# 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

Clementina Manera,[†] Giuseppe Saccomanni,*[,†] Barbara Adinolfi,[‡] Veronica Benetti,[†] Alessia Ligresti,[§] Maria Grazia Cascio,[§] Tiziano Tuccinardi,*[,†,||] Valentina Lucchesi,[†] Adriano Martinelli,[†] Paola Nieri,[‡] Emanuela Masini,[⊥] Vincenzo Di Marzo,[§] and Pier Luigi Ferrarini[†]

*Dipartimento di Scienze Farmaceutiche and Dipartimento di Psichiatria, Neurobiologia, Farmacologia e Biotecnologie, Università di Pisa, Via Bonanno 6, 56126 Pisa, Italy, Dipartimento di Farmacologia Preclinica e Clinica, Università di Firenze, Viale G. Pieraccini 6, 50139 Firenze, Italy, Endocannabinoid Research Group, Istituto di Chimica Biomolecolare, Consiglio Nazionale delle Ricerche, Via Campi Flegrei 34, 80078 Pozzuoli, Napoli, Italy, and Sbarro Institute for Cancer Research and Molecular Medicine, Center for Biotechnology, College of Science and Technology, Temple University, Philadelphia, Pennsylvania 19122*

*Received December 10, 2008*

The $CB_2$ 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(1*H*)-on-3-carboxamide derivatives, new 1,8-naphthyridin-2(1*H*)-on-3-carboxamide derivatives were designed, synthesized, and tested for their affinities toward the human $CB_1$ and $CB_2$ cannabinoid receptors. Some of the reported compounds showed a subnanomolar $CB_2$ affinity with a $CB_1/CB_2$ 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 $CB_2$-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(1*H*)-on-3-carboxamides represent a new scaffold very suitable for the development of new promising $CB_2$ 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, $CB_1$ ($CB_1R$) and $CB_2$ ($CB_2R$), have been identified. On the basis of pharmacological evidence of cannabinoid action in $CB_1R$- and $CB_2R$-deficient mice, recent studies support the presence of yet uncloned cannabinoid receptors.[3]

The $CB_1R$ 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, $CB_2R$ 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 $CB_2R$ 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 $CB_2$-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 $CB_2R$ 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 $CB_2R$ has been proposed in the treatment of cancers of different origin.[14,15] Finally, $CB_2$ selective agonists could be exploited for prevention of Alzheimer's disease by blocking $\beta$-amyloid peptide-induced activation of microglial cells[16] and possibly by providing neuroprotection through blockade of microglial activation.[17] For all these reasons, the development of potent, selective $CB_2$ molecules that are devoid of $CB_1R$ 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 $CB_2$ affinity, with a $K_i$ value in the nanomolar range. This affinity was accompanied by a high selectivity with respect to $CB_1R$. Moreover, the [$^{35}$S]GTP$\gamma$ binding assay and functional studies on human basophils indicated that these derivatives behaved as $CB_1$ and

* To whom correspondence should be addressed. For G.S.: phone, +39(0)502219580; fax, +39(0)502219605; e-mail: gsacco@farm.unipi.it. For T.T.: phone, +39(0)502219595; fax, +39(0)502219605; e-mail: tuccinardi@farm.unipi.it.

[†] Dipartimento di Scienze Farmaceutiche, Università di Pisa.

[‡] Dipartimento di Psichiatria, Neurobiologia, Farmacologia e Biotecnologie, Università di Pisa.

[§] Istituto di Chimica Biomolecolare.

[||] Temple University.

[⊥] Università di Firenze.

[a] Abbreviations: ECS, endocannabinoid system; $CB_1$, cannabinoid subtype 1 receptor; $CB_2$, cannabinoid subtype 2 receptor; RT-PCR, reverse transcription polymerase chain reaction.

10.1021/jm801563d CCC: $40.75 
Published on Web 05/12/2009

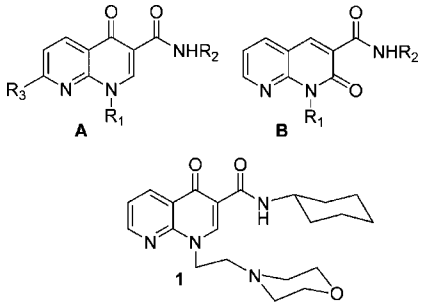



**Figure 1.** General structures of the 1,8-naphthyridin-4(1*H*)-on-3-carboxamide (**A**) and 1,8-naphthyridin-2(1*H*)-on-3-carboxamide (**B**) derivatives and structure of compound **1**.

$CB_2$ 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 $CB_2/CB_1$ selectivity and the improvement of the $CB_2$ 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 $CB_2R$ 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 $CB_2R$ with the nonconserved S3.31(112). The important role of 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 $CB_1$ ($hCB_1$) and $CB_2$ ($hCB_2$) 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 1,8-naphthyridin-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) ($CB_1R$ $K_i > 1000$ nM; $CB_2R$ $K_i = 48.6$ 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)-1,8-naphthyridin-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 $CB_2$ 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(1*H*)-on-3-carboxamide derivatives were synthesized.

## Chemistry

The synthetic routes to obtain the target 1,8-naphthyridin-2(1*H*)-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 $\mu$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

**$CB_1$ and $CB_2$ Receptor Affinity.** The binding affinities ($K_i$ values) of compounds **2**−**13** for human recombinant $CB_1$ and $CB_2$ 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 $CB_1$ and $CB_2$ cannabinoid receptors have also been included in Table 1.

Among the newly synthesized 1,8-naphthyridin-2(1*H*)-on-3-carboxamide 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 $CB_1$ 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 $CB_2R$, the new compounds **2**−**6** and **8**−**13** proved to be high affinity $CB_2R$ 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 $CB_2R$ affinity in this series, with $K_i$ values

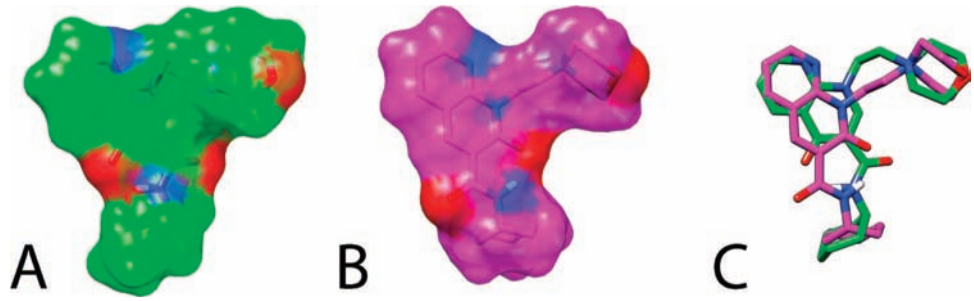
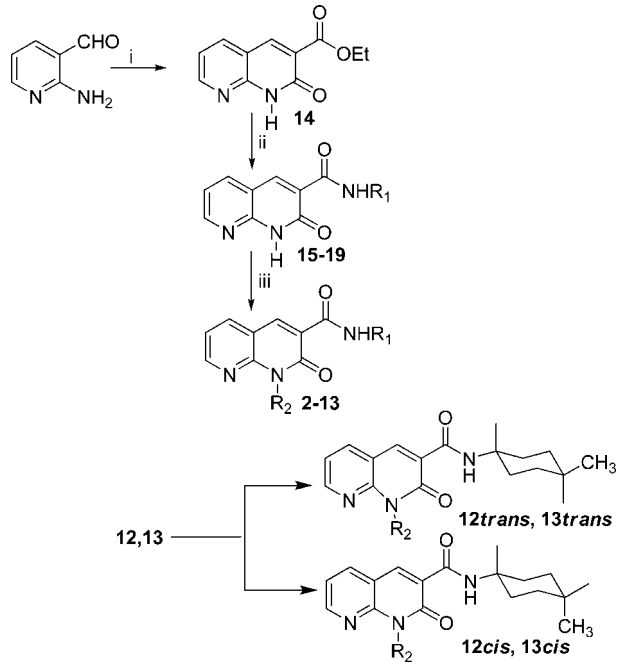


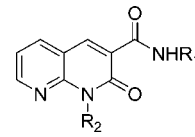

**Figure 2.** 3D structure and solvent-excluded molecular surface of compound **1** (A), **2** (B), and their superimposition (C).

**Scheme 1.** Synthesis of the 1,8-Naphthyridin-2(1*H*)-on-3-carboxamide Derivatives **2**−**13**[a]




[a] Reagents and conditions: (i) EMME, 120 °C, 20 h; (ii) $R_1NH_2$, microwave, 140 °C, 1 h 200 W; (iii) NaH, $R_2Cl$.

**Table 1.** Radioligand Binding Data of Compounds **2**−**13**[a]




| compd | $R_1$ | $R_2$ | $K_i$ (nM) $CB_1$[b] | $K_i$ (nM) $CB_2$[c] | $K_i(CB_1)/K_i(CB_2)$ |
|---|---|---|---|---|---|
| **2** | cyclohexyl | ethylmorpholino | 560 | 7.9 | 71 |
| **3** | cyclohexyl | benzyl | 560 | 31 | 18 |
| **4** | cyclohexyl | *p*-fluorobenzyl | 56 | 2.2 | 25 |
| **5** | cyclohexyl | *n*-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 | *p*-fluorobenzyl | 200 | 0.9 | 222 |
| *trans*-**13** | 4-methylcyclohexyl | *p*-fluorobenzyl | 300 | 9.0 | 33 |
| *cis*-**13** | 4-methylcyclohexyl | *p*-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 $CB_1$ and $CB_2$ 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 $CB_1$ receptor was evaluated using membranes from HEK-293 cells transfected with $CB_1$ receptor and [$^3$H]CP55,940. [c] Affinity of compounds for $CB_2$ receptor was evaluated using membranes from HEK-293 cells transfected with $CB_2$ receptor and [$^3$H]CP55,940.

of 0.7 nM. Finally, the compounds with an ethylmorpholino group in position 1 exhibited $CB_2R$ 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 $CB_2R$.

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 $CB_2R$. In fact, *cis*-**12** and *cis*-**13** showed 7-fold and 13-fold increases in their affinity for the $CB_2R$ 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 $CB_2R$ 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 $K_i(CB_1)/K_i(CB_2)$ ratios greater than 200. In particular, compound **6**, with ethylmorpholino and carboxy-4-methylcyclohexylamide substituents 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.

**$CB_2$ Functional Activity.** The functional activity of compound **12** was assessed by using functional studies on human basophils. In fact, activation of $CB_2$ 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 $\mu$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

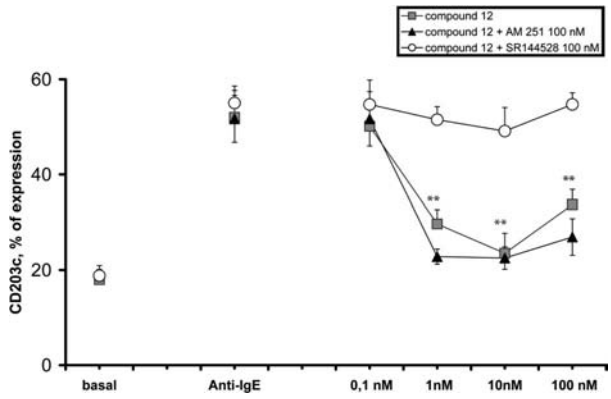



**Figure 3.** Expression of CD203c by human basophils activated with anti-IgE (1 $\mu$g/mL) is reduced by compound **12** in a dose-related fashion. The inhibitory effect of compound **12** on basophil CD203c expression is reverted by **20** (100 nM), a $CB_2$ antagonist, but not by **22** (100 nM), a $CB_1$ antagonist. The values are the mean ± SEM of six independent experiments performed in triplicate: (**) $P < 0.001$ vs compound **12** plus **22**.

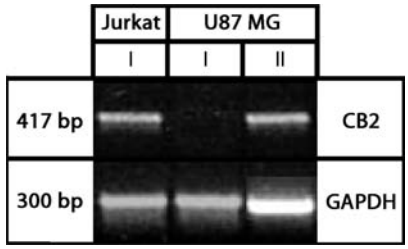



**Figure 4.** $CB_2$ and GAPDH mRNA levels were determined by RT-PCR on Jurkat and U87MG cell lines. On U87MG cells, $CB_2$ fragment was obtained after a further amplification of PCR product: (I) single amplification; (II) reamplification.

by the selective $CB_2$ antagonist **20** (100 nM, 30 min of preincubation, 37 °C) but not by the selective $CB_1$ 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 $CB_2$-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 $CB_2R$ agonist (2-methyl-1-propyl-1*H*-indol-3-yl)-1-naphthalenylmethanone (**23**, JWH015).[29] Jurkat cells are a cell line with a good level of $CB_2R$ expression (Figure 4). Exposure to **12** in the range 0.1−10 $\mu$M for 48 h induced a concentration-dependent decrease in cell viability. A similar behavior was observed with the known $CB_2$ agonist **23**, whose cytotoxicity matched that previously reported.[29] The 1,8-naphthyridin-2(1*H*)-on-3-carboxamide derivatives **12** and **23** showed $IC_{50}$ values that were not statistically different (Table 2). In addition, 1 $\mu$M [6-iodo-2-methyl-1-(2-morpholin-4-ylethyl)indol-3-yl]-(4-methoxyphenyl)methanone (**24**, AM630)[30] significantly antagonized the cytotoxic effect of **12** on Jurkat cells (Figure 5), confirming its $CB_2$-mediated mechanism.

On U87MG cells, which express $CB_2R$ at lower levels (a PCR electrophoretic band was evident only after reamplification) (Figure 4), **12** and **23** only induced a poor effect (about 20% of cell viability inhibition) and only at the highest dose used (10 $\mu$M).

**Table 2.** Cytotoxic Activity of **12** and **23** on Jurkat and U87MG Cell Lines[a]

| | $IC_{50}$ ($\mu$M) | |
|---|---|---|
| compd | Jurkat | U87MG |
| **12** | 5.98 ± 2.78 | NR |
| **23** | 9.60 ± 4.12 | NR |

[a] Data are the mean ± SE from three experiments carried out in triplicate. NR: not reached.

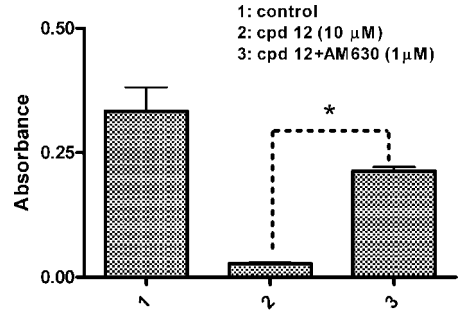



**Figure 5.** Effect of **12** (10 $\mu$M) and combination of **12**/**24** (1 $\mu$M) on Jurkat cell viability. Data are the mean ± SE of two experiments carried out in triplicate.

## Conclusions

In our study, we investigated possible modifications of the 1,8-naphthyridin-4(1*H*)-one central scaffold in order to improve $CB_2R$ 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 $CB_1R$ and $CB_2R$. The new compounds showed $CB_2R$ affinity and selectivity that were higher than corresponding 1,8-naphthyridin-4(1*H*)-on-3-carboxamide derivatives previously studied[19] and, in some cases (i.e., compound **6**), among the highest ever obtained for $CB_2R$ 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 $K_i(CB_1)/K_i(CB_2)$ 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(1*H*)-on-3-carboxamides possess agonist properties at $CB_2R$.

These results indicate that 1,8-naphthyridin-2(1*H*)-on-3-carboxamides 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. $^1$H NMR spectra were recorded with a Bruker AC-200 spectrometer in $\delta$ 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 ±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 × 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; $^1$H NMR (DMSO) $\delta$ 8.60 (dd, 1H, $H_7$), 8.50 (s, 1H, $H_4$), 8.26 (dd, 1H, $H_5$), 7.29 (m, 1H, $H_6$), 4.37 (q, 2H, $CH_2$), 1.29 (t, 3H, $CH_3$).

**General Procedure for the Preparation of $N_1$-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); $^1$H NMR (DMSO) $\delta$ 12.03 (br,1H, NH), 9.70 (d, 1H, NH), 8.85 (s, 1H, $H_4$), 8.66 (dd, 1H, $H_7$), 8.42 (dd, 1H, $H_5$), 7.36 (m, 1H, $H_6$), 3.85 (m, 1H, CH), 1.89−1.27 (m, 10H, cyclohexyl). Anal. ($C_{15}H_{17}N_3O_2$) C, H, N.

***N*-(4-Methylcyclohexyl)-1,8-naphthyridin-2(1*H*)-on-3-carboxamide (16).** Yield 87%; crystallized from ethyl acetate; $^1$H NMR (DMSO) $\delta$ 11.85 (br,1H, NH), 10.0 and 9.60 (2d, 1H, NH), 8.85 and 8.87 (2s, 1H, $H_4$), 8.66 (dd, 1H, $H_7$), 8.41 (dd, 1H, $H_5$), 7.36 (m, 1H, $H_6$), 4.11 and 3.85 (2m, 1H, CH), 2.00−1.05 (m, 9H, cyclohexyl), 0.92 and 0.89 (2d, 3H, $CH_3$). Anal. ($C_{16}H_{19}N_3O_2$) C, H, N.

***N*-Cycloheptyl-1,8-naphthyridin-2(1*H*)-on-3-carboxamide (17).** Yield 71%; mp 203−204 °C (crystallized from dichloromethane); $^1$H NMR (DMSO) $\delta$ 11.95 (br,1H, NH), 9.75 (d, 1H, NH), 8.84 (s, 1H, $H_4$), 8.65 (dd, 1H, $H_7$), 8.40 (dd, 1H, $H_5$), 7.35 (m, 1H, $H_6$), 4.10 (m, 1H, CH), 1.90−1.45 (m, 12H, cycloheptyl). Anal. ($C_{16}H_{19}N_3O_2$) 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); $^1$H NMR (DMSO) $\delta$ 12.15 (br,1H, NH), 9.71 (t, 1H, NH), 8.86 (s, 1H, $H_4$), 8.65 (dd, 1H, $H_7$), 8.42 (dd, 1H, $H_5$), 7.32 (m, 6H, $H_6$ + Ar), 3.59 (m, 2H, $CH_2$), 2.85 (t, 2H, $CH_2$). Anal. ($C_{17}H_{15}N_3O_2$) C, H, N.

***N*-(4-Fluorophenethyl)-1,8-naphthyridin-2(1*H*)-on-3-carboxamide (19).** Yield 84%; mp >300 °C (crystallized from ethanol); $^1$H NMR (DMSO) $\delta$ 11.15 (br,1H, NH), 9.68 (t, 1H, NH), 8.85 (s, 1H, $H_4$), 8.65 (dd, 1H, $H_7$), 8.40 (dd, 1H, $H_5$), 7.33 (m, 3H, $H_6$ + Ar), 7.12 (m, 2H, Ar), 3.58 (m, 2H, $CH_2$), 2.85 (t, 2H, $CH_2$). Anal. ($C_{17}H_{14}FN_3O_2$) C, H, N.

**General Procedure for the Synthesis of $N_1$-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 cooled, water was added and the solid obtained 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 ($M^+$); $^1$H NMR (DMSO) $\delta$ 9.75 (d, 1H, NH), 8.88 (s, 1H, $H_4$), 8.78 (dd, 1H, $H_7$), 8.50 (dd, 1H, $H_5$), 7.45 (m, 1H, $H_6$), 4.64 (t, 2H, $CH_2$), 3.90 (m, 1H, CH), 3.51 (m, 4H, morpholine), 2.57 (m, 6H, morpholine + $CH_2$), 1.89−1.22 (m, 10H, cyclohexyl). Anal. ($C_{21}H_{28}N_4O_3$) 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 ($M^+$); $^1$H NMR (DMSO) $\delta$ 9.58 (d, 1H, NH), 8.96 (s, 1H, $H_4$), 8.76 (dd, 1H, $H_7$), 8.54 (dd, 1H, $H_5$), 7.45 (m, 1H, $H_6$), 7.24 (m, 5H, Ar), 5.72 (s, 2H, $CH_2$), 3.85 (m, 1H, CH), 1.89−1.30 (m, 10H, cyclohexyl). Anal. ($C_{22}H_{23}N_3O_2$) 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 ($M^+$); $^1$H NMR (DMSO) $\delta$ 9.56 (d,1H, NH) 8.95 (s, 1H, $H_4$), 8.77 (dd, 1H, $H_7$), 8.53 (dd, 1H, $H_5$), 7.47 (m, 1H, $H_6$), 7.31 (m, 2H, Ar), 7.10 (m, 2H, Ar), 5.68 (s, 2H, $CH_2$), 3.80 (m, 1H, CH), 1.88−1.22 (m, 10H, cyclohexyl). Anal. ($C_{22}H_{22}FN_3O_2$) 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 ($M^+$); $^1$H NMR (DMSO) $\delta$ 9.69 (d, 1H, NH), 8.88 (s, 1H, $H_4$), 8.79 (dd, 1H, $H_7$), 8.49 (dd, 1H, $H_5$), 7.45 (m, 1H, $H_6$), 4.47 (t, 2H, $CH_2$), 3.82 (m, 1H, CH), 1.89−0.88 (m, 17H, cyclohexyl + $2CH_2$ + $CH_3$). Anal. ($C_{19}H_{25}N_3O_2$) 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 ($M^+$); $^1$H NMR (DMSO) $\delta$ 9.90 and 9.52 (2d, 1H, NH), 8.90 (s, 1H, $H_4$), 8.79 (dd, 1H, $H_7$), 8.50 (dd, 1H, $H_5$), 7.45 (m, 1H, $H_6$), 4.65 (t, 2H, $CH_2$), 4.00 and 3.75 (2m, 1H, CH), 3.51 (m, 4H, morpholine), 2.56 (m, 6H, morpholine + $CH_2$), 1.90−0.97 (m, 9H, cyclohexyl), 0.92 and 0.88 (2d, 3H, $CH_3$). Anal. ($C_{22}H_{30}N_4O_3$) 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 ($M^+$); $^1$H NMR (DMSO) $\delta$ 9.64 (t, 1H, NH), 8.96 (s, 1H, $H_4$), 8.77 (dd, 1H, $H_7$), 8.53 (dd, 1H, $H_5$), 7.47 (m, 1H, $H_6$), 7.23 (m, 10H, Ar), 5.72 (s, 2H, $CH_2$), 3.59 (m, 2H, $CH_2$), 2.86 (t, 2H, $CH_2$). Anal. ($C_{24}H_{21}N_3O_2$) 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 ($M^+$); $^1$H NMR (DMSO) $\delta$ 9.72 (brs, 1H, NH), 8.93 (s, 1H, $H_4$), 8.78 (dd, 1H, $H_7$), 8.43 (dd, 1H, $H_5$), 7.36 (m, 8H, Ar + $H_6$), 7.07 (m, 2H, Ar), 5.82 (s, 2H, $CH_2$) 3.69 (m, 2H, $CH_2$), 2.95 (t, 2H, $CH_2$). Anal. ($C_{24}H_{20}FN_3O_2$) 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 ($M^+$); $^1$H NMR (DMSO) $\delta$ 9.62 (d, 1H, NH), 8.95 (s, 1H, $H_4$), 8.76 (dd, 1H, $H_7$), 8.53 (dd, 1H, $H_5$), 7.46 (m, 1H, $H_6$), 7.25 (m, 5H, Ar), 5.72 (s, 2H, $CH_2$), 4.09 (m, 1H, CH), 1.90−0.85 (m, 12H, cycloheptyl). Anal. ($C_{23}H_{25}N_3O_2$) 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 ($M^+$); $^1$H NMR (DMSO) $\delta$ 9.78 (d, 1H, NH), 8.88 (s, 1H, $H_4$), 8.75 (dd, 1H, $H_7$), 8.49 (dd, 1H, $H_5$), 7.47 (m, 1H, $H_6$), 7.32 (m, 2H, Ar), 7.05 (m, 2H, Ar), 5.78 (s, 2H, $CH_2$), 4.10 (m, 1H, CH), 1.88−1.01 (m, 12H, cycloheptyl). Anal. ($C_{23}H_{24}FN_3O_2$) 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 ($M^+$); $^1$H NMR ($CDCl_3$) $\delta$ 9.77 (d, 1H, NH), 8.88 (s, 1H, $H_4$), 8.68 (dd, 1H, $H_7$), 8.08 (dd, 1H, $H_5$), 7.28 (dd, 1H, $H_6$), 4.78 (t, 2H, $CH_2$), 4.10 (m, 1H, CH), 3.70 (m, 4H, morpholine), 2.72 (m, 6H, morpholine + $CH_2$), 2.05−0.85 (m, 12H, cycloheptyl). Anal. ($C_{22}H_{30}N_4O_3$) 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 ($M^+$); $^1$H NMR (DMSO) $\delta$ 9.84 and 9.47 (2d, 1H, NH), 8.97 (s, 1H, $H_4$), 8.77 (dd, 1H, $H_7$), 8.53 (dd, 1H, $H_5$), 7.46 (m, 1H, $H_6$), 7.30 (m, 5H, Ar), 5.73 and 5.78 (2s, 2H, $CH_2$), 4.10 and 3.75 (2m, 1H, CH), 1.94−0.99 (m, 9H, cyclohexyl), 0.94 and 0.89 (2d, 3H, $CH_3$). Anal. ($C_{23}H_{25}N_3O_2$) 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 ($M^+$); $^1$H NMR (DMSO) $\delta$ 9.47 (d, 1H, NH), 8.97 (s, 1H, $H_4$), 8.77 (dd, 1H, $H_7$), 8.53 (dd, 1H, $H_5$), 7.46 (m, 1H, $H_6$), 7.30 (m, 5H, Ar), 5.73, (s, 2H, $CH_2$), 3.75 (m, 1H, CH), 1.94−0.99 (m, 9H, cyclohexyl), 0.89 (d, 3H, $CH_3$). Anal. ($C_{23}H_{25}N_3O_2$) C, H, N. *cis*-**12**: yield 20%; mp 157−159 °C (crystallized from hexane); MS *m*/*z* 375 ($M^+$); $^1$H NMR (DMSO) $\delta$ 9.84 (d, 1H, NH), 8.97 (s, 1H, $H_4$), 8.77 (dd, 1H, $H_7$), 8.53 (dd, 1H, $H_5$), 7.46 (m, 1H, $H_6$), 7.30 (m, 5H, Ar), 5.78 (s, 2H, $CH_2$), 4.10 (m, 1H, CH), 1.94−0.99 (m, 9H, cyclohexyl), 0.94 (d, 3H, $CH_3$). Anal. ($C_{23}H_{25}N_3O_2$) 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 ($M^+$); $^1$H NMR (DMSO) $\delta$ 9.85 and 9.43 (2d, 1H, NH), 8.95 (s, 1H, $H_4$), 8.77 (dd, 1H, $H_7$), 8.52 (dd, 1H, $H_5$), 7.47 (dd, 1H, $H_6$), 7.31 (m, 2H, Ar), 7.10 (m, 2H, Ar), 5.72 and 5.69 (2s, 2H, $CH_2$), 4.10 and 3.70 (2m, 1H, CH), 1.94−0.99 (m, 9H, cyclohexyl), 0.92 and 0.88 (2d, 3H, $CH_3$). Anal. ($C_{23}H_{24}FN_3O_2$) C, H, N.

***trans*-*N*-(4-Methylcyclohexyl)-1-(*p*-fluorobenzyl)-1,8-naphthyridin-2(1*H*)-on-3-carboxamide (*trans*-13) and *cis*-*N*-(4-Methylcyclohexyl)-1-(*p*-fluorobenzyl)-1,8-naphthyridin-2(1*H*)-on-3-carboxamide (*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 ($M^+$); $^1$H NMR (DMSO) $\delta$ 9.43 (d, 1H, NH), 8.95 (s, 1H, $H_4$), 8.77 (dd, 1H, $H_7$), 8.52 (dd, 1H, $H_5$), 7.47 (m, 1H, $H_6$), 7.31 (m, 2H, Ar), 7.10 (m, 2H, Ar) 5.69 (s, 2H, $CH_2$), 3.70 (2m, 1H, CH), 1.94−0.99 (m, 9H, cyclohexyl), 0.88 (d, 3H, $CH_3$). Anal. ($C_{23}H_{24}FN_3O_2$) C, H, N. *cis*-**13**: yield 18%; mp 170−172 °C (crystallized from hexane); MS *m*/*z* 393 ($M^+$); $^1$H NMR (DMSO) $\delta$ 9.85 (d, 1H, NH), 8.95 (s, 1H, $H_4$), 8.77 (dd, 1H, $H_7$), 8.52 (dd, 1H, $H_5$), 7.47 (m, 1H, $H_6$), 7.31 (m, 2H, Ar), 7.10 (m, 2H, Ar) 5.72 (s, 2H, $CH_2$), 4.10 (2m, 1H, CH), 1.94−0.99 (m, 9H, cyclohexyl), 0.92 (d, 3H, $CH_3$). Anal. ($C_{23}H_{24}FN_3O_2$) C, H, N.

**$CB_1$ and $CB_2$ Receptor Binding Assays.** The new compounds were evaluated in $CB_1R$ and $CB_2R$ 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 $CB_2R$ ($B_{max}$ = 4.7 pmol/mg protein) (Perkin-Elmer, Italy). These membranes were incubated with [$^3$H]-(−)-*cis*-3-[2-hydroxy-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 nM/$K_d$ = 0.31 nM for $CB_1$ and $CB_2$ receptors, respectively) as the high affinity ligand and displaced with 100 nM [(3*R*)-5-methyl-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 $CB_1R$ and $CB_2R$). 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 equation[34] 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 ± 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 420*g*. After removal of the supernatant plasma, the basophil-rich Ficoll-Paque layer was separated and the neutrophil-rich buffy coat was discarded. The suspension was washed twice with the washing buffer and centrifuged at 200*g* 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 $Na_3EDTA$, 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 high-purifying 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 200*g* 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 μg/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 μg/mL) (Roche Molecular Biochemicals, Milan, Italy). Exponentially growing Jurkat ($2 \times 10^4$) and U87MG cells ($4 \times 10^3$) 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 $CB_2R$ 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)-2*H*-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 $IC_{50}$ 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 ± SE. Statistical difference among $IC_{50}$ 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 $CB_2R$ Expression.** To confirm the expression of $CB_2R$ 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 ($5 \times 10^6$) Jurkat and ($2 \times 10^6$) U87MG cell lines using the "SV Total RNA Isolation System" kit (Promega S.r.l.; Milan, Italy). cDNAs were subsequently obtained from 1 $\mu$g of total RNA using the "Quantitect Reverse Transcription" kit (Qiagen; Milan, Italy). A DNase digestion step was included.

$CB_2$ forward (5′-CTGGCTCCTGTTCATCGCCT-3′) and $CB_2$ reverse (5′-GCTTCTTCTTTTGCCTCTGAC-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 $CB_2$, 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 Maestro[35] 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 bond[21] was maintained activating the fixfile parameter. The result of the docking study for compound **1** into the $CB_2$ receptor model[19] 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 Weinstein[40] 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.

**Acknowledgment.** Authors from University of Pisa thank Dr. Veronica Turini for technical assistance in HPLC. Authors from the CNR of Pozzuoli thank Marco Allarà for technical assistance. Molecular graphics images were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIH Grant P41 RR-01081).

**Supporting Information Available:** Chromatogram of **12**, *trans*-**12**, *cis*-**12** (Figure S1); chromatogram of **13**, *trans*-**13**, *cis*-**13** (Figure S2); elemental analysis data of the compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

## References

(1) Lambert, D. M.; Fowler, C. J. The endocannabinoid system: drug targets, lead compounds, and potential therapeutic applications. *J. Med. Chem.* **2005**, *48*, 5069–5087.
(2) Di Marzo, V. Targeting the endocannabinoid system: to enhance or reduce. *Nat. Rev. Drug Discovery* **2008**, *7*, 438–455.
(3) Begg, M.; Pacher, P.; Bátkai, S.; Osei-Hyiaman, D.; Offertáler, L.; Mo, F. M.; Liu, J.; Kunos, G. Evidence for novel cannabinoid receptors. *Pharmacol. Ther.* **2005**, *106*, 133–145.
(4) Compton, D. R.; Rice, K. C.; De Costa, B. R.; Razdan, R. K.; Melvin, L. S.; Johnson, M. R.; Martin, B. R. Cannabinoid structure−activity relationships: correlation of receptor binding in vivo activities. *J. Pharmacol. Exp. Ther.* **1993**, *265*, 218–226.
(5) Manera, C.; Tuccinardi, T.; Martinelli, A. Indoles and related compounds as cannabinoid ligands. *Mini-Rev. Med. Chem.* **2008**, *8*, 370–387.
(6) Lynn, A. B.; Herkenham, M. Localization of cannabinoid receptors and non saturable high-density cannabinoid binding sites in peripheral tissues of the rat: implications for receptor-mediated immune modulation by cannabinoids. *J. Pharmacol. Exp. Ther.* **1994**, *268*, 1612–1623.
(7) Stoll, G.; Jander, S. The role of microglia and macrophages in the pathophysiology of the CNS. *Prog. Neurobiol.* **1999**, *58*, 233–247.
(8) Guzmán, M.; Galve-Roperh, I.; Sánchez, C. Ceramide: a new second messenger of cannabinoid action. *Trends Pharmacol. Sci.* **2001**, *22*, 19–22.
(9) Oka, S.; Yanagimoto, S.; Ikeda, S.; Gokoh, M.; Kishimoto, S.; Waku, K.; Ishima, Y.; Sugiura, T. Evidence for the involvement of the cannabinoid CB2 receptor and its endogenous ligand 2-arachidonoylglycerol in 12-*O*-tetradecanoylphorbol-13-acetate-induced acute inflammation in mouse ear. *J. Biol. Chem.* **2005**, *280*, 18488–18497.
(10) Ibrahim, M. M.; Deng, H.; Zvonok, A.; Cockayne, D. A.; Kwan, J.; Mata, H. P.; Vanderah, T. W.; Lai, J.; Porreca, F.; Makriyannis, A.; Malan, T. P., Jr. Activation of the CB2 cannabinoid receptors by AM1241 inhibits experimental neuropathic pain: pain inhibition by receptors not present in the CNS. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 10529–10533.
(11) Bab, I.; Zimmer, A. Cannabinoid receptors and the regulation of bone mass. *Br. J. Pharmacol.* **2008**, *153*, 182–188.
(12) Maekawa, T.; Nojima, H.; Kuraishi, Y.; Aisaka, K. The cannabinoid CB2 receptor inverse agonist JTE-907 suppresses spontaneous itch-associated responses of NC mice, a model of atopic dermatitis. *Eur. J. Pharmacol.* **2006**, *542*, 179–183.
(13) Lotersztajn, S.; Teixeira-Clerc, F.; Julien, B.; Deveaux, V.; Ichigotani1, Y.; Manin, S.; Tran-Van-Nhieu, J.; Karsak, M.; Zimmer, A.; Mallat, A. CB2 receptors as new therapeutic targets for liver diseases. *Br. J. Pharmacol.* **2008**, *153*, 286–289.
(14) Sánchez, C.; De Ceballos, M. L.; Gómez del Pulgar, T.; Rueda, D.; Corbacho, C.; Velasco, G.; Galve-Roperh, I.; Huffman, J. W. Y.; Cajal, S. R.; Guzmán, M. Inhibition of glioma growth in vivo by selective activation of the CB2 cannabinoid receptor. *Cancer Res.* **2001**, *61*, 5784–5789.
(15) McKallip, R. J.; Lombard, C.; Fisher, M.; Martin, B. R.; Ryu, S.; Grants, S.; Nagarkatti, P. S.; Nagarkatti, M. Targeting CB2 cannabinoid receptors as a novel therapy to treat malignant lymphoblastic disease. *Blood* **2002**, *100*, 627–634.
(16) Ramírez, B. G.; Blázquez, C.; Gómez del Pulgar, T.; Guzmán, M.; de Ceballos, M. L. Prevention of Alzheimer's disease pathology by cannabinoids: neuroprotection mediated by blockade of microglial activation. *J. Neurosci.* **2005**, *25*, 1904–1913.
(17) Sagredo, O.; García-Arencibia, M.; de Lago, E.; Finetti, S.; Decio, A.; Fernández-Ruiz, J. Cannabinoids and neuroprotection in basal ganglia disorders. *Mol. Neurobiol.* **2007**, *36*, 82–91.
(18) Ferrarini, P. L.; Calderone, V.; Cavallini, T.; Manera, C.; Saccomanni, G.; Pani, L.; Ruiu, S.; Gessa, G. L. Synthesis and biological evaluation of 1,8-naphthyridin-4(1*H*)-on-3-carboxamide derivatives as new ligands of cannabinoid receptors. *Bioorg. Med. Chem.* **2004**, *12*, 1921–1933.
(19) Manera, C.; Benetti, V.; Castelli, M. P.; Cavallini, T.; Lazzarotti, S.; Pibiri, F.; Saccomanni, G.; Tuccinardi, T.; Vannacci, A.; Martinelli, A.; Ferrarini, P. L. Design, synthesis, and biological evaluation of new 1,8-naphthyridin-4(1*H*)-on-3-carboxamide and quinolin-4(1*H*)-on-3-carboxamide derivatives as CB2 selective agonists. *J. Med. Chem.* **2006**, *49*, 5947–5957.
(20) Manera, C.; Cascio, M. G.; Benetti, V.; Allarà, M.; Tuccinardi, T.; Martinelli, A.; Saccomanni, G.; Vivoli, E.; Ghelardini, C.; Di Marzo, V.; Ferrarini, P. L. New 1,8-naphthyridine and quinoline derivatives as CB2 selective agonists. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 6505–6510.

(21) Tuccinardi, T.; Ferrarini, P. L.; Manera, C.; Ortore, G.; Saccomanni, G.; Martinelli, A. Cannabinoid CB2/CB1 selectivity. Receptor modeling and automated docking analysis. *J. Med. Chem.* **2006**, *49*, 984–994.

(22) Vannacci, A.; Zagli, G.; Marzocca, C.; Pierpaoli, S.; Passani, M. B.; Mannaioni, P. F.; Masini, E. Down-regulation by cannabinoids of the immunological activation of human basophils and guinea pig mast cells. *Inflammation Res.* **2002**, *51*, S09–S10.

(23) Vannacci, A.; Giannini, L.; Passani, M. B.; Di Felice, A.; Pierpaoli, S.; Zagli, G.; Fantappie, O.; Mozzanti, R.; Masini, E.; Mannaioni, P. F. The endocannabinoid 2-arachidonylglycerol decreases the immunological activation of guinea pig mast cells: involvement of nitric oxide and eicosanoids. *J. Pharmacol. Exp. Ther.* **2004**, *311*, 256–264.

(24) Mogilaiah, K.; Rao, R. B. Synthesis of 1,8-naphthyridines under solvent free conditions. *Indian J. Chem., Sect. B* **2001**, *40B*, 713 714.

(25) Rinaldi-Carmona, M.; Barth, F.; Millan, J.; Derocq, J. M.; Casellas, P.; Congy, C.; Oustric, D.; Sarran, M.; Bouaboula, M.; Calandra, B.; Portier, M.; Shire, D.; Brelière, J. C.; Le Fur, G. L. SR144528, the first potent and selective antagonist of the CB2 cannabinoid receptor. *J. Pharmacol. Exp. Ther.* **1998**, *284*, 644–650.

(26) Huffman, J. W.; Liddle, J.; Yu, S.; Aung, M. M.; Abood, M. E.; Wiley, J. L.; Martin, B. R. 3-(1′,1′-Dimethylbutyl)-1-deoxy-delta8-THC and related compounds: synthesis of selective ligands for the CB2 receptor. *Bioorg. Med. Chem.* **1999**, *7*, 2905–2914.

(27) Boumiza, R.; Monneret, G.; Forissier, M. F.; Savoye, J.; Gutowski, M. C.; Powell, W. S.; Bievenu, J. Marked improvement of the basophil activation test by detecting CD203c instead of CD63. *Clin. Exp. Allergy* **2003**, *33*, 259–265.

(28) Gatley, S. J.; Gifford, A. N.; Volkow, N. D.; Lan, R.; Makriyannis, A. $^{123}$I-labeled AM251: a radioiodinated ligand which binds in vivo to mouse brain cannabinoid CB1 receptors. *Eur. J. Pharmacol.* **1996**, *307*, 331–338.

(29) McKallip, R. J.; Lombard, C.; Fisher, M.; Martin, B. R.; Ryu, S.; Grant, S.; Nagarkatti, P. S.; Nagarkatti, M. Targeting CB2 cannabinoid receptors as a novel therapy to treat malignant lymphoblastic disease. *Blood* **2002**, *100*, 627–634.

(30) Hosohata, K.; Quock, R. M.; Hosohata, Y.; Burkey, T. H.; Makriyannis, A.; Consroe, P.; Roeske, W. R.; Yamamura, H. I. AM630 is a competitive cannabinoid receptor antagonist in the guinea pig brain. *Life Sci.* **1997**, *61*, PL115–PL118.

(31) Gatley, S. J.; Lan, R.; Pyatt, B.; Gifford, A. N.; Volkow, N. D.; Makriyannis, A. Binding of the non-classical cannabinoid CP 55,940, and the diarylpyrazole AM251 to rodent brain cannabinoid receptors. *Life Sci.* **1997**, *61*, 191–197.

(32) Huffman, J. W.; Zengin, G.; Wu, M. J.; Lu, J.; Hynd, G.; Bushell, K.; Thompson, A. L.; Bushell, S.; Tartal, C.; Hurst, D. P.; Reggio, P. H.; Selley, D. E.; Cassidy, M. P.; Wiley, J. L.; Martin, B. R. Structure−activity relationships for 1-alkyl-3-(1-naphthoyl)indoles at the cannabinoid CB1 and CB2 receptors: steric and electronic effects of naphthoyl substituents. New highly selective CB2 receptor agonists. *Bioorg. Med. Chem.* **2005**, *13*, 89–112.

(33) Brizzi, A.; Brizzi, V.; Cascio, M. G.; Bisogno, T.; Siriani, R.; Di Marzo, V. Design, synthesis, and binding studies of new potent ligands of cannabinoid receptors. *J. Med. Chem.* **2005**, *48*, 7343–7350.

(34) Cheng, Y. C.; Prusoff, W. H. Relationship between the inhibition constant (Ki) and the concentration of inhibition which causes 50% inhibition (IC50) of an enzyme reaction. *Biochem. Pharmacol.* **1973**, *22*, 3099–3108.

(35) *Maestro*, version 7.5; Schrödinger Inc.: Portland, OR, 1999.

(36) *Macromodel*, version 8.5; Schrödinger Inc.: Portland, OR, 1999.

(37) Halgren, T. A.; Nachbar, R. B. Merck molecular force field. IV. conformational energies and geometries for MMFF94. *J. Comput. Chem.* **1996**, *17*, 587–615.

(38) Rush, T. S.; Grant, J. A.; Mosyak, L.; Nicholls, A. A shape-based 3-D scaffold hopping method and its application to a bacterial protein−protein interaction. *J. Med. Chem.* **2005**, *48*, 1489–1495.

(39) Boström, J.; Greenwood, J. R.; Gottfries, J. Assessing the performance of OMEGA with respect to retrieving bioactive conformations. *J. Mol. Graphics Modell.* **2003**, *21*, 449–462.

(40) Ballesteros, J. A.; Weinstein, H. W. Integrated methods for the construction of three-dimensional models and computational probing of structure−function relations in G-protein coupled receptors. *Methods Neurosci.* **1995**, *25*, 366–428.

JM801563D