emotions, hormonal function, and body temperature.

The human CB_2R is a 360-amino-acid protein that shares 44% homology with the CB_1R .⁵ Initial studies revealed that this

receptor was expressed exclusively in peripheral tissues, particularly under pathological conditions. Specifically, CB₂R has been demonstrated in cells and tissues of the immune system, such as the marginal zone of the spleen.⁶ In addition, it has been recently recognized that CB₂R may play a functionally relevant role in the central nervous system. This role is mediated primarily through microglia, a resident population of cells related to macrophages.⁷ Intracellular CB₂-dependent signaling pathways include Gi/o-dependent inhibition of adenylyl cyclase, stimulation of mitogen-activated protein kinase and phosphoinositide 3-kinase pathways, and activation of de novo ceramide production or cyclooxygenase-2 induction.⁸

Over the past few years, the CB₂R has emerged as a critical target for regulation of inflammation,⁹ pain,¹⁰ bone loss,¹¹ dermatitis,¹² and more recently, liver pathophysiology.¹³ Furthermore, the activation of CB₂R has been proposed in the treatment of cancers of different origin.^{14,15} Finally, CB₂ selective agonists could be exploited for prevention of Alzheimer's disease by blocking β -amyloid peptide-induced activation of microglial cells¹⁶ and possibly by providing neuroprotection through blockade of microglial activation.¹⁷ For all these reasons, the development of potent, selective CB₂ molecules that are devoid of CB₁R mediated psychotropic side effects has gained interest as a new target in drug discovery.

We have previously reported the synthesis and binding activity of a series of 1,8-naphthyridin-4(1*H*)-on-3-carboxamide derivatives of general structure **A** (Figure 1).^{18–20} These compounds generally exhibited a remarkable CB₂ affinity, with a K_i value in the nanomolar range. This affinity was accompanied by a high selectivity with respect to CB₁R. Moreover, the [³⁵S]GTP γ binding assay and functional studies on human basophils indicated that these derivatives behaved as CB₁ and

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^{*a*} Abbreviations: ECS, endocannabinoid system; CB₁, cannabinoid subtype 1 receptor; CB₂, cannabinoid subtype 2 receptor; RT-PCR, reverse transcription polymerase chain reaction.



Figure 1. General structures of the 1,8-naphthyridin-4(1H)-on-3-carboxamide (**A**) and 1,8-naphthyridin-2(1H)-on-3-carboxamide (**B**) derivatives and structure of compound **1**.

CB₂ receptor agonists. The docking study of this class of cannabinoid ligands highlighted the ligand—receptor interactions that determined an increase in affinity and selectivity.²¹ In particular, this analysis suggested that the preservation of a good CB₂/CB₁ selectivity and the improvement of the CB₂ affinity seemed to require the presence of a lipophilic central core connected with a nonaromatic carboxamide group in position 3 capable of interacting in the CB₂R with the nonconserved residue F5.46(197) and a lipophilic substituent in position 1 with an H-bond acceptor atom, capable of interacting in the CB₂R with the nonconserved S3.31(112) and F5.46(197) has also been confirmed by site-directed mutagenesis data.^{5,21}

Following this binding hypothesis, we investigated the possible modifications of the 1,8-naphthyridin-4(1*H*)-one central scaffold, and the present paper describes the synthesis and the pharmacological properties of a set of 1,8-naphthyridin-2(1*H*)-on-3-carboxamide derivatives of general structure **B** (Figure 1). The aliphatic carboxamide groups in position 3 and the substituents in position 1 have been selected on the basis of the binding results obtained for the derivatives of general structure **A**.^{18–20}

The new compounds were tested in competitive binding assays toward both human recombinant CB_1 (h CB_1) and CB_2 (h CB_2) cannabinoid receptors expressed in CHO cells and were found to be selective for CB_2R . Furthermore, a functional study on human basophils^{22,23} was performed to determine the functionality of one of the most interesting newly synthesized CB_2R ligands. Finally, the same compound was evaluated for its in vitro effect as anticancer agent on human cell lines expressing CB_2Rs , i.e., Jurkat and U87MG cells.

Theoretical Studies

In order to investigate possible modifications of the 1,8naphthyridin-4(1*H*)-one central scaffold, the published compound *N*-cyclohexyl-1-(2-morpholin-4-ylethyl)-1,8-naphthyridin-4(1*H*)-on-3-carboxamide (**1**, see Figure 1) (CB₁R $K_i > 1000$ nM; CB₂R $K_i = 48.6$ nM)¹⁹ was chosen as a lead for the development of new derivatives. The superimposition between this reference compound and the *N*-cyclohexyl-1-(2-morpholin-4-ylethyl)-1,8naphthyridin-2(1*H*)-on-3-carboxamide (**2**) analogue encouraged further experimental studies. As shown in Figure 2, the superimposition between the two compounds highlighted a satisfactory overlap of the structural features deemed important for the CB₂ affinity, i.e., the lipophilic central core, the cyclohexyl, and the morpholine substituents. On the basis of these results, a series of 1,8-naphthyridin-2(1H)-on-3-carboxamide derivatives were synthesized.

Chemistry

The synthetic routes to obtain the target 1,8-naphthyridin-2(1H)-on-3-carboxamide derivatives **2–13** are depicted in Scheme 1. All substituents are summarized in Table 1.

The heating of 2-aminopyridin-3-carboxaldehyde with ethyl malonate and a few drops of piperidine at 90 °C for 20 h afforded the ethyl 1,8-naphthyridin-2(1*H*)-on-3-carboxylate derivative 14.²⁴ The reaction of ethyl ester 14 with the appropriate amine in microwave for 1 h (power 200 W, pressure 100 psi, stirring on) provided the desired carboxamide derivatives 15-19. N-Alkylation of 15-10 in anhydrous DMF with the suitable benzylchloride or arylalkyl chloride or 4-(2-chloroethyl)morpholine in the presence of NaH afforded the desired 1,8-naphthyridin-2-one derivatives 2-13.

The separation of *cis* and *trans* isomers of **12** and **13** was obtained by flash chromatography on a silica gel using hexane/ AcOEt/MeOH, 10:1:0.1, to obtain *cis*-**12**, *trans*-**12**, *cis*-**13**, and *trans*-**13**. The faster eluting peak was identified as the *cis* isomer and the slower peak as the corresponding *trans*-isomer based on ¹H NMR spectroscopy data.

The purity of each isomer was determined by analytical HPLC. We analyzed the mixture to evaluate the best analysis condition and the retention times of the isomers. For both isomers, 1.0 mg was dissolved in 1.0 mL of MeOH and an amount of 20 μ L was injected in analytical HPLC. The retention times for the *cis*-12 and *trans*-12 isomers were about 28 and 32 min, and for the *cis*-13 and *trans*-13 isomers they were about 46 and 52 min, respectively. Comparing the chromatograms, we were able to estimate the purity of each isomer that appeared >96% (see Figures S1 and S2 in the Supporting Information).

Results and Discussion

CB₁ and **CB**₂ Receptor Affinity. The binding affinities (K_i values) of compounds 2–13 for human recombinant CB₁ and CB₂ receptors are reported in Table 1. The K_i values of 5-(4-chloro-3-methylphenyl)-1-[(4-methylphenyl)methyl]-N-[(1*S*,4*R*,6*S*)-1,5,5-trimethyl-6-bicyclo[2.2.1]heptanyl]pyrazole-3-carboxamide (**20**, SR144528)²⁵ and (6a*R*,10a*R*)-3-(1,1-dimethylbutyl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6*H*-dibenzo[*b*,*d*]pyran (**21**, JWH133)²⁶ as reference compounds for both CB₁ and CB₂ cannabinoid receptors have also been included in Table 1.

Among the newly synthesized 1,8-naphthyridin-2(1*H*)-on-3carboxamide derivatives, compound **7** possessed a solubility that hindered its biological testing in DMSO/water solution during the cannabinoid receptor binding assay. With regard to the CB₁ affinity, the other 1,8-naphthyridin-2(1*H*)-on-3-carboxamide derivatives **2**-**6** and **8**-**13** have high variability of affinity, with K_i values in the range of 6947 nM (**8**) to 9.6 nM (**10**).

Concerning the CB₂R, the new compounds **2–6** and **8–13** proved to be high affinity CB₂R ligands with K_i values ranging from 0.7 nM (**10** and *cis*-**13**) to 69.8 nM (**8**). The presence of a fluorine atom para to the benzyl group in position 1 of the 1,8-naphthyridine nucleus increased affinity. This finding was in agreement with a previous report concerning 1,8-naphthyridin-4(1*H*)-on-3-carboxamide derivatives.^{18–20} The increased affinity was evident when comparing compounds **4**, **10**, **13**, *trans*-**13**, and *cis*-**13** to their corresponding benzyl derivatives **3**, **9**, **12**, *trans*-**12**, and *cis*-**12**. In particular, the (*p*-fluorobenzyl)-1,8-naphthyridine derivatives **10** and *cis*-**13** proved to be the ones with the highest CB₂R affinity in this series, with K_i values



Figure 2. 3D structure and solvent-excluded molecular surface of compound 1 (A), 2 (B), and their superimposition (C).

Scheme 1. Synthesis of the 1,8-Naphthyridin-2(1H)on-3-carboxamide Derivatives $2-13^{a}$



 a Reagents and conditions: (i) EMME, 120 °C, 20 h; (ii) $R_1 N H_2,$ microwave, 140 °C, 1 h 200 W; (iii) NaH, $R_2 C I.$

of 0.7 nM. Finally, the compounds with an ethylmorpholino group in position 1 exhibited CB₂R affinity higher (K_i values lower) than that of the corresponding benzyl derivatives and lower (K_i values higher) than that of the corresponding *p*-fluorobenzyl derivatives. The presence of the *n*-butyl substituent (**5**) in the same position reduces the affinity for CB₂R.

With regard to the structural modification in position 3 of the 1,8-naphthyridine nucleus, the presence of the cycloheptylamido or the 4-methylcyclohexylamido substituents led to compounds that exhibited the highest affinity. Furthermore, the 1,8-naphthyridin-2(1*H*)-on-3-carboxamide derivatives **12** and **13** showed a stereoselectivity for the binding to the CB₂R. In fact, *cis*-**12** and *cis*-**13** showed 7-fold and 13-fold increases in their affinity for the CB₂R when compared with the corresponding *trans*-**12** and *trans*-**13**, respectively. Finally, the substitution of the cycloheptylamido or the 4-methylcyclohexylamido with cyclohexylamido or 4-fluorophenethylamido substituents reduced the affinity.

With regard to the CB₂R selectivity, the 1,8-naphthyridin-2(1*H*)-on-3-carboxamide derivatives **2**–**6** and **8**–**13** generally showed good selectivity. In particular, **6**, **12**, *cis*-**12**, and *cis*-**13** exhibited very significant selectivity, with K_i (CB₁)/ K_i (CB₂) ratios greater than 200. In particular, compound **6**, with ethylmorpholino and carboxy-4-methylcyclohexylamide substituents Table 1. Radioligand Binding Data of Compounds $2-13^a$

			K _i (nM)		
compd	R_1	R_2	CB_1^{b}	$CB_2^{\ c}$	$\frac{K_{i}(CB_{1})}{K_{i}(CB_{2})}$
2	cyclohexyl	ethylmorpholino	560	7.9	71
3	cyclohexyl	benzyl	560	31	18
4	cyclohexyl	<i>p</i> -fluorobenzyl	56	2.2	25
5	cyclohexyl	<i>n</i> -butyl	5600	70	80
6	4-methylcyclohexyl	ethylmorpholino	1000	1.9	526
7	phenethyl	benzyl	NT	NT	
8	4-fluorophenethyl	benzyl	6947	69.8	100
9	cycloheptyl	benzyl	960	22	44
10	cycloheptyl	p-fluorobenzyl	9.6	0.7	14
11	cycloheptyl	ethylmorpholino	18	3	6
12	4-methylcyclohexyl	benzyl	1600	7.8	205
trans-12	4-methylcyclohexyl	benzyl	5255	39.8	132
cis-12	4-methylcyclohexyl	benzyl	1519	5.8	262
13	4-methylcyclohexyl	<i>p</i> -fluorobenzyl	200	0.9	222
trans-13	4-methylcyclohexyl	<i>p</i> -fluorobenzyl	300	9.0	33
cis-13	4-methylcyclohexyl	<i>p</i> -fluorobenzyl	200	0.7	286
20 ²⁵		· ·	437	0.6	728
21 ²⁶			677	3	226

^{*a*} Data represent mean values for at least three separate experiments performed in duplicate and are expressed as K_i (nM) for CB₁ and CB₂ binding assays. Values of standard error of the mean (SEM) are not shown for the sake of clarity and were never higher than 5% of the means. NT = not tested, as it is insoluble in the solvent normally used in binding assays. ^{*b*} Affinity of compounds for CB₁ receptor was evaluated using membranes from HEK-293 cells transfected with CB₁ receptor and [³H]CP55,940.

bound in the 1 and 3 positions of the 1,8-naphthyridin-2-one nucleus, showed a CB_1/CB_2 selectivity ratio of 526. In no case was the $K_i(CB_1)/K_i(CB_2)$ ratio lower than 1. Therefore, no compound exhibited a reversed selectivity.

CB₂ **Functional Activity.** The functional activity of compound **12** was assessed by using functional studies on human basophils. In fact, activation of CB₂ receptors is known to down-regulate the immunological activation of guinea pig mast cells and human basophils.^{22,23} Human basophils pretreated with *N*-(4-methylcyclohexyl)-1-benzyl-1,8-naphthyridin-2(1*H*)-on-3-carboxamide (**12**) (0.1–100 nM, 30 min of preincubation, 37 °C) showed a reduced expression of CD203c in response to immunological stimulation (anti-IgE, 1 μ g/mL, 30 min, 37 °C). CD203c is a glycosilated type II transmembrane molecule that belongs to the family of ectanucleotide pyrophosphatase/phosphodiesterase enzymes. It has recently been demonstrated as a specific activation marker of basophils that is rapidly up-regulated after allergen challenge.²⁷ The inhibition was reversed



Figure 3. Expression of CD203c by human basophils activated with anti-IgE (1 μ g/mL) is reduced by compound **12** in a dose-related fashion. The inhibitory effect of compound **12** on basophil CD203c expression is reverted by **20** (100 nM), a CB₂ antagonist, but not by **22** (100 nM), a CB₁ antagonist. The values are the mean \pm SEM of six independent experiments performed in triplicate: (**) *P* < 0.001 vs compound **12** plus **22**.



Figure 4. CB_2 and GAPDH mRNA levels were determined by RT-PCR on Jurkat and U87MG cell lines. On U87MG cells, CB_2 fragment was obtained after a further amplification of PCR product: (I) single amplification; (II) reamplification.

by the selective CB₂ antagonist **20** (100 nM, 30 min of preincubation, 37 °C) but not by the selective CB₁ antagonist 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-piperidin-1-ylpyrazole-3-carboxamide (**22**, AM251)²⁸ (100 nM, 30 min of preincubation, 37 °C) (see Figure 3). These results suggest that the derivative **12** exerts a CB₂-mediated inhibitory action on immunological human basophil activation.

Cytotoxicity Assay. The derivative 12 was also evaluated for its anticancer activity on the human T cell leukemia line Jurkat and the human glioblastoma cell line U87MG. Both lines were obtained from the ICLC (Interlab Cell Line Collection) cell bank (Genoa, Italy). Compound 12 was evaluated in parallel with the known selective CB₂R agonist (2-methyl-1-propyl-1Hindol-3-yl)-1-naphthalenylmethanone (23, JWH015).²⁹ Jurkat cells are a cell line with a good level of CB₂R expression (Figure 4). Exposure to 12 in the range $0.1-10 \,\mu\text{M}$ for 48 h induced a concentration-dependent decrease in cell viability. A similar behavior was observed with the known CB₂ agonist 23, whose cytotoxicity matched that previously reported.²⁹ The 1,8naphthyridin-2(1H)-on-3-carboxamide derivatives 12 and 23 showed IC₅₀ values that were not statistically different (Table 2). In addition, 1 µM [6-iodo-2-methyl-1-(2-morpholin-4ylethyl)indol-3-yl]-(4-methoxyphenyl)methanone (24, AM630)³⁰ significantly antagonized the cytotoxic effect of 12 on Jurkat cells (Figure 5), confirming its CB₂-mediated mechanism.

On U87MG cells, which express CB_2R at lower levels (a PCR electrophoretic band was evident only after reamplification)

Table 2. Cytotoxic Activity of **12** and **23** on Jurkat and U87MG Cell Lines^{*a*}

	IC ₅₀ (µ)	IC ₅₀ (µM)		
compd	Jurkat	U87MG		
12	5.98 ± 2.78	NR		
23	9.60 ± 4.12	NR		

 a Data are the mean \pm SE from three experiments carried out in triplicate. NR: not reached.



Figure 5. Effect of **12** (10 μ M) and combination of **12/24** (1 μ M) on Jurkat cell viability. Data are the mean \pm SE of two experiments carried out in triplicate.

(Figure 4), **12** and **23** only induced a poor effect (about 20% of cell viability inhibition) and only at the highest dose used (10 μ M).

Conclusions

In our study, we investigated possible modifications of the 1,8-naphthyridin-4(1*H*)-one central scaffold in order to improve CB₂R affinity and selectivity. On the basis of theoretical studies, a series of 1,8-naphthyridin-2(1*H*)-on-3-carboxamides was designed, synthesized, and tested on CB₁R and CB₂R. The new compounds showed CB₂R affinity and selectivity that were higher than corresponding 1,8-naphthyridin-4(1*H*)-on-3-carboxamide derivatives previously studied¹⁹ and, in some cases (i.e., compound **6**), among the highest ever obtained for CB₂R ligands. In particular, the (*p*-fluorobenzyl)-1,8-naphthyridine derivatives **10** and *cis*-**13** exhibited the highest affinity, with *K*_i values of 0.7 nM. The derivatives **6**, **12**, *cis*-**12**, **13**, and *cis*-**13** showed very remarkable selectivity with a *K*_i(CB₁)/*K*_i(CB₂) ratio >200.

Furthermore, the concentration-dependent activity shown by derivative 12 in human basophils and in Jurkat cells strongly suggests that this compound and the other 1,8-naphthyridin-2(1H)-on-3-carboxamides possess agonist properties at CB₂R.

These results indicate that 1,8-naphthyridin-2(1*H*)-on-3carboxamides represent new scaffolds that may be suitable for the development of new potent and selective CB_2R agonists to be used in several therapeutic areas.

Experimental Section

Chemistry. Melting points were determined on a Kofler hot stage apparatus and are uncorrected. IR spectra in Nujol mulls were recorded on an ATI Mattson Genesis series FTIR spectrometer. ¹H NMR spectra were recorded with a Bruker AC-200 spectrometer in δ units with TMS as an internal standard. Microwave-assisted reactions were run in a CEM microwave synthesizer. Mass spectra were performed with a Hewlett-Packard MS system 5988. Elemental analyses (C, H, N) were within $\pm 0.4\%$ of theoretical values and were performed on a Carlo Erba elemental analyzer model 1106 apparatus.

The analytical HPLC system consisted of a Thermo Finnigan SpectraSystem SN4000 system controller, coupled to a P2000 pump, a SCM1000 degasser, and a UV2000 UV detector at an operation wavelength of 280 nm (Thermo Finnigan, Waltham, MA). Separation was performed on a 150 mm \times 4.6 mm ChromoSep column packed with 5 μm C18 particles, preceded by a ChromSep guard column Inertsil 5 ODS-3 (Varian). The mobile phase, delivered at a flow rate of 1.0 mL/min, consisted of methanol and water (75:25, v/v). HPLC grade methanol was acquired from Sigma-Aldrich (Sydney, Australia), and the water used was of Milli-Q grade purified by a Milli-Q UV purification system (Millipore Corporation, MA).

1,8-Naphthyridin-2(1*H***)-on-3-carboxylic Acid Ethyl Ester (14).²⁴ A solution of 1.0 g (8.19 mmol) of 2-aminopyridin-3-carboxaldehyde, 1.85 mL of diethyl malonate (12.18 mmol), and few drops of piperidine was heated at 120 °C for 20 h. After the mixture was cooled, the precipitate formed was treated with ethyl ether and crystallized from ethyl acetate to obtain 14 (1.26 g, 76%) mp 182–185 °C; ¹H NMR (DMSO) \delta 8.60 (dd, 1H, H₇), 8.50 (s, 1H, H₄), 8.26 (dd, 1H, H₅), 7.29 (m, 1H, H₆), 4.37 (q, 2H, CH₂), 1.29 (t, 3H, CH₃).**

General Procedure for the Preparation of N₁-Substituted 1,8-Naphthyridin-2(1*H*)-on-3-carboxamides 15–19. A mixture of 1 mmol of ethyl ester 14 and 10 mmol of the appropriate amine was heated in microwave at 140 °C for 1 h (power 200 W, pressure 100 psi, stirring on). After cooling, the reaction mixture was treated with ethyl ether to give a solid residue which was collected by filtration and purified by crystallization.

N-Cyclohexyl-1,8-naphthyridin-2(1*H*)-on-3-carboxamide (15). Yield 88%; mp 278–280 °C (crystallized from ethyl acetate); ¹H NMR (DMSO) δ 12.03 (br,1H, NH), 9.70 (d, 1H, NH), 8.85 (s, 1H, H₄), 8.66 (dd, 1H, H₇), 8.42 (dd, 1H, H₅), 7.36 (m, 1H, H₆), 3.85 (m, 1H, CH), 1.89–1.27 (m, 10H, cyclohexyl). Anal. (C₁₅H₁₇N₃O₂) C, H, N.

N-(4-Methylcyclohexyl)-1,8-naphthyridin-2(1*H*)-on-3-carboxamide (16). Yield 87%; crystallized from ethyl acetate; ¹H NMR (DMSO) δ 11.85 (br,1H, NH), 10.0 and 9.60 (2d, 1H, NH), 8.85 and 8.87 (2s, 1H, H₄), 8.66 (dd, 1H, H₇), 8.41 (dd, 1H, H₅), 7.36 (m, 1H, H₆), 4.11 and 3.85 (2m, 1H, CH), 2.00–1.05 (m, 9H, cyclohexyl), 0.92 and 0.89 (2d, 3H, CH₃). Anal. (C₁₆H₁₉N₃O₂) C, H, N.

N-Cycloheptyl-1,8-naphthyridin-2(1*H*)-on-3-carboxamide (17). Yield 71%; mp 203–204 °C (crystallized from dichloromethane); ¹H NMR (DMSO) δ 11.95 (br,1H, NH), 9.75 (d, 1H, NH), 8.84 (s, 1H, H₄), 8.65 (dd, 1H, H₇), 8.40 (dd, 1H, H₅), 7.35 (m, 1H, H₆), 4.10 (m, 1H, CH), 1.90–1.45 (m, 12H, cycloheptyl). Anal. (C₁₆H₁₉N₃O₂) C, H, N.

N-Phenethyl-1,8-naphthyridin-2(1*H*)-on-3-carboxamide (18). Yield 87%; mp 270–272 °C (crystallized from ethyl acetate); ¹H NMR (DMSO) δ 12.15 (br,1H, NH), 9.71 (t, 1H, NH), 8.86 (s, 1H, H₄), 8.65 (dd, 1H, H₇), 8.42 (dd, 1H, H₅), 7.32 (m, 6H, H₆ + Ar), 3.59 (m, 2H, CH₂), 2.85 (t, 2H, CH₂). Anal. (C₁₇H₁₅N₃O₂) C, H, N.

N-(4-Fluorophenethyl)-1,8-naphthyridin-2(1*H*)-on-3-carboxamide (19). Yield 84%; mp >300 °C (crystallized from ethanol); ¹H NMR (DMSO) δ 11.15 (br,1H, NH), 9.68 (t, 1H, NH), 8.85 (s, 1H, H₄), 8.65 (dd, 1H, H₇), 8.40 (dd, 1H, H₅), 7.33 (m, 3H, H₆ + Ar), 7.12 (m, 2H, Ar), 3.58 (m, 2H, CH₂), 2.85 (t, 2H, CH₂). Anal. (C₁₇H₁₄FN₃O₂) C, H, N.

General Procedure for the Synthesis of N₁-Substituted 1,8-Naphthyridin-2(1*H*)-on-3-carboxamides 2–13. NaH (0.10 g, 2.00 mmol, 50% in mineral oil) was added to a solution of suitable 1,8-naphthyridin-2(1*H*)-on-3-carboxamide derivatives 15-19 (0.81 mmol) in 6.5 mL of dry DMF. After 1 h, a suitable chloride (0.81 mmol) was added and the mixture was stirred for 24 h at room temperature for compounds 2–5, 7–9, 12, and 13 or at 50 °C for compounds 6 and 10 or at 70 °C for compound 11. After the mixture was collected by filtration and was purified by crystallization.

N-Cyclohexyl-1-(2-morpholin-4-ylethyl)-1,8-naphthyridin-2(1*H*)-on-3-carboxamide (2). Yield 56%; mp 148–151 °C (crystallized from hexane); MS *m/z* 384 (M⁺); ¹H NMR (DMSO) δ 9.75 (d, 1H, NH), 8.88 (s, 1H, H₄), 8.78 (dd, 1H, H₇), 8.50 (dd, 1H, H₅), 7.45 (m, 1H, H₆), 4.64 (t, 2H, CH₂), 3.90 (m, 1H, CH), 3.51 (m, 4H, morpholine), 2.57 (m, 6H, morpholine + CH₂), 1.89–1.22 (m, 10H, cyclohexyl). Anal. (C₂₁H₂₈N₄O₃) C, H, N. *N*-Cyclohexyl-1-benzyl-1,8-naphthyridin-2(1*H*)-on-3-carboxamide (3). Yield 61%; mp 154–156 °C (crystallized from hexane); MS *m*/z 361 (M⁺); ¹H NMR (DMSO) δ 9.58 (d, 1H, NH), 8.96 (s, 1H, H₄), 8.76 (dd, 1H, H₇), 8.54 (dd, 1H, H₅), 7.45 (m, 1H, H₆), 7.24 (m, 5H, Ar), 5.72 (s, 2H, CH₂), 3.85 (m, 1H, CH), 1.89–1.30 (m, 10H, cyclohexyl). Anal. (C₂₂H₂₃N₃O₂) C, H, N.

N-Cyclohexyl-1-(*p*-fluorobenzyl)-1,8-naphthyridin-2(1*H*)-on-3-carboxamide (4). Yield 8.44%; mp 203–205 °C (crystallized from hexane); MS *m*/z 379 (M⁺); ¹H NMR (DMSO) δ 9.56 (d,1H, NH) 8.95 (s, 1H, H₄), 8.77 (dd, 1H, H₇), 8.53 (dd, 1H, H₅), 7.47 (m, 1H, H₆), 7.31 (m, 2H, Ar), 7.10 (m, 2H, Ar), 5.68 (s, 2H, CH₂), 3.80 (m, 1H, CH), 1.88–1.22 (m, 10H, cyclohexyl). Anal. (C₂₂H₂₂FN₃O₂) C, H, N.

N-Cyclohexyl-1-butyl-1,8-naphthyridin-2(1*H*)-on-3-carboxamide (5). Yield 55%; mp 152–154 °C (crystallized from hexane); MS *m*/z 327 (M⁺); ¹H NMR (DMSO) δ 9.69 (d, 1H, NH), 8.88 (s, 1H, H₄), 8.79 (dd, 1H, H₇), 8.49 (dd, 1H, H₅), 7.45 (m, 1H, H₆), 4.47 (t, 2H, CH₂), 3.82 (m, 1H, CH), 1.89–0.88 (m, 17H, cyclohexyl + 2CH₂ + CH₃). Anal. (C₁₉H₂₅N₃O₂) C, H, N.

N-(4-Methylcyclohexyl)-1-(2-morpholin-4-ylethyl)-1,8-naphthyridin-2(1*H*)-on-3-carboxamide (6). Yield 33%; crystallized from ethyl acetate; MS *m*/*z* 398 (M⁺); ¹H NMR (DMSO) δ 9.90 and 9.52 (2d, 1H, NH), 8.90 (s, 1H, H₄), 8.79 (dd, 1H, H₇), 8.50 (dd, 1H, H₅), 7.45 (m, 1H, H₆), 4.65 (t, 2H, CH₂), 4.00 and 3.75 (2m, 1H, CH), 3.51 (m, 4H, morpholine), 2.56 (m, 6H, morpholine + CH₂), 1.90–0.97 (m, 9H, cyclohexyl), 0.92 and 0.88 (2d, 3H, CH₃). Anal. (C₂₂H₃₀N₄O₃) C, H, N.

N-(β-Phenylethyl)-1-benzyl-1,8-naphthyridin-2(1*H*)-on-3-carboxamide (7). Yield 98%; mp 220–222 °C (crystallized from ethyl acetate); MS *m*/*z* 383 (M⁺); ¹H NMR (DMSO) δ 9.64 (t, 1H, NH), 8.96 (s, 1H, H₄), 8.77 (dd, 1H, H₇), 8.53 (dd, 1H, H₅), 7.47 (m, 1H, H₆), 7.23 (m, 10H, Ar), 5.72 (s, 2H, CH₂), 3.59 (m, 2H, CH₂), 2.86 (t, 2H, CH₂). Anal. (C₂₄H₂₁N₃O₂) C, H, N.

N-(4-Fluorophenylethyl)-1-benzyl-1,8-naphthyridin-2(1*H*)-on-3-carboxamide (8). Yield 87%; mp 183–185 °C (crystallized from ethyl acetate); MS m/z 401 (M⁺); ¹H NMR (DMSO) δ 9.72 (brs, 1H, NH), 8.93 (s, 1H, H₄), 8.78 (dd, 1H, H₇), 8.43 (dd, 1H, H₅), 7.36 (m, 8H, Ar + H₆), 7.07 (m, 2H, Ar), 5.82 (s, 2H, CH₂) 3.69 (m, 2H, CH₂), 2.95 (t, 2H, CH₂). Anal. (C₂₄H₂₀FN₃O₂) C, H, N.

N-Cycloheptyl-1-benzyl-1,8-naphthyridin-2(1*H*)-on-3-carboxamide (9). Yield 88%; mp 171–173 °C (crystallized from ethanol); MS *m*/z 375 (M⁺); ¹H NMR (DMSO) δ 9.62 (d, 1H, NH), 8.95 (s, 1H, H₄), 8.76 (dd, 1H, H₇), 8.53 (dd, 1H, H₅), 7.46 (m, 1H, H₆), 7.25 (m, 5H, Ar), 5.72 (s, 2H, CH₂), 4.09 (m, 1H, CH), 1.90–0.85 (m, 12H, cycloheptyl). Anal. (C₂₃H₂₅N₃O₂) C, H, N.

N-Cycloheptyl-1-(*p*-fluorobenzyl)-1,8-naphthyridin-2(1*H*)-on-3-carboxamide (10). Yield 97.%; mp 161–163 °C (crystallized from ethanol); MS *m*/*z* 393 (M⁺); ¹H NMR (DMSO) δ 9.78 (d, 1H, NH), 8.88 (s, 1H, H₄), 8.75 (dd, 1H, H₇), 8.49 (dd, 1H, H₅), 7.47 (m, 1H, H₆), 7.32 (m, 2H, Ar), 7.05 (m, 2H, Ar), 5.78 (s, 2H, CH₂), 4.10 (m, 1H, CH), 1.88–1.01 (m, 12H, cycloheptyl). Anal. (C₂₃H₂₄FN₃O₂) C, H, N.

N-Cycloheptyl-1-(2-morpholin-4-ylethyl)-1,8-naphthyridin-2(1*H*)-on-3-carboxamide (11). Yield 64%; mp 154–158 °C (crystallized from hexane); MS *m*/z 398 (M⁺); ¹H NMR (CDCl₃) δ 9.77 (d, 1H, NH), 8.88 (s, 1H, H₄), 8.68 (dd, 1H, H₇), 8.08 (dd, 1H, H₅), 7.28 (dd, 1H, H₆), 4.78 (t, 2H, CH₂), 4.10 (m, 1H, CH), 3.70 (m, 4H, morpholine), 2.72 (m, 6H, morpholine + CH₂), 2.05–0.85 (m, 12H, cycloheptyl). Anal. (C₂₂H₃₀N₄O₃) C, H, N.

N-(4-Methylcyclohexyl)-1-benzyl-1,8-naphthyridin-2(1*H*)-on-3-carboxamide (12). Yield 75%; crystallized from hexane; MS m/z 375 (M⁺); ¹H NMR (DMSO) δ 9.84 and 9.47 (2d, 1H, NH), 8.97 (s, 1H, H₄), 8.77 (dd, 1H, H₇), 8.53 (dd, 1H, H₅), 7.46 (m, 1H, H₆), 7.30 (m, 5H, Ar), 5.73 and 5.78 (2s, 2H, CH₂), 4.10 and 3.75 (2m, 1H, CH), 1.94–0.99 (m, 9H, cyclohexyl), 0.94 and 0.89 (2d, 3H, CH₃). Anal. (C₂₃H₂₅N₃O₂) C, H, N.

trans-N-(4-Methylcyclohexyl)-1-benzyl-1,8-naphthyridin-2(1*H*)on-3-carboxamide (*trans*-12) and *cis-N*-(4-Methylcyclohexyl)-1-benzyl-1,8-naphthyridin-2(1*H*)-on-3-carboxamide (*cis*-12). Compounds *trans*-12 and *cis*-12 were obtained from derivative 12 by flash chromatography on a silica gel using hexane/AcOEt/MeOH, 10:1:0.1. *trans*-**12**: yield 25%; mp 152–154 °C (crystallized from hexane); MS *m*/z 375 (M⁺); ¹H NMR (DMSO) δ 9.47 (d, 1H, NH), 8.97 (s, 1H, H₄), 8.77 (dd, 1H, H₇), 8.53 (dd, 1H, H₅), 7.46 (m, 1H, H₆), 7.30 (m, 5H, Ar), 5.73, (s, 2H, CH₂), 3.75 (m, 1H, CH), 1.94–0.99 (m, 9H, cyclohexyl), 0.89 (d, 3H, CH₃). Anal. (C₂₃H₂₅N₃O₂) C, H, N. *cis*-**12**: yield 20%; mp 157–159 °C (crystallized from hexane); MS *m*/z 375 (M⁺); ¹H NMR (DMSO) δ 9.84 (d, 1H, NH), 8.97 (s, 1H, H₄), 8.77 (dd, 1H, H₇), 8.53 (dd, 1H, H₃), 7.46 (m, 1H, H₆), 7.30 (m, 5H, Ar), 5.78 (s, 2H, CH₂), 4.10 (m, 1H, CH), 1.94–0.99 (m, 9H, cyclohexyl), 0.94 (d, 3H, CH₃). Anal. (C₂₃H₂₅N₃O₂) C, H, N.

N-(4-Methylcyclohexyl)-1-(*p*-fluorobenzyl)-1,8-naphthyridin-2(1*H*)-on-3-carboxamide (13). Yield 80%; crystallized from hexane; MS *m*/*z* 393 (M⁺); ¹H NMR (DMSO) δ 9.85 and 9.43 (2d, 1H, NH), 8.95 (s, 1H, H₄), 8.77 (dd, 1H, H₇), 8.52 (dd, 1H, H₅), 7.47 (dd, 1H, H₆), 7.31 (m, 2H, Ar), 7.10 (m, 2H, Ar), 5.72 and 5.69 (2s, 2H, CH₂), 4.10 and 3.70 (2m, 1H, CH), 1.94–0.99 (m, 9H, cyclohexyl), 0.92 and 0.88 (2d, 3H, CH₃). Anal. (C₂₃H₂₄FN₃O₂) C, H, N.

trans-N-(4-Methylcyclohexyl)-1-(p-fluorobenzyl)-1,8-naphthyridin-2(1H)-on-3-carboxamide (trans-13) and cis-N-(4-Methylcyclohexyl)-1-(p-fluorobenzyl)-1,8-naphthyridin-2(1H)-on-3carboxamide (cis-13). Compounds trans-13 and cis-13 were obtained from derivative 13 by flash chromatography on a silica gel using hexane/AcOEt/MeOH, 10:1:0.1. trans-13: yield 22%; mp 161–163 °C (crystallized from hexane); MS m/z 393 (M⁺); ¹H NMR (DMSO) δ 9.43 (d, 1H, NH), 8.95 (s, 1H, H₄), 8.77 (dd, 1H, H₇), 8.52 (dd, 1H, H₅), 7.47 (m, 1H, H₆), 7.31 (m, 2H, Ar), 7.10 (m, 2H, Ar) 5.69 (s, 2H, CH₂), 3.70 (2m, 1H, CH), 1.94–0.99 (m, 9H, cyclohexyl), 0.88 (d, 3H, CH₃). Anal. (C₂₃H₂₄FN₃O₂) C, H, N. cis-13: yield 18%; mp 170-172 °C (crystallized from hexane); MS *m*/*z* 393 (M⁺); ¹H NMR (DMSO) δ 9.85 (d, 1H, NH), 8.95 (s, 1H, H₄), 8.77 (dd, 1H, H₇), 8.52 (dd, 1H, H₅), 7.47 (m, 1H, H₆), 7.31 (m, 2H, Ar), 7.10 (m, 2H, Ar) 5.72 (s, 2H, CH₂), 4.10 (2m, 1H, CH), 1.94-0.99 (m, 9H, cyclohexyl), 0.92 (d, 3H, CH₃). Anal. (C₂₃H₂₄FN₃O₂) C, H, N.

CB1 and CB2 Receptor Binding Assays. The new compounds were evaluated in CB₁R and CB₂R binding assays using membranes from HEK-293 cells transfected with cDNAs encoding the human recombinant CB_1R ($B_{max} = 2.5$ pmol/mg protein) and human recombinant CB₂R ($B_{\text{max}} = 4.7$ pmol/mg protein) (Perkin-Elmer, Italy). These membranes were incubated with $[^{3}H]$ -(-)-*cis*-3-[2hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol (25, $[^{3}H]CP55,940)^{31}$ (0.14 nM/ K_{d} = 0.18 nM and $0.084 \text{ nM}/K_d = 0.31 \text{ nM}$ for CB₁ and CB₂ receptors, respectively) as the high affinity ligand and displaced with 100 nM [(3R)-5methyl-3-(morpholin-4-ylmethyl)-2,3-dihydro[1,4]oxazino[2,3,4-hi]indol-6-yl](2-naphthyl)methanone (26, WIN55212-2)³² as the heterologous competitor for nonspecific binding (K_i values 9.2 and 2.1 nM, respectively, for CB₁R and CB₂R). All compounds were tested following the procedure described by the cell membrane manufacturer.³³ Displacement curves were generated by incubating drugs with 25 for 90 min at 30 °C. K_i values were calculated by applying the Cheng-Prusoff equation³⁴ to the IC_{50} values (obtained by GraphPad) for the displacement of the bound radioligand by increasing concentrations of the test compound. Data are the mean \pm SEM of at least n = 3 experiments.

Preparation of Basophil-Rich Leukocyte Samples. Twenty healthy donors were recruited in the transfusion unit of Careggi General Hospital (Florence, Italy). The subjects did not suffer from allergic diseases and had not taken any drug during the previous 4 weeks. They gave explicit informed consent to their enrollment in this study. About 400 mL of venous blood was collected from each subject, and 64 mL of a citrate solution (CPD) was added as an anticoagulant. The blood was centrifuged at 3500 rpm (11 min, 20 °C) in a slow-stop centrifuge (Sorvall RC 12 BP, Kendro Laboratory Products). Plasma was removed by an automatic press (NPBI Compomat 64). After 24 h of gentle stirring in a platelet incubator (Helmer) at 22 °C to reduce cell stress, the buffy coat was centrifuged at 900 rpm (9 min, 20 °C). Platelet-rich plasma was removed by the same automatic press. A volume of 30 mL of the

residual leukocyte-rich preparation was diluted 1:4 with a buffer with the following composition: 20 mM HEPES, 130 mM NaCl, 5 mM KCl, 5 IU/mL sodium heparin, and 1.5 mg/mL bovine serum albumin (BSA), at a pH of 7.4 (washing buffer). Aliquots of 10 mL were then carefully layered over 10 mL of Ficoll-Paque in 30 mL conical tubes (25 mm diameter) and centrifuged at room temperature at 420g. After removal of the supernatant plasma, the basophil-rich Ficoll-Paque layer was separated and the neutrophilrich buffy coat was discarded. The suspension was washed twice with the washing buffer and centrifuged at 200g at 20 °C for 10 min. The pellets were then resuspended in a calcium-free maintenance buffer composed of 20 mM HEPES, 130 mM NaCl, 5 mM KCl, 5 mM Na₃EDTA, and 1.5 mg/mL bovine serum albumin (BSA) at a pH of 7.4. The pellets were then further processed as described below. Upon isolation, cell viability (determined by trypan blue exclusion) was always greater than 95%. The procedure used resulted in the low handling of basophils. Low handling prevented their aspecific activation, which could have occurred if highpurifying procedures such as specific antibody-coated magnetic beads had been used. Before the experiments were started, samples from each basophil-rich leukocyte preparation were challenged for their ability to respond to anti-IgE by flow cytometric assay (described below). Poorly responsive preparations were discarded.

Flow Cytometric Analysis. Basophil-rich leukocyte pellets were labeled with a saturating concentration of anti-IgE fluoresceine isothiocyanate (FITC) conjugated antibodies and anti-CD203c phycoerythrin (PE) conjugated antibodies. The fluorescent antibodies were incubated with the pellets for 20 min at 4 °C. The cells were then washed with buffer, centrifuged at 200g for 10 min at room temperature, and resuspended in buffer. After the lysis of residual erythrocytes, the leukocyte suspensions were analyzed by a flow cytometer (Coulter XL, Coulter Cytometry, Hialeah, FL). Because the separation technique provides a leukocyte preparation with no more than 70% basophils, it was necessary to sort the basophil-related events using appropriate electronic gates. Basophils were recognized by their high expression of membrane-bound IgE resulting in a high signal related to FITC fluorescence (emission peak at 530 nm). IgE-negative cells were then gated out by electronic subtraction. The fluorescent signal of PE (emission peak at 575 nm) was used to characterize activated and nonactivated cells. Before activation human basophils showed a low expression of CD203c, which was strongly up-regulated after the activation of the cells.

Cytotoxicity Assay. Jurkat cells were maintained in RPMI 1640 medium supplemented with L-glutamine (2 mM), 15% fetal bovine serum, and 1% of a 1:1 mixture of penicillin (50 IU/mL) and streptomycin (50 μ g/mL) (Roche Molecular Biochemicals, Milan, Italy). U87MG cells were maintained in DMEM medium supplemented with 10% fetal bovine serum, 1% of a 1:1 mixture of penicillin (50 IU/mL) and streptomycin (50 μ g/mL) (Roche Molecular Biochemicals, Milan, Italy). U87MG cells were maintained in DMEM medium supplemented with 10% fetal bovine serum, 1% of a 1:1 mixture of penicillin (50 IU/mL) and streptomycin (50 μ g/mL) (Roche Molecular Biochemicals, Milan, Italy). Exponentially growing Jurkat (2 × 10⁴) and U87MG cells (4 × 10³) were seeded into 96-well plates in serum-free medium to avoid interactions of serum proteins and cannabinoid molecules (Z. M. Zheng et al., 1993). After 2 h of incubation (for in suspension Jurkat cells) and 24 h of incubation (for adherent U87MG cells), they were exposed to compound **12** or to the known selective CB₂R agonist **23**.

Compounds **12** (10 mM), **23** (10 mM), and **24** (1 mM) were dissolved in DMSO. Mother solutions were diluted to working concentrations with the appropriate medium (without serum). Cell viability was measured using a method based on the cleavage of the 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) to formazan by mitochondrial dehydrogenase activity (cell proliferation reagent WST-1; Roche, Milan, Italy). Following drug exposure, WST-1 was added to each well. After 60 min of incubation at 37 °C, the absorbance at 450 nm was measured by a microplate reader (Wallac Victor II, Perkin-Elmer, MA). Inhibition of cell viability was calculated after 48 h of drug exposure, by comparing the number of viable cells after treatment to the number of viable cells exposed to solvent alone (controls). The IC₅₀ value represents the drug concentration at which

the absorbance subtracted from the relative blank (drug/solvent in medium w/o cells) is 50% of that in controls. All experiments were performed in triplicate, and results are expressed as the mean \pm SE. Statistical difference among IC₅₀ values was evaluated by means of ANOVA analysis of variance and Bonferroni post-test. A *p*-value less than 0.05 was taken to be significant.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) Analysis of CB₂R Expression. To confirm the expression of CB₂R in the specific clones of both Jurkat and U87MG cell lines used in this study, we performed an RT-PCR analysis. RNA was isolated from (5×10^6) Jurkat and (2×10^6) U87MG cell lines using the "SV Total RNA Isolation System" kit (Promega S.r.l.; Milan, Italy). cDNAs were subsequently obtained from 1 μ g of total RNA using the "Quantitect Reverse Transcription" kit (Qiagen; Milan, Italy). A DNase digestion step was included.

CB₂ forward (5'-CTGGCTCCTGTTCATCGCCT-3') and CB₂ reverse (5'-GCTTCTTCTTTGCCTCTGAC-3') primers yielding a 417 bp product (705–1121 position in Genbank accession NM_001841.1) were used. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an expression standard and was amplified with forward (5'-GTGAAGGTCGGTGTCAACG-3') and reverse (5'-GGTGAAGACGCCAGTAGACTC-3') primers. This process yielded a 300 bp product (85–384 position in Genbank accession NM_002046.2).

PCR reactions were performed using the following parameters: for CB₂, 95 °C 15' (one cycle), 95 °C 1', 58.4 °C 1', 72 °C 1' (35 cycles), and 72 °C 10' (one cycle); for GAPDH, 95 °C 15' (one cycle), 95 °C 1', 55 °C 2', 74 °C 1' (36 cycles), and 74 °C 9' (one cycle). The "HotStarTaq Master Mix" kit (Qiagen; Milan, Italy) was used for all PCR reactions. All protocols were carried out in a MyCycler thermal cycler (Bio-Rad, Milan, Italy), and PCR products were run on ethidium bromide-stained 1% agarose gel. Gel images were acquired under UV (Euroclone SpA, Siziano PV, Italy) transillumination. The identity of PCR-products was confirmed by sequencing.

Ligands Superposition. The ligands were built by means of Maestro³⁵ and were then minimized in a water environment (using the generalized-Born/surface-area model) by means of Macromodel.³⁶ They were minimized using the conjugate gradient, the MMFFs force field,³⁷ and a distance-dependent dielectric constant of 1.0 until they reached a convergence value of 0.05 kcal/(Å·mol). The superimposition of the two compounds was developed using the ROCS 2.2 software,³⁸ which is a shape-similarity method based on the Tanimoto-like overlap of volumes. The alignment was developed using the combo score, which combines the Tanimoto shape score with the color score for the appropriate overlap of groups with similar properties (donor, acceptor, hydrophobe, cation, anion, and ring). All the other parameters were used as ROCS default values. For compound 2, the number of conformations was about 800, generated using Omega 2.1.0.39 Default parameters were used with the following exceptions: the rms parameter was set to 0.2 and the intramolecular H bond²¹ was maintained activating the fixfile parameter. The result of the docking study for compound 1 into the CB₂ receptor model¹⁹ was used as a reference structure for the superimposition.

Amino Acid Numbering. In referring to specific amino acids, both the sequence number (in parentheses) and the numbering system proposed by Ballesteros and Weinstein⁴⁰ were employed. For the latter, the most highly conserved residue in each TM helix (TMH) was assigned a value of 0.50, and this number was preceded by the TMH number. The other residues in the helix were given a locant value relative to this.

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