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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 CB_2 Selective Agonists

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On the basis of docking studies carried out using the recently published cannabinoid receptor models,[35] new 1,8-naphthyridin-4(1*H*)-on-3-carboxamide and quinolin-4(1*H*)-on-3-carboxamide derivatives were designed, synthesized, and tested for their affinities toward the cannabinoid CB_1 and CB_2 receptors. Compound **10**, which presented *p*-fluorobenzyl and carboxycycloheptylamide substituents bound in the 1 and 3 positions of the 1,8-naphthyiridine-4-one nucleus, showed a high CB_2 affinity with a K_i of 1.0 nM. The substitution of the naphthyridine-4-one nucleus with the quinoline-4-one system determined a general increase in CB_2 affinity. In particular, the *N*-cyclohexyl-7-chloro-1-(2-morpholin-4-ylethyl)quinolin-4(1*H*)-on-3-carboxamide (**40**) possessed a remarkable affinity, with K_i of 3.3 nM, which was also accompanied by a high selectivity for the CB_2 receptor ($K_i(CB_1)/K_i(CB_2)$ ratio greater than 303). Moreover, the [^{35}S]GTPγ binding assay and functional studies on human basophils indicated that the 1,8-naphthyridin-4(1*H*)-on-3-carboxamide derivatives behaved as CB_1 and CB_2 receptor agonists.

Introduction

Cannabinoids are present in Indian hemp, *Cannabis sativa* L., and have been used since antiquity as medicinal agents.[1] Interest in cannabinoid pharmacology has rapidly increased since the discovery of the endocannabinoid system (ECS), which includes cannabinoid receptors, the endocannabinoids (anandamide[2] and 2-arachidonoylglycerol[3]), metabolizing enzymes (fatty acid amide hydrolase[4] and monoglyceride lipase[5]), and a specific cellular uptake system (the anandamide transporter protein[6]).

To date, two distinct cannabinoid receptors, CB_1 and CB_2, have been identified in mammalian tissues and cloned.[7,8] These receptors belong to the superfamily of G-protein-coupled receptor (GPCR) seven-transmembrane receptors, which negatively regulate adenylate cyclase. The CB_1 receptor is mainly located in the central nervous system, with the highest density in the cerebellum, the basal ganglia, the substantia nigra pars compacta, and some regions of the globus pallidus. It is also present in peripheral organs such as the adrenal glands, bone marrow, lung, testis, and uterus.[9] Unlike CB_1, the CB_2 receptor is limited essentially to the cells associated with the immune system, like spleen, thymus, and tonsils.[10] Because of the virtually exclusive peripheral expression of CB_2 and its presence only in microglial cells in the central nervous system (CNS), selective CB_2 ligands should be devoid of the undesired central nervous system effects typical of (−)-*trans*-Δ^9-tetrahydrocannabinol (Δ^9-THC), the major psychoactive component of *Cannabis sativa* L.[11]

The finding of endogenous agonists at these receptors, the endocannabinoids, opened new therapeutic possibilities through the modulation of the activity of the CB receptor. Moreover, the molecular characterization of these receptors allowed the development of synthetic compounds with cannabinoid and noncannabinoid structures, which have found pharmaceutical application.[12,13] Although the physiological role of CB receptors is not yet completely explained, these receptors seem to be involved in several pathophysiological diseases.[14] In particular, selective CB_1 receptor antagonists such as rimonabant[15] are currently under investigation in clinical human studies for treating obesity through the control of appetite[16,17] and may be a helpful tool to stop smoking.[18] In contrast, Δ^9-THC and nabilone are currently marketed to reduce emesis and/or prevent cachexia in AIDS or cancer patients.[19] Unlike the CB_1 receptor, the physiological and putative therapeutic potential of the CB_2 receptor largely remains unexplored. However, selective ligands could be useful for the treatment of pain,[20] inflammation,[21] osteoporosis,[22] growth of malignant gliomas,[23] tumors of immune origin,[24] and immunological disorders such as multiple sclerosis.[25] Furthermore, CB_2 agents could be exploited for prevention of Alzheimer's disease pathology, given of the presence of the CB_2 receptor in the brain microglial cells.[26,27] Finally, it has recently been shown that CB_2 receptor agonists might provide neuroprotection by blockade of microglial activation.[28]

Cannabinoid ligands are currently classified into different structural classes, namely, classic cannabinoids (tricyclic dibenzopyran derivatives produced by the *Cannabis* plant and their synthetic derivatives) such as Δ^9-THC, nonclassic cannabinoids (bicyclic or tricyclic THC derivatives) such as [^{3}H]CP-55,-940,[29,30] endocannabinoids such as arachidonylethanolamide (AEA) and their synthetic derivatives,[31] indoles (typified by WIN-55,212−2), pyrazoles,[32] and indenes.[33]

We have previously reported the synthesis and the binding activity at mouse cannabinoid receptors of a series of 1,8-naphthyridin-4(1*H*)-on-3-carboxamide derivatives whose general structure is **A** (Figure 1).[34]

The binding results showed that the naphthyridine derivatives generally exhibit a higher affinity for the CB_2 than for the CB_1 receptor, and for some of these compounds the $K_i(CB_1)/K_i(CB_2)$

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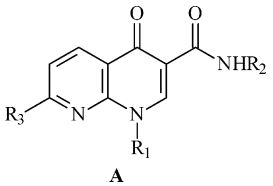


A

Figure 1. General structure of 1,8-naphthyridin-4(1*H*)-on-3-carboxamide derivatives.

ratio was higher than 20. Furthermore, some of these compounds exhibit a CB_2 affinity value in the nanomolar range.[34]

We recently constructed the three-dimensional models of the CB_1 and CB_2 receptors by means of a molecular modeling procedure, and a series of CB_2 ligands were docked into both receptors, showing that the CB_2 model was reliable and predictive.[35]

The docking study of structure **A** naphthyridine derivatives[34] highlighted the ligand−receptor interactions that determine an increase in affinity and selectivity. In particular, this analysis suggested that the preservation of good CB_2/CB_1 selectivity and the improvement of the CB_2 affinity seemed to require (i) the presence of a nonaromatic R_2 substituent capable of interacting in the CB_2 receptor with the nonconserved residue F5.46(197) and (ii) a lipophilic R_1 substituent with an H bond acceptor atom capable of interacting in the CB_2 receptor with the nonconserved S3.31(112).[35]

Bearing this in mind, new 7-methyl-1,8-naphthyridin-4(1*H*)-on-3-carboxamide derivatives were synthesized and tested. For eight compounds in this series, the virtual screening in the CB_2 model predicted an affinity lower than 13 nM, whereas for one compound a poor affinity was calculated.

Furthermore, our studies suggested that some features of the 1,8-naphthyridine derivatives did not seem to be important for interaction with the CB_2 receptor. In particular, the methyl group (R_3) did not seem to interact strongly with any lipophilic residues of the CB_2 receptor, and the N8 atom of the naphthyridine ring played a secondary role, since it did not interact with any polar residue (see Figure 3).

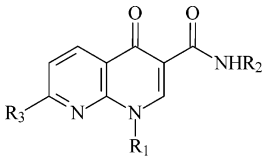
The virtual screening in the CB_2 receptor of compounds in which the methyl group (R_3) was removed or substituted with a chlorine atom and of compounds in which the naphthyridin-4-one ring was substituted with a quinolin-4-one as its central nucleus revealed that these ligands seemed to maintain good CB_2 affinity, and in some cases the affinity seemed to be greater than that of their methyl-substituted and naphthyridine analogues.

In light of these considerations, we synthesized new quinolin-4(1*H*)-on-3-carboxamide derivatives and some new 1,8-naphthyridin-4(1*H*)-on-3-carboxamide in which the R_3 methyl group was removed or substituted with a chlorine atom.

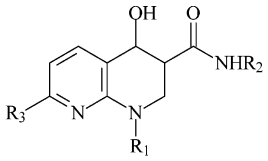
Finally, all the 1,8-naphthyridine derivatives tested could form an intramolecular H bond between the carbonylic oxygen and the amidic NH, creating a pseudocycle planar with the naphthyridine ring, and our studies suggested that this interaction was quite strong.[35]

To verify the ability of our CB_2 model to discriminate between active and inactive ligands and also to verify whether the formation of a planar pseudocycle was important for

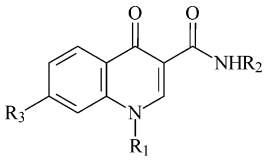
Table 1. Radioligand Binding Data of Compounds **6**−**20**, **24**−**26**, **29**, **33**, **38**−**40**



6-20, 29, 33



24-26

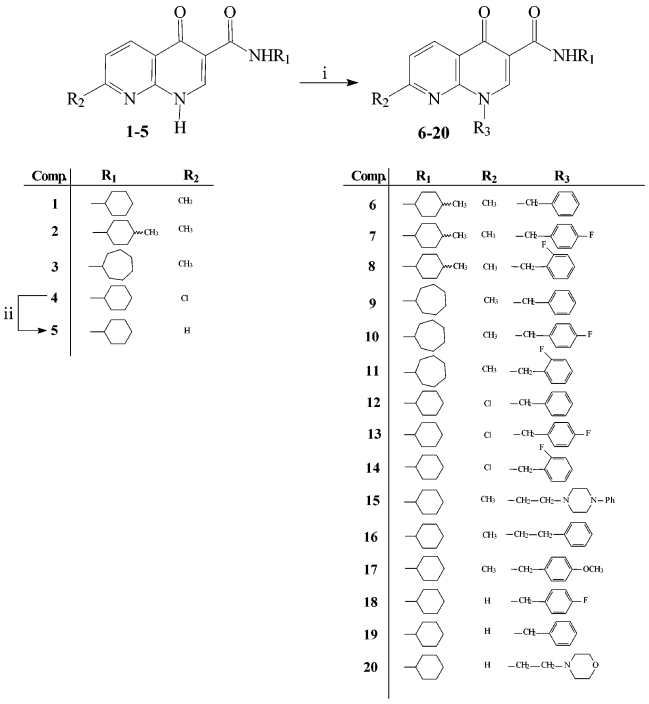


38-40

compd	R_1	R_2	R_3	K_i (nM) CB_1[a,c]	K_i (nM) CB_2[b,c]	$K_i(CB_1)/K_i(CB_2)$	predicted $K_i(CB_2)$ (nM)
6	benzyl	4-methylcyclohexyl	methyl	NT	NT	NT	3.6
7	*p*-fluorobenzyl	4-methylcyclohexyl	methyl	8.7 ± 1.6	1.4 ± 0.1	6	4.0
8	*o*-fluorobenzyl	4-methylcyclohexyl	methyl	37.5 ± 5.4	8.4 ± 0.3	4	5.8
9	benzyl	cycloheptyl	methyl	143.2 ± 9.1	5.1 ± 1.3	28	7.8
10	*p*-fluorobenzyl	cycloheptyl	methyl	4.3 ± 0.6	1.0 ± 0.1	4.3	2.7
11	*o*-fluorobenzyl	cycloheptyl	methyl	149.4 ± 1.8	13.4 ± 4.7	11	3.6
12	benzyl	cyclohexyl	cloro	463.6 ± 1.1	24.6 ± 4.7	19	63.6
13	*p*-fluorobenzyl	cyclohexyl	cloro	495.0 ± 39.4	21.4 ± 1.0	23	9.3
14	*o*-fluorobenzyl	cyclohexyl	cloro	171.2 ± 12.3	18.1 ± 2.7	9.5	7.8
15	1-ethyl-4-phenylpip	cyclohexyl	methyl	>1000	>1000		2956.9
16	phenethyl	cyclohexyl	methyl	>1000	16.3 ± 1.2	>62	4.5
17	*p*-methoxybenzyl	cyclohexyl	methyl	>1000	35.8 ± 2.1	>28	12.1
18	*p*-fluorbenzyl	cyclohexyl	H	384.1 ± 25.3	13.0 ± 1.4	29	73.7
19	benzyl	cyclohexyl	H	>1000	48.6 ± 12.0	>21	102.8
20	ethylmorph	cyclohexyl	H	>1000	67.2 ± 11.6	>15	57.8
24	*o*-fluorobenzyl	cyclohexyl	methyl	>1000	>1000		1013.7
25	ethylmorph	4-methylcyclohexyl	methyl	>1000	>1000		777.4
26	benzyl	cyclohexyl	methyl	>1000	>1000		1763.6
29	*o*-fluorobenzyl	cyclohexyl	*o*-fluorobenzyloxy	NT	NT		1312.6
33	ethylmorph	4-methylcyclohexyl	Cl	>1000	40.5 ± 7.7	>25	125.4
38	ethylmorph	cyclohexyl	H	NT	NT		70.0
39	benzyl	cyclohexyl	H	>1000	4.8 ± 0.4	>210	39.4
40	ethylmorph	cyclohexyl	Cl	>1000	3.3 ± 0.4	>303	17.9
SDEP							0.69
41[32]	ethylmorph	cyclopentyl	methyl	>1000	50 ± 4	>20	
42[32]	benzyl	cyclohexyl	methyl	127 ± 10	10 ± 0.5	13	
ACEA				3.9 ± 0.2	120.8 ± 14.5	0.03	
JWH-133				458.0± 15.1	65 ± 8.7	7.0	

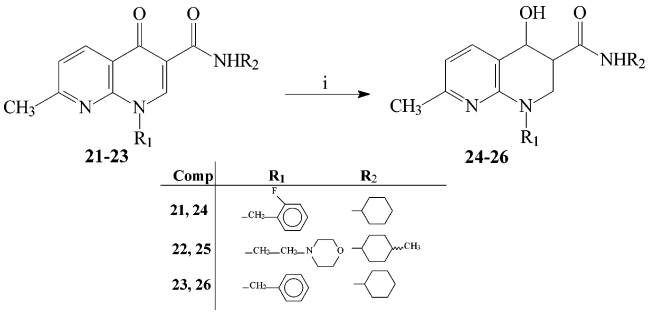
[a] Affinity of compounds for CB_1 receptor was evaluated using mouse brain membranes and [^{3}H]CP-55,940. [b] Affinity of compounds for CB_2 receptor was evaluated using mouse spleen and [^{3}H]CP-55,940. [c] NT = not tested because of insolubility in the solvent normally used in binding assays.

Scheme 1. Synthesis of N_1-Substituted 1,8-Naphthyridin-4(1*H*)-on-3-carboxamide Derivatives **6-20**[a]



[a] Reagents and conditions: (i) DMF, NaH, R_3Cl; (ii) MeOH, H_2, Pd/C, 3 h.

Scheme 2. Synthesis of N_1-Substituted 4-Hydroxy-7-methyl-1,2,3,4-tetrahydro-1,8-naphthyridin-3-carboxamides **24**−**26**[a]



[a] Reagents and conditions: (i) $NaBH_4$, room temp, 12 h.

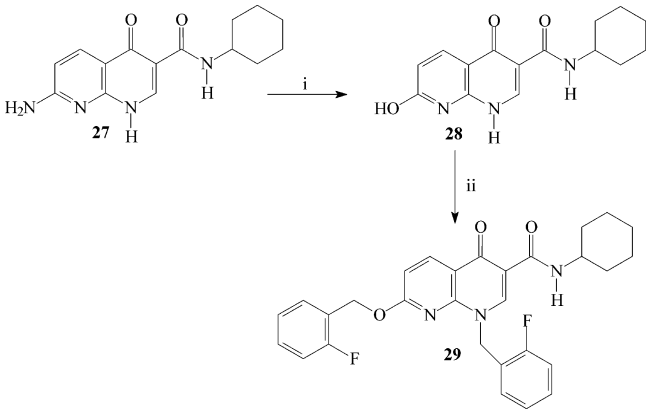
interaction inside the CB_2 receptor, we synthesized and tested some new compounds characterized by the presence of a hydroxy group in position 4 of the naphthyridine nucleus, instead of the carbonyl oxygen atom, and by partial removal of the aromaticity of the cycle (for which, as shown in Table 1, virtual screening predicted a CB_2 affinity greater than 750 nM).

Chemistry

The compounds described in this study are shown in Table 1, and their syntheses are outlined in Schemes 1−5. The treatment of carboxamide derivative **1**, **2**, **3**, **4**,[34] and **5** in anhydrous DMF with NaH for 1 h and then with the appropriate benzyl chloride or arylalkyl chloride or 4-(2-chloroethyl)-morpholine provided the desired 1,8-naphthyridin-4-one derivatives **6**−**20** (Scheme 1). 1-(2-Chloroethyl)-4-phenylpiperazine, which was needed to prepare **15**, was synthesized following the method reported in the literature.[36] The carboxamide **5** was obtained by dehalogenation of **4**[34] with H_2 in the presence of Pd/C as a catalyst.

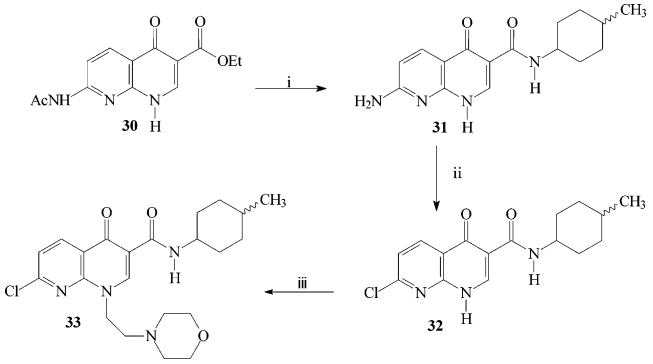
The reaction of the 1,8-naphthyridin-4-one derivatives **21**−**23**[34] with sodium borohydride in anhydrous ethanol gave the

Scheme 3. Synthesis of 1-(*o*-Fluorbenzyl)-7-(*o*-fluorobenzyloxy)-1,8-naphthyridin-4(1*H*)-on-3-carboxamide Derivative **29**[a]



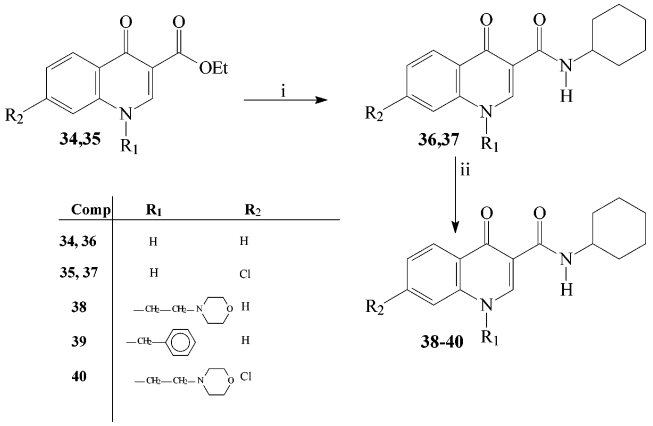
[a] Reagents and conditions: (i) $NaNO_2$, H_2SO_4, 4 h, room temp; (ii) *o*-fluorobenzyl chloride, NaH, DMF, 72 h, 80 °C.

Scheme 4. Synthesis of 7-Chloro-1,8-naphthyridin-4(1*H*)-on-3-carboxamide Derivative **33**[a]



[a] Reagents and conditions: (i) 4-methylcyclohexylamine, 120 °C, 24 h; (ii) $NaNO_2$, HCl, 40 °C, 3 h; (iii) 4-(2-chloroethyl)morpholine, NaH, DMF, 50 °C, 24 h.

Scheme 5. Synthesis of N-Substituted Quinolin-4(1*H*)-on-3-Carboxamides **38**−**40**[a]



[a] Reagents and conditions: (i) cyclohexylamine, 120 °C, 24 h; (ii) NaH, DMF, R_1Cl, 50 or 80 °C, 24 h.

N_1-substituted 4-hydroxy-7-methyl-1,2,3,4-tetrahydro-1,8-naphthyridin-3-carboxamides **24**−**26** (Scheme 2). As reported in Scheme 3, the diazotization of compound **27**[34] with $NaNO_2$ in aqueous 96% sulfuric acid gave the 7-hydroxy derivative **28**, which, by reaction with *o*-fluorobenzyl chloride under the same conditions described above, gave *N*-cyclohexyl-1-(*o*-fluorbenzyl)-7-(*o*-fluorobenzyloxy)-1,8-naphthyridin-4(1*H*)-on-3-carboxamide (**29**).

Table 2. Effect of 1,8-Naphthyridine Derivatives **7** and **8** on [^{35}S]GTPγS Binding in Mouse Brain Membranes

compd	R_1	R_2	R_3	EC_{50} [a] (nM)	E_{max} [a] (%)
7	*p*-fluorobenzyl	4-methylcyclohexyl	methyl	27 ± 4.7*	172 ± 9.6
8	*o*-fluorobenzyl	4-methylcyclohexyl	methyl	138 ± 21**	161 ± 2.3
WIN-55,212-2				204 ± 24	187 ± 19

[a] Data are the mean ± SEM of at least four experiments, each performed in duplicate. Compound-mediated [^{35}S]GTPγS binding data represent percentage of stimulation over basal values (set as 100%). E_{max} and EC_{50} were determined by nonlinear regression curve fit (GraphPad Prism). ANOVA: $F(2,8) = 21.97$, $P < 0.002$; (*) $P < 0.01$ with respect to WIN-55212,2 and **7**; (**) $P < 0.05$ with respect to WIN-55212,2 (Newman−Keuls test).

The 7-acetamido-1,8-naphthyridin-4(1*H*)-on-3-carboxylic acid ethyl ester **30**[37] was heated at 120 °C in a sealed tube with 4-methylcyclohexylamine (Scheme 4). Under these conditions, the hydrolysis of the acetamido group also takes place, and thus, the 7-amino-3-carboxamide derivative **31** was obtained. Diazotization of this compound carried out in aqueous 37% hydrochloride acid afforded the 7-chloro derivative **32**, which, by reaction with 4-(2-chloroethyl)morpholine at 50 °C, gave the desired compound **33** (Scheme 4). As reported in Scheme 5, the reaction of quinolin-4(1*H*)-on-3-carboxylic acid ethyl ester **34** or **35**[38] in a sealed tube with cychlohexylamine at 120 °C afforded the corresponding 3-carboxamide derivatives **36** or **37**, respectively, which by treatment with NaH and then with benzyl chloride or 4-(2-chloroethyl)morpholine gave the desired compounds **38**−**40**.

Results and Discussion

Pharmacology. Affinities at CB_1 and CB_2 receptors for the 1,8-naphthyridin-4(1*H*)-on-3-carboxamide derivatives **6**−**20**, **24**−**26**, **29**, and **33** and for the quinolin-4(1*H*)-on-3-carboxamide derivatives **38**−**40** were determined by measuring their ability to displace [^{3}H]CP-55,940 from its binding site in a membrane preparation from mouse brain (minus cerebellum) and mouse spleen homogenate, respectively. [^{3}H]CP-55,940 binding was carried out following a modified version of the method previously described.[39] The results of these determinations are summarized in Table 1. The K_i values of ACEA and JWH-133, as reference compounds at the CB_1 and CB_2 receptors, respectively, are also included in Table 1.

Finally, the question of the 1,8-naphthyridine-4-one derivatives functionality at the CB_1 and CB_2 receptors was investigated by using a [^{35}S]GTPγ binding assay[40,41] and functional studies on human basophils, respectively.[42,43]

CB_1 Receptor Affinity. The results reported in Table 1 for the 1,8-naphthyridin-4(1*H*)-on-3-carboxamide derivatives **6**−**20**, **29**, and **33** show that, as previously reported,[34] the compounds with an ethylmorpholino group in position 1 (**20** and **33**), regardless of the nature of the carboxyamido substituent in position 3 and of the substituent in position 7 of the heterocyclic nucleus, exhibited a poor affinity, with K_i values greater than 1000. Analogously, compounds **15**, **16**, and **17**, which bear in position 1 of the naphthyridine nucleus a 1-ethyl-4-phenylpiperazinyl group, a phenethyl group, and a *p*-methoxybenzyl group, respectively, possess a very low affinity, with K_i values greater than 1000. The presence in position 1 of a benzyl group, whether substituted or not, led to compounds with an interesting affinity. In particular, the *p*-fluorobenzyl derivatives **7** and **10** showed the highest affinity toward the CB_1 receptor, with K_i of 8.7 and 4.3 nM, respectively.

As regards the structural modifications in position 3 of the 1,8-naphthyridine nucleus, the substitution of the carboxycyclohexylamide group with a carboxy-4-methylcyclohexylamide group or a carboxycyclohepthylamide group leads to compounds that exhibit an increase in affinity toward the CB_1 receptor, as is clear from a comparison of compounds **7**−**11** with the corresponding 3-carboxycyclohexylamide derivatives previously studied.[34]

Furthermore, the substitution of the methyl group in position 7 of the 1,8-naphthyridine nucleus with an atom of chlorine, or the lack of any substituent in the same position, reduces the CB_1 receptor affinity, as can be seen from a comparison of compounds **12**−**14**, **18**−**20**, and **33** with the corresponding 7-methyl-1,8-naphthyridine derivatives previously studied.[34]

Finally, the 4-hydroxy-1,2,3,4-tetrahydro-1,8-naphthyridine derivatives **24**−**26** and the quinolin-4(1*H*)-one derivatives **39** and **40** lack any affinity toward the CB_1 receptor, with a K_i value greater than 1000.

These results show that some of the compounds studied possess an interesting affinity at the CB_1 receptor. In particular, the 1,8-naphthyridine derivatives **7**, **8**, and **10** exhibit a considerable affinity but are not selective for this receptor.

Influence of CB_1 Ligands on [^{35}S]GTPγS Binding. Among all compounds showing a high affinity for CB_1 receptors, **7** and **8** were selected with the aim of investigating their agonistic or antagonistic functions at the CB_1 receptor by means of a [^{35}S]-GTPγS binding assay, using mouse brain membranes. This assay provides a functional measure of the interaction of the receptor and the G protein. The first step in the activation of intracellular signaling by the G-protein-coupled receptor is the induction of an exchange of GDP for GTP on the guanine nucleotide binding site of the α subunit of a heterotrimeric G protein. The effects of various cannabinoid receptor agonists on GDP-GTP exchange can be determined from agonist-induced binding of the non-hydrolyzable GTP analogue [^{35}S]GTPγS.[40,41]

WIN-55,212-2, a CB_1 receptor agonist, was used as the reference compound. [^{35}S]GTPγS binding was stimulated in a concentration-dependent and saturable manner by **7**, **8**, and WIN-55,212-2 (see Table 2). Maximal stimulation (E_{max}) of [^{35}S]GTPγS binding by WIN-55,212-2 was 187 ± 19%, with an EC_{50} of 204 ± 24 nM. The E_{max} values for **7** and **8** were 172 ± 9.6% and 161 ± 2.3%, respectively, with no significant difference from the E_{max} produced by WIN-55,212-2 (ANOVA: $F(2,8) = 1.04$, $P = 0.4068$). The EC_{50} values for **7** and **8** were 27 ± 4.7 and 138 ± 21 nM, respectively, both of which were more potent than WIN-55,212-2 ($F(2,8) = 21.97$, $P < 0.002$]. These findings clearly indicate that **7** and **8** are agonists at the CB_1 receptor. Furthermore, we may hypothesize that the other 1,8-naphthyridin-4(1*H*)-on-3-carboxamide derivatives **6**, **9**−**20**, **29**, and **33**, which are structurally analogous to **7** and **8**, possess the same kind of activity.

CB_2 Receptor Affinity. The results obtained indicate that, in agreement with previous report,[34] the *N*-benzyl-1,8-naphthyridine derivatives possess a higher affinity than the *N*-ethylmorpholino derivatives, as is clear from a comparison of compounds **7**, **8**, **18**, and **19** with **20** and **33**. For the *N*-benzyl-1,8-naphthyridine derivatives (**7**−**14**, **17**−**19**), the presence of an atom of fluorine on the benzyl increases the affinity, above all if the substitution is in the para position. In particular, the *p*-fluorobenzyl-1,8-naphthyridine derivatives **7** ($K_i = 1.4$ nM)

and **10** (K_i = 1.0 nM) proved to be the compounds with the highest affinity in this series. Furthermore, the *N*-phenethyl-1,8-naphthyridine derivative **16** showed a good affinity, with a K_i of 16.3 nM. In contrast, the compound bearing a 1-ethyl-4-phenylpiperazinyl group in position 1 of the naphthyridine nucleus (**15**) possesses a very low affinity, with a K_i value greater than 1000.

As had previously been found for the CB_1 receptor, the substitution of the carboxycyclohexylamide group in position 3 of the 1,8-naphthyridine nucleus with a carboxy-4-methylcyclohexylamide or a carboxycyclohepthylamide group determines an increase in the affinity toward the CB_2 receptor, as confirmed by a comparison of compounds **7**−**11** with the corresponding 3-carboxycyclohexylamide derivatives previously studied.[34]

Furthermore, the substitution of the methyl group in position 7 of the 1,8-naphthyridine nucleus with an atom of chlorine (**12**−**14** and **33**), or the lack of any substituent in the same position (**18**−**20**), generally determines the maintenance or an increase in the affinity (except for compounds **13** and **19**, which showed a 4-fold and 5-fold decrease in affinity compared with the methyl analogues[34]).

As in the case of the CB_1 receptor, 4-hydroxy-1,2,3,4-tetrahydro-1,8-naphthyridine derivatives **24**−**26** exhibit a poor affinity toward the CB_2 receptor, with K_i values greater than 1000.

Finally, compounds **39** and **40** in which the naphthyridin-4-one system was substituted by the quinoline-4-one, possess a remarkable affinity, as suggested by virtual screening, with K_i values of 4.8 and 3.3 nM, respectively.

Very recently, quinolin-4-one derivatives,[44] with structures similar to those of **39** and **40**, were synthesized; however, our compounds were characterized by different substituents in positions 1, 3, and 7 of the heterocyclic nucleus.

Most compounds showed a good affinity for the CB_2 receptor. In particular, the 1,8-naphthyridin-4-one derivatives **7**−**11** and the quinolin-4-one derivatives **39** and **40** possess a very high affinity, with K_i values less than 10 nM.

As for the selectivity toward the CB_2 receptor, the 1,8-naphthyridine derivatives **16**−**19** and **33** show good selectivity, with $K_i(CB_1)/K_i(CB_2) > 21$. Furthermore, quinolin-4-one derivatives **39** and **40** exhibited very significant CB_2 receptor selectivity, with $K_i(CB_1)/K_i(CB_2)$ greater than 210 and greater than 303, respectively, which were higher than reports for the analogous compounds ($K_i(CB_1)/K_i(CB_2)$ = 143 for the best compound).[44]

Test for CB_2 Functionality. To assess the functionality of the studied compounds at CB_2 receptors, functional studies on human basophils were performed. Activation of CB_2 receptors is known to down-regulate the immunological activation of guinea pig mast cells and human basophils.[42,43]

N-Cyclohexyl-1-benzylquinolin-4(1*H*)-on-3-carboxamide (**39**) and *N*-cyclopentyl-7-methyl-1-(2-morpholin-4-ylethyl)-1,8-naphthyridin-4(1*H*)-on-3-carboxamide (**41**),[34] which is structurally analogous to the 1,8-naphthyridin-4(1*H*)-on-3-carboxamide derivatives studied in this paper, were used for functional studies. Human basophils, pretreated with these compounds (1 nM to 1 μM, 30 min of preincubation, 37 °C), showed a reduced expression of CD203c in response to immunological stimulation (anti-IgE 1 μg/mL, 30 min, 37 °C). The inhibition was reversed by the selective CB_2 antagonist SR 144528 (SR, 100 nM, 30 min of preincubation, 37 °C) but not by AM251 (AM, 100 nM, 30 min of preincubation, 37 °C), a selective CB_1 antagonist (see Figure 2).

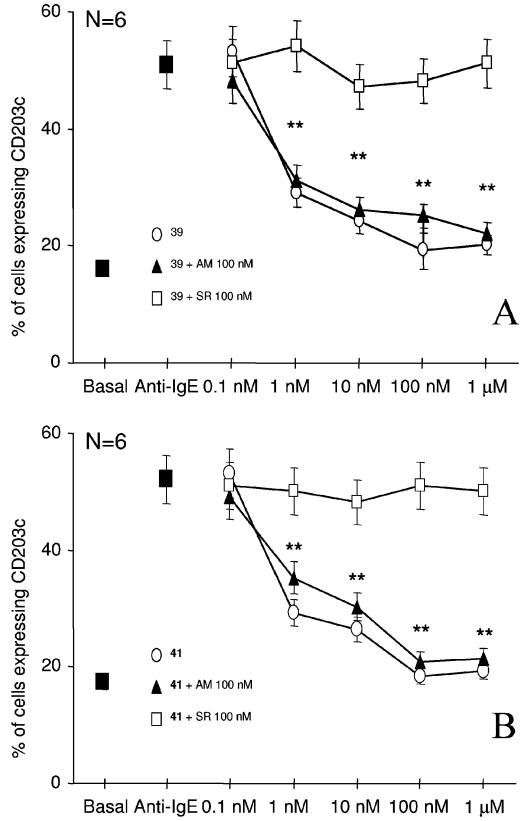


Figure 2. Expression of CD203c by human basophils activated with anti-IgE (1 μg/mL) is reduced by compounds **39** (A) and **41** (B) in a dose-related fashion. The inhibitory effect of compounds **39** and **41** on basophil CD203c expression is reverted by SR 144528 (100 nM), a CB_2 antagonist, but not by AM 251 (100 nM), a CB_1 antagonist. The values are the mean ± SEM of six independent experiments performed in triplicate: (**) $P < 0.001$.

These results show that compounds **39** and **41** exert a CB_2-mediated inhibitory action on immunological human basophil activation. Furthermore, we hypothesize that both the quinolin-4(1*H*)-on-3-carboxamide **40** and the 1,8-naphthyridin-4(1*H*)-on-3-carboxamide derivatives reported in this work possess the same kind of activity.

Molecular Modeling. With the AUTODOCK 3.0 program,[45] the compounds shown in Table 1 were docked into the CB_2 receptor, and their activities were predicted on the basis of the published computational model[35] (see Experimental Section for details). As indicated by the SDEP value (0.69) reported in Table 1, there was quite a good correlation between the experimental and the calculated K_i. Furthermore, all the ligands with a CB_2 affinity higher than 1000 nM were predicted to have an affinity higher than 750 nM.

As suggested by our model, the compound with the best CB_2 affinity was **10**. The docking showed that the CB_2 binding pocket was delimited by TM3, TM4, TM5, and TM6, and the cycloheptyl substituent of compound **10** was directed toward the intracellular side of the receptor, interacting with W5.43-(194) and F5.46(197) (see Figure 3). As for the *p*-F-benzyl group, it interacted in a lipophilic pocket formed by L3.27-(108), P4.60(168), and L4.61(169), and the fluorine atom formed an H bond with S3.31(112).

The docking of compound **40** (see Figure 4), the most CB_2 selective ligand in this series, also revealed that the presence of the chlorine atom might contribute to the increase in CB_2 affinity and CB_2 selectivity, since it might interact with the nonconserved S6.58(268) (aspartate in the CB_1 receptor).

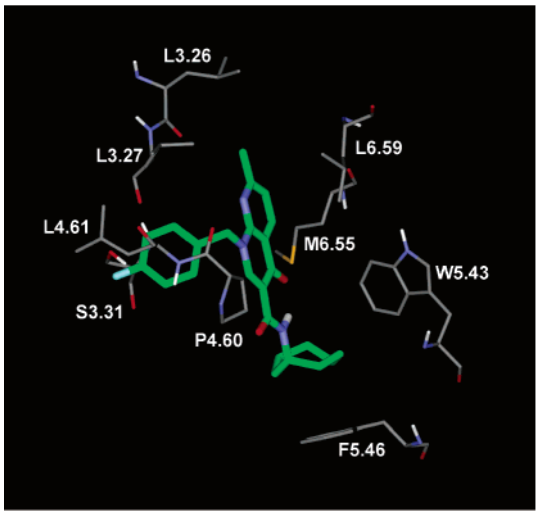


Figure 3. Compound **10** docked into the CB_2 receptor model.

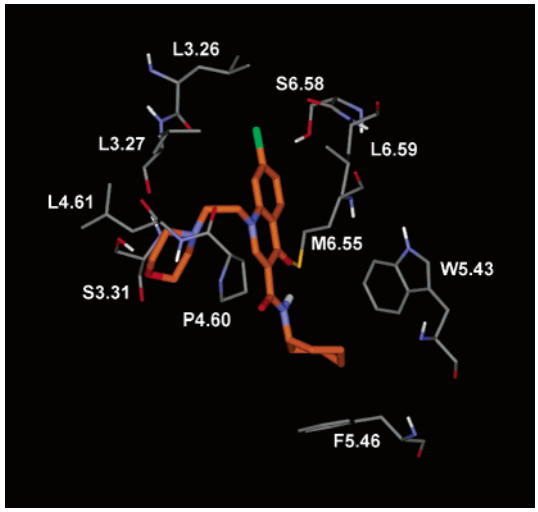


Figure 4. Compound **40** docked into the CB_2 receptor model.

Finally, the docking of the inactive compounds **24**−**26** revealed that the lack of planarity determines a different position of the central lipophilic core, determining weaker interactions with the receptor. As shown in Figure 5, the central core of **26**, compared with the position of the naphthyridine ring of *N*-cyclohexyl-7-methyl-1-benzyl-1,8-naphthyridin-4(1*H*)-on-3-carboxamide (**42**),[34] was shifted further way from TM3 and directed toward TM5, determining weaker interactions with M6.55(265) and L3.27(107); furthermore, this arrangement determined a weaker interaction of the cyclohexyl ring with F5.46(197), at a distance of 4.7 Å (while for **42** the distance is 4.0 Å).

Conclusions

In the present study, by means of a structure-based approach, we tried to improve the activity and selectivity of 1,8-naphthyridin-4(1*H*)-on-3-carboxamide derivatives, which had proved to be a new class of CB_2 ligands.[34]

For this purpose, following the suggestion obtained from the docking of ligands into a CB_2 receptor model,[35] new 1,8-naphthyridin-4(1*H*)-on-3-carboxamide derivatives and quinolin-4(1*H*)-on-3-carboxamide derivatives were designed, synthesized, and tested on the CB_1 and CB_2 receptors.

Some of these compounds showed a good selectivity toward the CB_2 receptor and a high CB_2 affinity, in agreement with

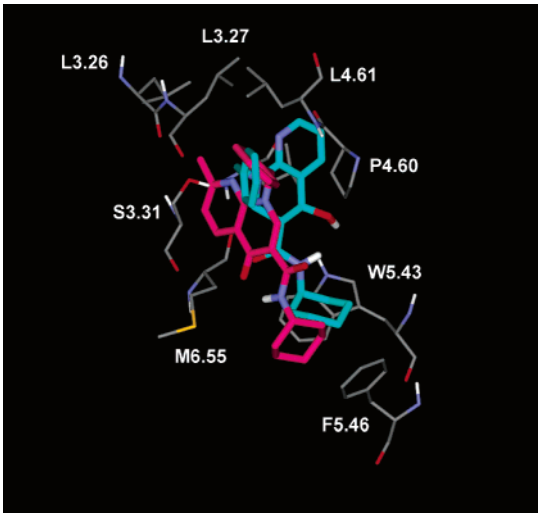


Figure 5. Compounds **42** (magenta) and **25** (sky-blue) docked into the CB_2 receptor model.

the values predicted by the docking study. In particular, compound **10**, which presented *p*-fluorobenzyl and carboxycycloheptylamide substituents bound in the 1 and 3 positions of the 1,8-naphthyiridine-4-one nucleus, showed a high CB_2 affinity with a K_i value of 1 nM.

The substitution of the naphthyridine-4-one nucleus with the quinoline-4-one system determined a general increase in CB_2 affinity. For compound **40**, the good CB_2 affinity was also accompanied by a high selectivity toward the CB_2 receptor, and the docking studies suggested that the interaction of the chlorine atom in position 7 of the heterocyclic nucleus with the nonconserved residue S6.58(268) in the CB_2 receptor seemed to be one of the reasons for the high selectivity value.

Finally, the low affinity shown by the new 4-hydroxytetrahydro-1,8-naphthyridine derivatives confirmed the hypothesis about the fundamental role of the presence of a planar pseudocycle with the naphthyridine nucleus obtained by an intramolecular H bond between the carbonylic oxygen and the amidic NH.

These results provide an interesting addition to currently available structure−activity relationships for cannabinoid agonist ligands, opening up a new field of research for designing new cannabinoid receptor agonists characterized by a high selectivity toward the CB_2 receptor.

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 δ units from TMS as an internal standard. Mass spectra were obtained with a Hewlett-Packard MS system 5988. Elemental analysis results (C, H, N) were within ±0.4% of theoretical values and were performed on a Carlo Erba elemental analyzer model 1106 apparatus.

General Procedure for the Synthesis of N_1-Substituted 1,8-Naphthyridine Derivatives (6−20 and 33). An amount of 1.2 mmol of NaH was added to a solution of 1 mmol of **5** or of 7-methyl- (**1**, **2**, and **3**[34]) or of 7-chloro-1,8-naphthyridine-3-carboxamide derivative (**4**[34] and **32**) in 10 mL of dry *N*,*N*-dimethylformamide. After 1 h, the appropriate chloride (1 mmol) was added and the mixture was stirred for 24 h at room temperature for compounds **6**−**11**, **15**, **18**, and **19** or at 50 °C for compounds **12**−**14**, **16**, **17**, and **33** or at 50 °C for 48 h for **20**. The reaction mixture, after cooling in the cases of **12**−**14**, **17**, and **33**, was treated

with water, and the precipitate formed was collected by filtration, whereas for **16**, the solvent was evaporated in vacuo and the solid obtained was treated with water and collected by filtration.

***N*-(4′-Methylcyclohexyl)-1-benzyl-7-methyl-1,8-naphthyridin-4(1*H*)-on-3-carboxamide (6).** Yield 0.310 g, 80%; mp 239−241 °C (crystallized from ethyl acetate); MS *m/z* 389 (M^+); ^{1}H NMR δ 10.20, 9.75 (2d, 1H, NH), 9.08, 9.06 (2s, 1H, H_2), 8.52, 8.60 (2d, 1H, H_5), 7.48 (d, 1H, H_6), 7.33 (m, 5H, Ar), 5.80 (s, 2H, CH_2), 4.12, 3.69 (2m, 1H, CH), 2.63 (s, 3H, CH_3), 2.20−0.86 (m, 12H, cyclohexyl + CH_3). Anal. ($C_{24}H_{27}N_3O_2$) C, H, N.

***N*-(4′-Methylcyclohexyl)-1-(*p*-fluorobenzyl)-7-methyl-1,8-naphthyridin-4(1*H*)-on-3-carboxamide (7).** Yield 0.270 g, 66%; mp 167−169 °C (crystallized from *n*-hexane); MS *m/z* 407 (M^+); ^{1}H NMR δ 10.18, 9.77 (2d, 1H, NH), 9.12, 9.10 (2s, 1H, H_2), 8.59, 8.52 (2d, 1H, H_5), 7.50 (m, 3H, Ar + H_6), 7.16 (m, 2H, Ar), 5.77 (s, 2H, CH_2), 4.18, 3.70 (2m, 1H,CH), 2.65 (s, 3H, CH_3), 1.98−0.86 (m, 12H, cyclohexyl + CH_3). Anal. ($C_{24}H_{26}FN_3O_2$) C, H, N.

***N*-(4′-Methylcyclohexyl)-1-(*o*-fluorobenzyl)-7-methyl-1,8-naphthyridin-4(1*H*)-on-3-carboxamide (8).** Yield 0.245 g, 60%; mp 183−185 °C (crystallized from *n*-hexane); MS *m/z* 407 (M^+); ^{1}H NMR δ 10.18, 9.77 (2d, 1H, NH), 9.09, 9.07 (2s, 1H, H_2), 8.58, 8.52 (2d, 1H, H_5), 7.46 (d, 1H, H_6), 7.36−7.14 (m, 4H, Ar), 5.81 (s, 2H, CH_2), 4.15, 3.70 (2m, 1H, CH), 2.61 (s, 3H, CH_3), 2.00−0.86 (m, 12H, cyclohexyl + CH_3). Anal. ($C_{24}H_{26}FN_3O_2$) C, H, N.

***N*-Cycloheptyl-1-benzyl-7-methyl-1,8-naphthyridin-4(1*H*)-on-3-carboxamide (9).** Yield 0.225 g, 58%; mp 198−200 °C (crystallized from cyclohexane); MS *m/z* 389 (M^+); ^{1}H NMR δ 9.88 (d, 1H, NH), 9.07 (s, 1H, H_2), 8.55 (d, 1H, H_5), 7.48 (d, 1H, H_6), 7.33 (m, 5H, Ar), 5.79 (s, 2H, CH_2), 4.15 (m, 1H, CH), 2.64 (s, 3H, CH_3), 1.85−1.39 (m, 12H, cycloheptyl). Anal. ($C_{24}H_{27}N_3O_2$) C, H, N.

***N*-Cycloheptyl-1-(*p*-fluorobenzyl)-7-methyl-1,8-naphthyridin-4(1*H*)-on-3-carboxamide (10).** Yield 0.300 g, 75%; mp 194−196 °C (crystallized from hexane); MS *m/z* 407 (M^+); ^{1}H NMR δ 9.91 (d, 1H, NH), 9.10 (s, 1H, H_2), 8.55 (d, 1H, H_5), 7.48 (m, 3H, Ar +H_6), 7.16 (m, 2H, Ar), 5.77 (s, 2H, CH_2), 4.15 (m, 1H, CH), 2.64 (s, 3H, CH_3), 1.85−1.39 (m, 12H, cycloheptyl). Anal. ($C_{24}H_{26}FN_3O_2$) C, H, N.

***N*-Cycloheptyl-1-(*o*-fluorbenzyl)-7-methyl-1,8-naphthyridin-4(1*H*)-on-3-carboxamide (11).** Yield 0.255 g, 63%; mp 188−189 °C (crystallized from cyclohexane); MS *m/z* 407 (M^+); ^{1}H NMR δ 9.90 (d, 1H, NH), 9.07 (s, 1H, H_2), 8.55 (d, 1H, H_5), 7.46 (d, 1H, H_6), 7.32−7.14 (m, 4H, Ar), 5.81 (s, 2H, CH_2), 4.15 (m, 1H, CH), 2.61 (s, 3H, CH_3), 1.85−1.39 (m, 12H, cycloheptyl). Anal. ($C_{24}H_{26}FN_3O_2$) C, H, N.

***N*-Cyclohexyl-1-benzyl-7-chloro-1,8-naphthyridin-4(1*H*)-on-3-carboxamide (12).** Yield 0.275 g, 70%; mp 258−260 °C (crystallized from hexane); MS *m/z* 395 (M^+); ^{1}H NMR δ 9.72 (d, 1H, NH), 9.12 (s, 1H, H_2), 8.66 (d, 1H, H_5), 7.67 (d, 1H, H_6), 7.32 (m, 5H, Ar), 5.73 (s, 2H, CH_2), 3.85 (m, 1H, CH), 1.85−1.32 (m, 10H, cyclohexyl). Anal. ($C_{22}H_{22}ClN_3O_2$) C, H, N.

***N*-Cyclohexyl-7-chloro-1-(*p*-fluorbenzyl)-1,8-naphthyridin-4(1*H*)-on-3-carboxamide (13).** Purified by flash chromatography (ethyl acetate/hexane, 5:6), yield 0.100 g, 25%; mp 198−200 °C (crystallized from cyclohexane); MS *m/z* 413 (M^+); ^{1}H NMR δ 9.72 (d, 1H, NH), 9.14 (s, 1H, H_2), 8.65 (d, 1H, H_5), 7.68 (d, 1H, H_6), 7.44 (m, 2H, Ar), 7.18 (m, 2H, Ar), 5.71 (s, 2H, CH_2), 3.89 (m, 1H, CH), 1.90−1.17 (m, 10H, cyclohexyl). Anal. ($C_{22}H_{21}ClFN_3O_2$) C, H, N.

***N*-Cyclohexyl-7-chloro-1-(*o*-fluorbenzyl)-1,8-naphthyridin-4(1*H*)-on-3-carboxamide (14).** Yield 0.300 g, 73%; mp 198−200 °C (crystallized from cyclohexane); MS *m/z* 413 (M^+); ^{1}H NMR δ 9.72 (d, 1H, NH), 9.11 (s, 1H, H_2), 8.65 (d, 1H, H_5), 7.67 (d, 1H, H_6), 7.41−7.12 (m, 4H, Ar), 5.76 (s, 2H, CH_2), 3.85 (m, 1H, CH), 1.98−1.32 (m, 10H, cyclohexyl). Anal. ($C_{22}H_{21}ClFN_3O_2$) C, H, N.

***N*-Cyclohexyl-7-methyl-1-[2-(4-phenylpiperazin-1-yl)ethyl]-1,8-naphthyridin-4(1*H*)-on-3-carboxamide (15).** Purified by flash chromatography (ethyl acetate/hexane/triethylamine, 10:1:0.2), yield 0.120 g, 25%; mp 147−149 °C (crystallized from hexane); MS *m/z* 473 (M^+); ^{1}H NMR δ 9.87 (d, 1H, NH), 8.94 (s, 1H, H_2), 8.53 (d, 1H, H_5), 7.46 (d, 1H, H_6), 7.18 (m, 2H, Ar), 6.90−6.71 (m, 3H, Ar), 4.70 (m, 2H, CH_2), 3.80 (m, 1H, CH), 3.03 (m, 4H, piperazinyl), 2.73 (m, 2H, CH_2), 2.66 (s, 3H, CH_3), 2.60 (m, 4H, piperazinyl), 1.85−1.08 (m, 10H, cyclohexyl). Anal. ($C_{28}H_{35}N_5O_2$) C, H, N

***N*-Cyclohexyl-7-methyl-1-phenethyl-1,8-naphthyridin-4(1*H*)-on-3-carboxamide (16).** Purified by flash chromatography (ethyl acetate/hexane, 2:3), yield 0.110 g, 28%; mp 148−150 °C (crystallized from hexane); MS *m/z* 389 (M^+); ^{1}H NMR δ 9.86 (d, 1H, NH), 8.85 (s, 1H, H_2), 8.54 (d, 1H, H_5), 7.48 (d, 1H, H_6), 7.24 (m, 5H, Ar), 4.76 (m, 2H, CH_2), 3.90 (m, 1H, CH), 3.10 (m, 2H, CH_2), 2.69 (s, 3H, CH_3), 1.85−1.23 (m, 10H, cyclohexyl). Anal. ($C_{24}H_{27}N_3O_2$) C, H, N.

***N*-Cyclohexyl-7-methyl-1-(4-methoxybenzyl)-1,8-naphthyridin-4(1*H*)-on-3-carboxamide (17).** Purified by flash chromatography (ethyl acetate/hexane, 2: 1), 0.283 g, 70%; mp 170−172 °C (crystallized from hexane); MS *m/z* 405 (M^+); ^{1}H NMR: δ 9.90 (d, 1H, NH), 9.05 (s, 1H, H_2), 8.54 (d, 1H, H_5), 7.47 (d, 1H, H_6), 7.35 (d, 2H, Ar), 6.89 (d, 2H, Ar), 5.70 (s, 2H, CH_2), 3.80 (m, 1H, CH), 3.70 (s, 3H, OCH_3), 2.67 (s, 3H, CH_3), 1.90−1.30 (m, 10H, cyclohexyl). Anal. ($C_{24}H_{27}N_3O_3$) C, H, N.

***N*-Cyclohexyl-1-(*p*-fluorbenzyl)-1,8-naphthyridin-4(1*H*)-on-3-carboxamide (18).** Yield 0.185 g, 49%; mp 193−195 °C (crystallized from hexane); MS *m/z* 379 (M^+); ^{1}H NMR δ 9.80 (d, 1H, NH), 9.15 (s, 1H, H_2), 8.90 (dd, 1H, H_7), 8.67 (dd, 1H, H_5), 7.62 (m, 1H, H_6), 7.37 (m, 2H, Ar), 7.15 (m, 2H, Ar), 5.81 (s, 2H, CH_2), 3.85 (m, 1H, CH), 1.86−1.23 (m, 10H, cyclohexyl). Anal. ($C_{22}H_{22}FN_3O_2$) C, H, N.

***N*-Cyclohexyl-1-benzyl-1,8-naphthyridin-4(1*H*)-on-3-carboxamide (19).** Yield 0.200 g, 55%; mp 181−183 °C (crystallized from hexane); MS *m/z* 361 (M^+); ^{1}H NMR δ 9.95 (d, 1H, NH), 9.12 (s, 1H, H_2), 8.90 (dd, 1H, H_7), 8.70 (dd, 1H, H_5), 7.70 (m, 1H, H_6), 7.29 (m, 5H, Ar), 5.84 (s, 2H, CH_2), 3.85 (m, 1H, CH), 1.86−1.30 (m, 10H, cyclohexyl). Anal. ($C_{22}H_{23}N_3O_2$) C, H, N.

***N*-Cyclohexyl-1-(2-morpholin-4-yl-ethyl)-1,8-naphthyridin-4(1*H*)-on-3-carboxamide (20).** Yield 0.155 g, 40%; mp 142−144 °C (crystallized from hexane); MS *m/z* 384 (M^+); ^{1}H NMR δ 9.84 (d, 1H, NH), 8.98 (s, 1H, H_2), 8.90 (dd, 1H, H_7), 8.68 (dd, 1H, H_5), 7.60 (m, 1H, H_6), 4.66 (t, 2H, CH_2), 3.86 (m, 1H, CH), 3.49 (m, 4H, morpholine), 2.70 (m, 2H, CH_2), 2.44 (m, 4H, morpholine), 1.85−0.82 (m, 10H, cyclohexyl). Anal. ($C_{21}H_{28}N_4O_3$) C, H, N.

***N*-(4′-Methylcyclohexyl)-7-chloro-1-(2-morpholin-4-yl-ethyl)-1,8-naphthyridin-4(1*H*)-on-3-carboxamide (33).** Purified by flash chromatography (ethyl acetate/hexane, 1: 1), yield 0.130, 30%; mp 191−193 °C (crystallized from cyclohexane); MS *m/z* 432 (M^+); ^{1}H NMR δ 10.20, 10.00 (2d, 1H, NH), 8.65, 8,61 (2s, 1H, H_2), 8.20, 8,18 (2d, 1H, H_5), 6.87 (d, 1H, H_6), 4.51 (m, 2H, CH_2), 4.00 (m, 1H, CH), 3.51 (m, 4H, morpholine), 2.67 (m, 2H, CH_2), 2.44 (m, 4H, morpholine), 1.89−0.76 (m, 12H, cyclohexyl + CH_3). Anal. ($C_{22}H_{29}ClN_4O_3$) C, H, N.

***N*-Cyclohexyl-1,8-naphthyridin-4(1*H*)-on-3-carboxamide (5).** A solution of 7-chloronaphthyridine **4**[34] (0.40 g, 1.31 mmol) in methanol (20 mL) was submitted to hydrogenation in the presence of 10% Pd/C (0.04 g) at room pressure and temperature for 3 h. The catalyst was filtered off, and the solvent was evaporated to dryness under reduced pressure to give a residual solid, which was purified by flash chromatography (ethyl acetate) and crystallized from hexane to give **5** (0.110 g, 31%): mp 215−218 °C; ^{1}H NMR δ 9.85 (d, 1H, NH), 8.84 (dd, 1H, H_7), 8.68 (s, 1H, H_2), 8.65 (dd, 1H, H_5), 7.55 (m, 1H, H_6), 3.85 (m, 1H, CH), 1.86−1.22 (m, 10H, cyclohexyl). Anal. ($C_{15}H_{17}N_3O_2$) C, H, N.

General Procedure for the Preparation of N_1-Substituted 4-Hydroxy-7-methyl-1,2,3,4-tetrahydro-1,8-naphthyridin-3-carboxamides 24−26. $NaBH_4$ (0.30 g, 8 mmol) was added to a solution of the appropiate 7-methyl-1,8-naphthyridin-4(1*H*)-on-3-carboxamide derivatives **21**−**23**[34] (0.38 mmol) in absolute ethanol (7.5 mL), and the mixture was stirred at room temperature for 12 h. The organic solvent was evaporated from the reaction mixture under reduced pressure to obtain a residue, which was treated with H_2O. In the cases of **24** and **26**, the solid precipitate obtained was collected by filtration and purified by crystallization from hexane, whereas for **25** the mixture was extracted with chloroform, the

organic solution was dried ($MgSO_4$) and evaporated to dryness under reduced pressure, and the crude solid was purified by crystallization from cyclohexane.

***N*-Cyclohexyl-1-(*o*-fluorbenzyl)-4-hydroxy-7-methyl-1,2,3,4-tetrahydro-1,8-naphthyridin-3-carboxamide (24).** Yield 0.170 g, 87%; MS *m*/*z* 397 (M^+); ^{1}H NMR δ 7.82 (m, 1H, NH), 7.40 (d, 1H, H_5), 7.30−7.08 (m, 4H, Ar), 6.44 (d, 1H, H_6), 5.63 (d, 1H, OH), 4.95−4.69 (m, 3H, CH_2 + H_4), 3.56 (m, 1H, CH), 3.30 (m, 2H, $2H_2$), 2.23 (s, 3H, CH_3), 1.68−1.11 (m, 11H, cyclohexyl + H_3). Anal. ($C_{23}H_{28}FN_3O_2$) C, H, N.

4-Hydroxy-*N*-(4-methylcyclohexyl)-1-(2-morpholin-4-ylethyl)-7-methyl-1,2,3,4-tetrahydro-1,8-naphthyridin-3-carboxamide (25). Yield 0.100 g, 61%; MS *m*/*z* 416 (M^+).^{1}H NMR δ 7.81 (m, 1H, NH), 7.34 (d, 1H, H_5), 6.35 (d, 1H, H_6), 5.55 (m, 1H, OH), 4.61 (m, 1H, H_4), 3.78 (m, 1H, CH), 3.57−3.33 (m, 8H, morpholine + NCH_2 + $2H_2$), 2.45 (m, 6H, morpholine + CH_2), 2.22 (s, 3H, CH_3), 1.70−1.05 (m, 13H, cyclohexyl + CH_3 + H_3). Anal. ($C_{23}H_{36}N_4O_3$) C, H, N.

***N*-Cyclohexyl-1-benzyl-4-hydroxy-7-methyl-1,2,3,4-tetrahydro-1,8-naphthyridin-3-carboxamide (26).** Yield 0.080 g, 52%; MS *m*/*z* 379 (M^+); ^{1}H NMR: δ 7.48 (d, 1H, H_5), 7.31 (m, 5H, Ar), 6.48 (m, 1H, H_6), 6.00 (d, 1H, OH), 4.92 (m, 3H, CH_2 + H_4), 3.74 (m, 1H, CH), 3.40 (m, 2H, $2H_2$), 2.57 (m, 1H, H_3), 2.38 (s, 3H, CH_3), 1.84−0.83 (m, 10H, $5CH_2$). Anal. ($C_{23}H_{29}N_3O_2$) C, H, N.

***N*-Cyclohexyl-7-hydroxy-1,8-naphthyridin-4(1*H*)-on-3-carboxamide (28).** Sodium nitrite (0.55 g, 8.0 mmol) was added portionwise to a cooled solution (−10 °C) of 7-amino-1,8-naphthyridine-3-carboxamide **27**[34] (0.44 g, 1.6 mmol) in 7 mL of concentrated sulfuric acid. After standing for 4 h at room temperature, the mixture was poured over crushed ice and the pH was adjusted to 8 with aqueous concentrated ammonium hydroxide. The solid obtained was collected by filtration, washed with water, and purified by crystallization from toluene to obtain **28** (0.490 g, 94%): mp 303−305 °C; ^{1}H NMR δ 10.49 (d, 1H, NH), 8.45 (s, 1H, H_2), 8.10 (d, 1H, H_5), 6.25 (d, 1H, H_6), 3.78 (m, 1H, CH), 1.82−1.26 (m, 10H, cyclohexyl). Anal. ($C_{15}H_{17}N_3O_3$) C, H, N.

***N*-Cyclohexyl-1-(*o*-fluorobenzyl)-7-(*o*-fluorobenzyloxy)-1,8-naphthyridin-4(1*H*)-on-3-carboxamide (29).** NaH (0.05 g, 1.08 mmol, 50% in mineral oil) was added to a solution of 7-hydroxy-1,8-naphthyridine **28** (0.25 g, 0.87 mmol) in 6 mL of dry DMF. After 1 h, 2-fluorobenzyl chloride (0.125 g, 0.87 mmol) was added and the mixture was stirred for 3 days at 80 °C. After the mixture was cooled, water was added and the solid obtained was collected by filtration, purified by flash chromatography (ethyl acetate/hexane, 1:1), and crystallized from cyclohexane to give **29** (0.130 g, 30%): mp 198−200 °C; MS *m*/*z* 503 (M^+); ^{1}H NMR δ 9.94 (d, 1H, NH), 9.03 (s, 1H, H_2), 8.52 (d, 1H, H_5), 7.47−7.10 (m, 8H, 2 Ar), 7.03 (d, 1H, H_6), 5.80 (s, 2H, CH_2), 5.43 (s, 2H, CH_2), 3.85 (m, 1H, CH), 1.32 (m, 10H, cyclohexyl). Anal. ($C_{29}H_{27}F_2N_3O_3$) C, H, N.

***N*-(4-Methylcyclohexyl)-7-amino-1,8-naphthyridin-4(1*H*)-on-3-carboxamide (31).** A mixture of 1,8-naphthyridine-3-carboxylic acid ethyl ester **30**[37] (0.276 g, 1 mmol) and 4-methylcyclohexylamine (1.130 g, 10 mmol) was heated in a sealed tube at 120 °C for 24 h. After cooling, the reaction mixture was treated with ethyl ether to give a solid residue, which was collected by filtration and purified by crystallization from ethyl acetate to obtain **31** (0.250 g, 83%): mp 198−200 °C; ^{1}H NMR δ 10.45, 10.18 (2d, 1H, NH), 8.33 (s, 1H, H_2), 8.13 (d, 1H, H_5), 7.12 (s, 2H, NH_2), 6.55 (d, 1H, H_6), 3.80 (m, 1H, CH), 1.86−0.85 (m, 12H, cyclohexyl + CH_3). Anal. ($C_{16}H_{20}N_4O_2$) C, H, N.

***N*-(4-Methylcyclohexyl)-7-chloro-1,8-naphthyridin-4(1*H*)-on-3-carboxamide (32).** Sodium nitrite (0.34 g, 5.0 mmol) was added portionwise to a cooled solution (−5 °C) of 7-amino-1,8-naphthyridine-3-carboxamide **31** (0.3 g, 1.0 mmol) in 54.5 mL of concentrated hydrochloric acid. The mixture was stirred for 3 h at 40 °C and, after cooling, was poured over crushed ice. The pH was adjusted to 4−5 with aqueous concentrated ammonium hydroxide. The solid obtained was collected by filtration, washed with water, and purified by flash chromatography (ethyl acetate/hexane, 1:1) to obtain **32** (0.100 g, 31%): mp 271−273 °C (crystallized from ethyl acetate); ^{1}H NMR δ 10.00, 9.63 (2d, 1H, NH), 8.64 (m, 2H, H_2 + H_5), 7.62 (d, 1H, H_6), 3.80 (m, 1H, CH), 1.97−1.00 (m, 9H, cyclohexyl), 0.89, 0.92 (2d, 3H, CH_3). Anal. ($C_{16}H_{18}ClN_3O_2$) C, H, N.

***N*-Cyclohexyl-quinolin-4(1*H*)-on-3-carboxamide (36) and *N*-Cyclohexyl-7-chloroquinolin-4(1*H*)-on-3-carboxamide (37).** A mixture of 1 mmol of quinoline-3-carboxylic acid ethyl ester **34** or **35**[38] and 10 mmol of cyclohexylamine in a sealed tube was heated at 120 °C for 24 h. After cooling, the reaction mixture was treated with ethyl ether to give a solid residue, which was collected by filtration and purified by crystallization from ethyl acetate. **36**: yield 0.230 g, 88%; mp 112−114 °C; ^{1}H NMR δ 10.10 (d, 1H, NH), 8.72 (s, 1H, H_2), 8.24 (d, 1H, Ar), 7.70 (m, 2H, Ar), 7.47 (m, 1H, Ar), 3.82 (m, 1H, CH), 1.86−1.31 (m, 10H, $5CH_2$). Anal. ($C_{16}H_{18}N_2O_2$) C, H, N. **37**: yield 0.275 g, 90%; mp 132−135 °C; ^{1}H NMR δ 10.18 (d, 1H, NH), 8.72 (s, 1H, H_2), 8.21 (d, 1H, Ar), 7.69 (s, 1H, Ar), 7.40 (d, 1H, Ar), 3.81 (m, 1H, CH), 1.85−1.08 (m, 10H, cyclohexyl). Anal. ($C_{16}H_{17}ClN_2O_2$) C, H, N.

General Procedure for the Synthesis of N_1-Substituted *N*-Cyclohexylquinoline-3-carboxamide Derivatives 38−40. NaH (4.36 mmol, 50% in mineral oil) was added to a hot solution (50 °C) of *N*-cyclohexylquinoline-3-carboxamide derivatives **36** or **37** (0.92 mmol) in 9.2 mL of dry DMF. After 1 h, 4-(2-chloroethyl)-morpholine hydrochloride or benzyl chloride (0.92 mmol) was added, and the mixture was stirred for 24 h at 50 °C (**38** and **40**) or at 80 °C (**39**). After the mixture was cooled (3−5 °C), the addition of water caused the precipitation of the title compounds, which were purified by crystallization.

***N*-Cyclohexyl-1-(2-morpholin-4-ylethyl)-quinolin-4(1*H*)-on-3-carboxamide (38).** Yield 0.180 g, 50%; mp 169−170 °C (crystallized from ethyl acetate); MS *m*/*z* 383 (M^+); ^{1}H NMR δ 10.04 (d, 1H, NH), 8.80 (s, 1H, H_2), 8.34 (d, 1H, Ar), 7.90 (m, 2H, Ar), 7.52 (m, 1H, Ar), 4.58 (m, 2H, CH_2), 3.90 (m, 1H, CH), 3.49 (m, 4H, morpholine), 2.65 (m, 2H, CH_2), 2.42 (m, 4H, morpholine), 1.88−1.22 (m, 10H, cyclohexyl). Anal. ($C_{22}H_{29}N_3O_3$) C, H, N.

***N*-Cyclohexyl-1-benzylquinolin-4(1*H*)-on-3-carboxamide (39).** Yield 62%; mp 239−240 °C (crystallized from hexane); MS *m*/*z* 360 (M^+); ^{1}H NMR δ 10.05 (d, 1H, NH), 8.97 (s, 1H, H_2), 8.54 (d, 1H, Ar), 7.61−7.15 (m, 8H, Ar), 5.48 (s, 2H, CH_2), 3.98 (m, 1H, CH), 2.02−1.44 (m, 10H, cyclohexyl). Anal. ($C_{23}H_{24}N_2O_2$) C, H, N.

***N*-Cyclohexyl-7-chloro-1-(2-morpholin-4-ylethyl)-quinolin-4(1*H*)-on-3-carboxamide (40).** Yield 0.200 g, 52%; mp 229−231 °C (crystallized from ethyl acetate); MS *m*/*z* 417 (M^+). ^{1}H NMR δ 10.00 (d, 1H, NH), 8.80 (s, 1H, H_2), 8.35 (d, 1H, Ar), 8.05 (m, 1H, Ar), 7.60 (m, 1H, Ar), 4.50 (s, 2H, CH_2), 3.80 (m, 1H, CH), 3.47 (m, 4H, morpholine), 2.45 (m, 6H, CH_2 + morpholine), 1.40−1.05 (m, 10H, cyclohexyl). Anal. ($C_{22}H_{28}ClN_3O_3$) C, H, N.

Pharmacology. Male DBA/J2 mice (Charles River, Como, Italy), weighing 20−25 g, were maintained on ad libitum food and water and were used in all experiments. [^{3}H]CP-55,940 (168 Ci/mmol) and [^{35}S]GTPγS (1250 Ci/mmol) were purchased from Perkin-Elmer Life Science (Boston, MA). [^{3}H]CP-55,940 and WIN-55,-212-2 were obtained from Tocris (Ballwin, MO). Guanosine 5′-diphosphate (GDP) and guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) were obtained from Sigma/RBI (St. Louis, MO). For biochemical experiments, drugs were dissolved in dimethyl sulfoxide (DMSO). DMSO concentration in the different assays never exceeded 0.1% (v/v) and had no effects on [^{3}H]CP-55,940 binding and [^{35}S]GTPγS binding assay.

[^{3}H]CP-55,940 Binding Assay. Mice were sacrificed by decapitation, and the brain (minus cerebellum) and spleen were rapidly removed and placed on an ice-cold plate. After thawing, tissues were homogenated in 20 volumes (w/v) of ice-cold TME buffer (50 mM Tris-HCl, 1 mM EDTA, and 3 mM $MgCl_2$, pH 7.4). The homogenates were centrifuged at 1000*g* for 10 min at 4 °C, and the resulting supernatants were centrifuged at 45000*g* for 30 min at 4 °C. Aliquots of membranes were frozen at −80 °C until the day of experiment.

The Bradford[46] protein assay was used for protein determination using bovine serum albumin (BSA) as a standard in accordance with the supplier protocol (Bio-Rad, Milan, Italy).

[^{3}H]CP-55,940 binding was carried out following a modified version of the method previously described.[39] Briefly, the brain or spleen membranes (40−60 μg of protein) were incubated for 1 h at 30 °C with [^{3}H]CP-55,940 (0.5 nM) in a final volume of 0.5 mL of TME buffer containing 5 mg/mL BSA. Nonspecific binding was determined in the presence of 10 μM [^{3}H]CP-55,940. Incubation was terminated by rapid filtration through Whatman GF/C filters pretreated with 0.5% (w/v) polyethyleneimine (PEI), using a Brandell 24-sample harvester (Gaithersburg, MD). Filters were washed three times with ice-cold Tris-HCl buffer (pH 7.4) containing 1 mg/mL BSA. Filter-bound radioactivity was counted in a liquid scintillation counter (Packard Tricarb1600 TR, Packard, Meridien, CT), using 3 mL of scintillation fluid (Ultima Gold Packard, MV, Meridien, CT).

[^{3}H]CP-55,940 displacement curves were plotted using serial dilutions ranging from 10^{-9} to 10^{-3} M unlabeled compounds and [^{3}H]CP-55,940 (0.5 nM). Independent experiments were repeated on membrane preparations from at least three different experiments.

The calculation of the IC_{50} (the concentration that inhibits 50% of specific radioligand binding) was performed by nonlinear curve fitting of the concentration-effect curves using the GraphPad Prism program, San Diego, CA. The *F*-test was used to determine the best approximation of a nonlinear curve fitting to a one- or two-site model ($P < 0.05$). IC_{50} values were converted to K_i values by means of the Cheng and Prusoff equation.[47]

[^{35}S]GTPγS Binding Assay. Brain tissue (minus cerebellum) was suspended in 20 volumes of cold centrifugation buffer (50 mM Tris-HCl, 3 mM $MgCl_2$, 1 mM EDTA, pH 7.4) and homogenized using a homogenizer system (Glas-Col, Terre Haute, IN). The homogenate was centrifuged at 48000*g* for 10 min at 4 °C. The pellet was then resuspended in the same buffer, homogenized, and centrifuged as previously. The final P2 pellet was subsequently resuspended in assay buffer (50 mM Tris-HCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 100 mM NaCl, pH 7.4), homogenized, and diluted to a concentration of ∼2 mg/mL with assay buffer. Membrane aliquots were then stored at −80 °C until use.

[^{35}S]GTPγS binding is measured as previously described.[40] Briefly, mouse brain membranes (5−10 μg of protein) were incubated with compounds for 60 min at 30 °C in assay buffer containing 0.1% BSA in the presence of 0.05 nM [^{35}S]GTPγS and 30 μM GDP in a final volume of 1 mL. The reaction was terminated by rapid filtration using a Packard Unifilter-GF/B, washed 2 times with 1 mL of ice-cold 50 mM Tris-HCl, pH 7.4 buffer, and dried for 1 h at 30 °C. The radioactivity on the filters was counted in a liquid microplate scintillation counter (TopCount NXT, Packard, Meridien, CT) using 30 μL of scintillation fluid (Microscint 20, Packard, Meridien, CT).

Stock solution of compounds were prepared in DMSO and were then diluted in assay buffer. The final concentration of DMSO was <0.01%, which had no effect on basal or stimulated [^{35}S]GTPγS binding. Concentration-effect curves were determined by incubating membranes with various concentrations of compounds (1−5000 nM) in the presence of 0.05 nM [^{35}S]GTPγS and 30 μM GDP.

Nonspecific binding was measured in the presence of 10 μM unlabeled GTPγS. Basal binding was assayed in the absence of agonist and in the presence of GDP. Stimulation by the agonist was defined as a percentage increase above basal levels (i.e., {[dpm(agonist) − dpm(no agonist)]/dpm(no agonist)} × 100).

Nonlinear regression analysis of concentration-response data was performed using Prism 2.0 software (GraphPad Prism program, San Diego, CA) to calculate E_{max} (maximal stimulation over basal levels) and EC_{50} (concentration of agonist to obtain 50% of the maximal effect) values.

Data are reported as the mean ± SEM of three to six experiments, performed in triplicate. Data were statistically evaluated by one-way analysis of variance followed by the Newman−Keuls test for multiple comparisons.

Preparation of Basophil-Rich Leukocyte Samples. Twenty healthy donors were recruited in the transfusion unit of Careggi General Hospital (Florence). 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 of them; 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. An amount 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, 1.5 mg/ml bovine serum albumin (BSA), at pH 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, 1.5 mg/mL bovine serum albumin (BSA), pH 7.4, and were further processed as described below. Upon isolation, cell viability, determined by trypan blue exclusion, was always greater than 95%. The procedure used, as a result of the low handling of basophils, also prevented their aspecific activation, as might have occurred using high-purifying procedures, such as specific antibody-coated magnetic beads. Before the experiments were started, samples from each basophil-rich leukocyte preparation were challenged for their ability to respond to anti-IgE by the 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 then 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. Human basophils before activation showed a low expression of CD203c, which was strongly up-regulated after the activation of the cells.

Docking Studies. With the Macromodel program,[48] the ligands were submitted to a conformational search of 1000 steps with an energy window, for saving the structure, of 10 kJ/mol. The algorithm used was the Monte Carlo method with MMFFs as the force field and a distance-dependent dielectric constant of 1.0. The ligands were then minimized using the conjugated gradient method until a convergence value of 0.05 kcal $Å^{-1}$ mol^{-1}, using the same force field and dielectric constant used for the conformational search.

Automated docking was carried out by means of the program AUTODOCK 3.0.[45] AUTODOCK TOOLS[49] was used to identify the torsion angles in the ligands, to add the solvent model and to assign partial atomic charges (Gasteiger for the ligands and Kollman for the receptors). The regions of interest used by AUTODOCK were defined by considering the previously published WIN-55,-212-2 docked into the CB_2 receptor[35] as the central group of a grid of 54, 50, and 52 points in the *x*, *y*, and *z* directions. A grid spacing of 0.375 Å and a distance-dependent function of the dielectric

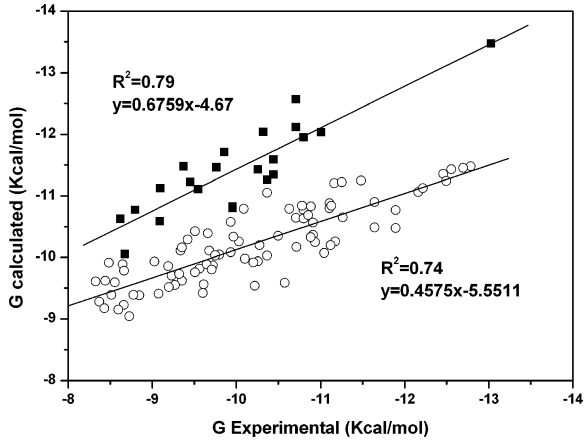


Figure 6. Plot of the average estimated binding free energy of the chosen cluster vs experimental binding energy. Ligands with a morpholinic group are indicated by ■, while all the others are indicated by ○.

constant were used for the energy map calculations. Because all the compounds can form an intramolecular H bond and our previous study suggested that the interaction was quite strong,[35] this H bond was also considered to be maintained during interaction in the binding site. For this reason, during the AUTODOCK protocol, we blocked the torsions involved in this intramolecular bond, to prevent the loss of this interaction.

With the Lamarckian genetic algorithm, all docked compounds were subjected to 100 runs of the AUTODOCK search, in which the default values of the other parameters were used. Cluster analysis was performed on the docked results using an rms tolerance of 1.0 Å, and the cluster with the best average of estimated free energy was chosen.

To predict the binding affinity of the ligands, we used the correlation between the calculated and experimental binding free energy obtained in our previous paper.[35] Figure 6 shows a plot of experimental binding energy versus the average estimated binding free energy used for the prediction calculation. It differs slightly from the data of our published paper because in this study the test set previously used to validate the predictivity of the model was incorporated in the training set.

Supporting Information Available: Elemental analysis results for all target compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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