# Flavonoids as Opioid Receptor Ligands: Identification and Preliminary Structure−Activity Relationships

Peter L. Katavic,[‡] Kenneth Lamb,[‡] Hernan Navarro,[⊥] and Thomas E. Prisinzano*[,‡]

*Division of Medicinal & Natural Products Chemistry, College of Pharmacy, The University of Iowa, Iowa City, Iowa 52242, and Research Triangle Institute, Research Triangle Park, North Carolina 27709*

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Flavonoids have been recognized as the active ingredients of many medicinal plant extracts due to interactions with proteins via phenolic groups and low toxicity. Here, we report the investigation of the flavonoid core as a potential new scaffold for the development of opioid receptor ligands. Biological results suggest that stereochemistry of the C2 and C3 positions is important for antagonist activity and selectivity. Our results also suggest that the actions of *Hypericum perforatum* may be mediated in part by opioid receptors.

At present, effective pharmacotherapies have yet to be developed for cocaine and methamphetamine dependence. A large body of evidence in animal models indicates that $\kappa$ receptors (and their endogenous opioid peptide ligands, e.g., the dynorphins) modulate the effects of these CNS stimulants.[1,2] The $\kappa$ receptor/dynorphin system is thought to be part of the brain's counter-regulatory response to enhanced dopaminergic activity, which is thought to be a major initial event underlying drug-induced reinforcement and abuse potential. In particular, $\kappa$ antagonists attenuate the reinstatement of extinguished drug-taking behavior (a model of relapse).[3]

Among the first nonpeptide $\kappa$ antagonists identified were those derived from the nonselective antagonist naltrexone (**1**) such as nor-BNI (nor-binaltorphimine, **2a**) and GNTI (5′-guanidinonaltrindole, **2b**) (Chart 1).[4,5] While **2a** has been extensively used to study $\kappa$ opioid receptors, its pharmacological properties are not optimal, and it exhibits a much longer than expected half-life in vivo.[6] Further study[7−11] of its structure−activity relationships identified **2b** as a $\kappa$ antagonist.[12,13] Compound **2b** has increased potency in vivo compared to **2a**, but unfortunately also has a slow onset of action and a long half-life in vivo.[14]

Recently, Thomas et al. identified several novel $\kappa$ opioid receptor antagonists from several classes of opioids.[15−18] $\kappa$-Selective antagonists were identified from the 4-phenylpiperidine and the 5-phenylmorphan classes of opioids. In particular, JDTic ((3*R*)-7-hydroxy-*N*-(1*S*)-1-[(3*R*,4*R*)-4-(3-hydroxyphenyl)-3,4-dimethyl-1-piperidinyl]methyl-2-methylpropyl-1,2,3,4-tetrahydro-3-isoquinolinecarboxamide, **3**) was shown to be a more potent $\kappa$ antagonist than **2a**.[16] Biological studies have shown that **3** blocks $\kappa$-agonist-induced antinociception in mice and squirrel monkey and antagonizes $\kappa$-agonist-induced diuresis in rats.[19] A more recent study illustrated that **3** is effective in decreasing withdrawal signs in rodents, indicating **3** may find some application in the treatment of opiate abuse.[20] Furthemore, **3** significantly reduced foot-shock-induced reinstatement of cocaine responding in rats and decreased immobility and increased swimming time in the forced swim stress test similar to the antidepressant desipramine.[3] However, like other $\kappa$ antagonists mentioned above, **3** has a slow onset and extremely long duration of action.[21]

One approach to circumventing the problems of slow onset and long duration of action seen with $\kappa$ antagonists is to identify novel structural scaffolds for chemical development. Here, we describe the identification of flavonoids as a novel structural scaffold for opioid receptor ligands.

## Results and Discussion

Recent work has indicated that *Hypericum perforatum* L. (St. John's Wort) may possess antiaddictive properties. For example, extracts of *H. perforatum* have been shown to attenuate alcohol self-administration in different strains of alcohol-preferring rats.[22,23] Endogenous opioids play a key role in the rewarding properties of alcohol, and opioid receptor antagonists, such as **1**, are used clinically to treat alcohol abuse.[24,25] Interestingly, *H. perforatum* extracts have also been shown to act synergistically with opioid receptor antagonists to attenuate ethanol intake in rats.[26] This further supports the idea that the attenuation of alcohol self-administration caused by extracts of *H. perforatum* is likely due to effects on opioid receptors. Extracts of *H. perforatum* inhibit bladder contractility in the rat in part through interaction with opioid receptors,[27] indicating that *H. perforatum* could be a novel treatment for urinary incontinence. Extracts of *H. perforatum* have also been evaluated for their anti-inflammatory and analgesic properties in rodents[28−31] and found to be effective in a carrageenan-induced edema model of inflammation and formalin-induced pain model for nociception. These effects are also mediated in part by opioid receptors.[32,33] Furthermore, in vitro receptor screens have indicated that extracts of *H. perforatum* inhibited binding of [$^3$H]naloxone and [$^3$H]deltorphin to opioid receptors.[34,35] Furthermore, amentoflavone (**4**), a biflavone present in extracts of *H. perforatum*, competed for binding to opioid receptors.[36]

Additional screening showed that **4** had significant $\delta$ opioid receptor affinity ($K_i$ = 36 nM) in vitro.[36] However, it was not known whether **4** was an agonist or an antagonist at $\delta$ opioid receptors. A recent report has shown that **4** is able to pass the blood brain barrier by passive diffusion in vitro,[37] so it is possible that some of the CNS effects of *H. perforatum* are the result of its interaction with opioid receptors.

As a first step toward identifying opioid receptor ligands in *H. perforatum*, studies were initiated to determine whether **4** had activity at opioid receptors. Amentoflavone was characterized for intrinsic and antagonist activity at the human $\kappa$, $\mu$, and $\delta$ opioid receptors using the [$^{35}$S]GTP$\gamma$S functional binding assay as described previously.[38] Up to 10 $\mu$M of **4** had no intrinsic activity at any of the opioid receptors (not shown). It was then assayed for antagonist activity by determining the ability of a single concentration of **4** to cause a rightward shift in the concentration−response curve of the opioid receptor selective agonists (D-Ala$^2$,MePhe$^4$,-Gly-ol$^5$)enkephalin (DAMGO, $\mu$), [D-Pen$^2$,D-Pen$^5$]enkephalin (DPDPE, $\delta$ receptor), and U69,593 ($\kappa$ receptor). Biflavone **4** was inactive as an antagonist at the $\mu$ opioid receptor ($K_e$ > 10 000 nM) and weakly active at the $\delta$ opioid receptor ($K_e$ = 6000 nM) (Table 1). In contrast, **4** had good activity at the $\kappa$ opioid receptor

* To whom correspondence should be addressed. Tel: (319) 335-6920. Fax: (319) 335-8766. E-mail: thomas-prisinzano@uiowa.edu.
[‡] University of Iowa.
[⊥] Research Triangle Institute.

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**Chart 1.** Structures of Naltrexone (**1**), nor-BNI (**2a**), GNTI (**2b**), JDTic (**3**), Amentoflavone (**4**), Apigenin (**5**), Hyperoside (**6**), 7,4′-Dihydroxyflavone (**7**), and Naringenin (**8**)

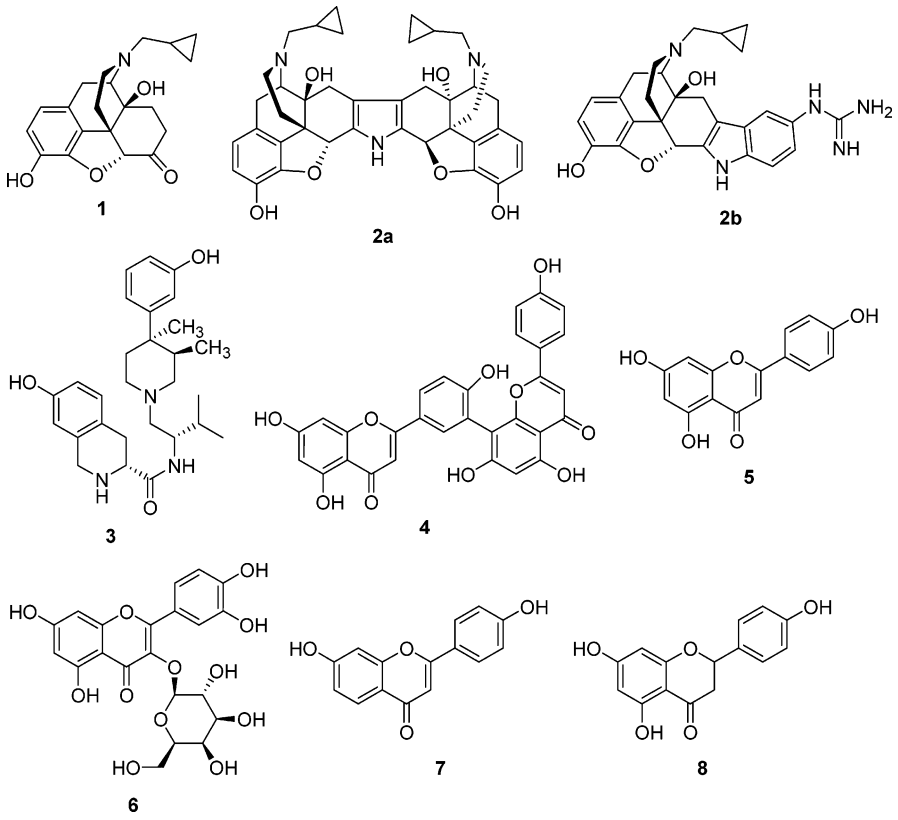



**Table 1.** Results from [$^{35}$S]GTP-$\gamma$-S Functional Assay Carried out in Stably Transfected CHO Cells Containing DNA for Human $\mu$, $\delta$, and $\kappa$ Receptors

| | $K_e \pm$ SD, nM | | | selectivity | |
|---|---|---|---|---|---|
| compd | $\mu$ | $\delta$ | $\kappa$ | $\mu/\kappa$ | $\delta/\kappa$ |
| **4** | >10 000 | 6000 ± 960 | 490 ± 150 | >20 | 12 |
| **5** | 410 ± 30 | 970 ± 300 | 410 ± 90 | 1.0 | 2.4 |
| **6** | 390 ± 20 | 9070 ± 3350 | 340 ± 100 | 1.1 | 27 |
| **7** | 480 ± 160 | 2090 ± 690 | 1610 ± 800 | 0.30 | 1.3 |
| **8** | 2300 ± 1800 | 3680 ± 80 | 1700 ± 910 | 1.4 | 2.2 |
| **9** | 590 ± 280 | 3490 ± 1230 | 550 ± 20 | 1.1 | 6.3 |
| **10** | >10 000 | >10 000 | >10 000 | ND | ND |
| **11** | 3070 ± 700 | 3820 ± 2170 | 3280 ± 250 | 0.93 | 1.2 |
| **12** | >10 000 | 1180 ± 250 | 320 ± 20 | >31 | 3.7 |
| **13** | 2400 ± 500 | 1770 ± 130 | 3600 ± 1100 | 0.67 | 0.49 |
| **15** | 3020 ± 620 | 2170 ± 480 | 5810 ± 110 | 0.52 | 0.37 |
| **16** | 210 ± 70 | 950 ± 180 | 220 ± 4 | 0.95 | 4.3 |
| **17** | 1500 ± 600 | 1700 ± 500 | 1900 ± 100 | 0.79 | 0.89 |
| **18** | 300 ± 30 | 1990 ± 880 | 250 ± 150 | 1.2 | 8.0 |
| naltrexone | 3.6 ± 1.5 | 60.7 ± 10.6 | 4.6 ± 1.5 | 0.78 | 13 |
| nor-BNI[a] | 26 ± 7 | 29 ± 8 | 0.05 ± 0.02 | 520 | 580 |
| JDTic[a] | 25 ± 4 | 76 ± 3 | 0.02 ± 0.01 | 1250 | 3800 |

[a] Data from ref 53.

with a $K_e$ of 490 ± 150 nM. Figure 1 shows that amentoflavone at 1000 nM caused nearly a 4-fold shift in the U69,593 concentration−response curve. Moreover, **4** was more than 10-fold selective for the $\kappa$ over the $\delta$ opioid receptor. This is the first report of a flavonoid with $\kappa$ antagonist activity and opens a new structural scaffold for the development of opioid antagonists. This also adds evidence that the actions of *H. perforatum* may be mediated in part by opioid receptors.

Efforts were then begun to investigate structural modifications to the core structure of **4**. Removal of one of the flavone ring creates apigenin (**5**). Up to 10 $\mu$M, **5** had no intrinsic activity at any of the opioid receptors. It was then assayed for antagonist activity as described above. Apigenin was roughly equipotent to **4** as a $\kappa$ antagonist ($K_e$ = 410 nM vs $K_e$ = 490 nM). This indicates the second flavone moiety is not required for $\kappa$ antagonism. Moreover, this modification increased activity 6-fold at $\delta$ receptors ($K_e$ = 970 nM vs $K_e$ = 6000 nM) and at $\mu$ receptors ($K_e$ = 410 nM vs $K_e$ > 10 000 nM) compared to **4**. Replacement of the second flavone moiety with a 3$\beta$-D-galactose sugar affords hyperoside (**6**). This compound is also found in extracts of *H. perforatum*, and a previous study showed that **6** is active in the forced swim stress test.[39] Addition of a 3$\beta$-D-galactose sugar had little effect on activity at $\mu$ and $\kappa$ receptors but increased selectivity 9-fold over $\delta$ receptors ($K_e$ = 9070 nM vs $K_e$ = 970 nM). The removal of the 5-hydroxyl group from **5** creates 7,4′-dihydroxyflavone (**7**). This change had little effect on activity at $\mu$ receptors ($K_e$ = 480 nM vs $K_e$ = 410 nM) compared to **5**. However, activity at $\delta$ receptors was reduced 2-fold ($K_e$ = 2090 nM vs $K_e$ = 970 nM), and activity at $\kappa$ receptors

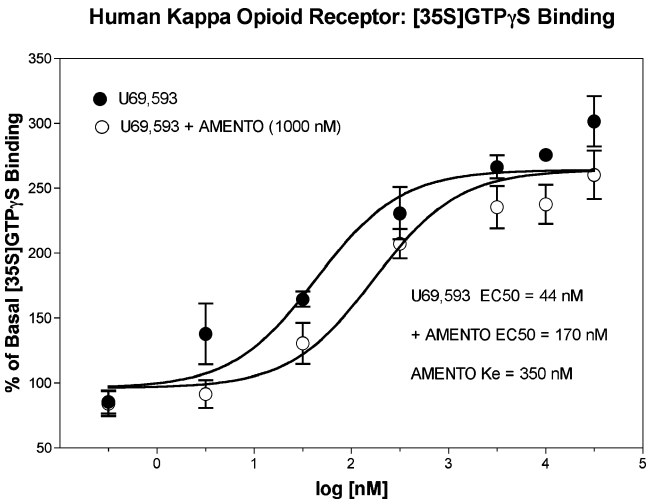
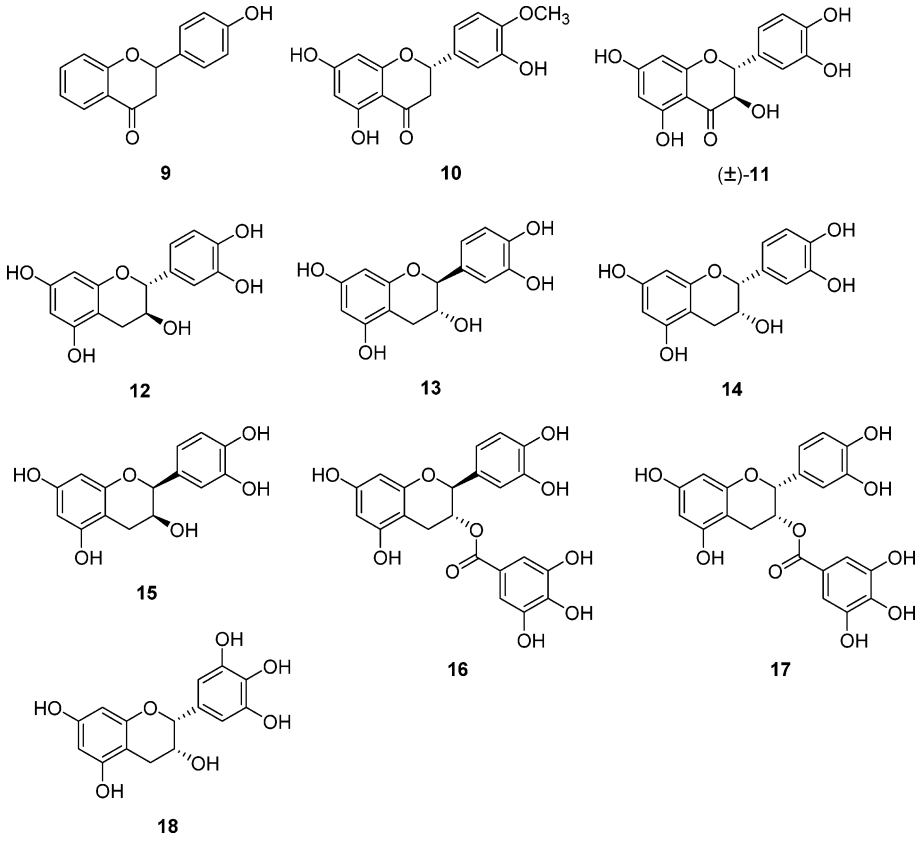



**Figure 1.** Representative data from [$^{35}$S]GTP$\gamma$S assay at $\kappa$ opioid receptors.

**Chart 2.** Structures of 4′-Hydroxyflavanone (**9**), Hesperetin (**10**), Taxifolin (**11**), (+)-Catechin (**12**), (−)-Catechin (**13**), (−)-Epicatechin (**14**), (+)-Epicatechin (**15**), (−)-Catechin Gallate (**16**), (−)-Epicatechin Gallate (**17**), and (−)-Epigallocatechin (**18**)




was reduced 4-fold ($K_e$ = 1610 nM vs $K_e$ = 410 nM). This indicates that the 5-hydroxyl group is more important for activity at $\kappa$ receptors. Reduction of the alkene in **5** creates racemic naringenin (**8**). This change decreased activity 6-fold at $\mu$ receptors ($K_e$ = 2300 nM vs $K_e$ = 410 nM) and 4-fold at both $\delta$ receptors ($K_e$ = 3680 nM vs $K_e$ = 970 nM) and $\kappa$ receptors ($K_e$ = 1700 nM vs $K_e$ = 410 nM). The removal of the 3- and 5-hydroxyl groups of **8** creates 4′-hydroxyflavanone (**9**) (Chart 2). This change increased activity 4-fold at $\mu$ receptors ($K_e$ = 590 nM vs $K_e$ = 2300 nM) and 3-fold at $\kappa$ receptors ($K_e$ = 550 nM vs $K_e$ = 1700 nM) but had little effect on activity at $\delta$ receptors ($K_e$ = 3490 nM vs $K_e$ = 3680 nM) compared to **8**. These results would indicate that the 3- and 5-hydroxy groups are not necessary for activity at opioid receptors. Addition of a 3′-hydroxy group and methylation of the 4′-hydroxyl of **8** affords hesperetin (**10**). These changes, however, were not tolerated and antagonist activity at $\mu$, $\delta$, and $\kappa$ receptors was abolished ($K_e$ > 10 000 nM). This would suggest that the 4′-hydroxyl group is essential for antagonist activity at opioid receptors. Addition of 3- and 3′-hydroxy groups to **8** creates racemic taxifolin (**11**). These changes had little effect on activity at $\mu$ receptors ($K_e$ = 3070 nM vs $K_e$ = 2300 nM) and $\delta$ receptors ($K_e$ = 3820 nM vs $K_e$ = 3680 nM) but decreased activity 2-fold at $\kappa$ receptors ($K_e$ = 3280 nM vs $K_e$ = 1700 nM) compared to **8**. It was unclear whether this reduction in activity was the result of the addition of the 3-hydroxyl or the 3′-hydroxyl group. To further address this issue, we evaluated several additional flavonoids.

Removal of the 4-keto group from **11** creates a flavan-3-ol, or a catechin. Given that there are two asymmetric centers, there are four possible stereoisomers, (+)-catechin (**12**), (−)-catechin (**13**), (−)-epicatechin (**14**), and (+)-epicatechin (**15**). Catechins **12**−**15** were readily available and were evaluated for opioid receptor activity to give insight as to the role of stereochemistry on activity. With the exception of **13**, which had weak partial agonist activity ($E_{max}$ = 18% of U69,593), **12**−**15** had no intrinsic activity at opioid receptors. (+)-Catechin (**12**) was inactive as an antagonist at the $\mu$ opioid receptor but was active at the $\delta$ opioid receptor ($K_e$ = 1180 nM). In contrast, **12** had good antagonist activity at the $\kappa$ opioid receptor with a $K_e$ of 320 nM. (−)-Catechin (**13**) was less active at $\kappa$ receptors ($K_e$ = 3600 nM vs $K_e$ = 320 nM) and $\delta$ receptors ($K_e$ = 1770 nM vs $K_e$ = 1180 nM) compared to **12**. However, **13** had increased activity at $\mu$ receptors ($K_e$ = 2400 nM vs $K_e$ > 10 000 nM). (−)-Epicatechin (**14**) was less active at $\kappa$ receptors and $\delta$ receptors compared to **12** (data not shown). Furthermore, (+)-epicatechin (**15**) was found to have the same activity at $\mu$ receptors ($K_e$ = 3020 nM vs $K_e$ = 2400 nM), $\kappa$ receptors ($K_e$ = 5810 nM vs $K_e$ = 3600 nM), and $\delta$ receptors ($K_e$ = 2170 nM vs $K_e$ = 1770 nM) compared to **13**. However, **15** is more potent at $\mu$ receptors ($K_e$ = 3020 nM vs $K_e$ > 10 000 nM) and less potent at $\kappa$ receptors ($K_e$ = 5810 nM vs $K_e$ = 320 nM) than **12**. This would indicate that the configuration of the 3-hydroxyl group influences selectivity.

On the basis of the above results, we sought to explore an additional structural modification to (−)-catechin (**13**) and (−)-epicatechin (**14**). The addition of a trihydroxybenzoyl group or galloyl group to **13** and **14** affords (−)-catechin gallate (**16**) and (−)-epicatechin gallate (**17**), respectively. This was based in part on a previous report that indicated catechin 3-*O*-gallate had affinity for the opiate receptor ($IC_{50}$ = 36 nM).[40] Up to 10 $\mu$M, **16** and **17** had no intrinsic activity at any of the opioid receptors. (−)-Catechin gallate (**16**) is greater than 47-fold more active at $\mu$ receptors ($K_e$ = 210 nM vs $K_e$ > 10 000 nM) than **13**. Moreover, **16** is also more potent at $\kappa$ receptors (approximately 16-fold, $K_e$ = 220 nM vs $K_e$ = 3600 nM) and $\delta$ receptors (2-fold, $K_e$ = 950 nM vs $K_e$ = 1770 nM). A change in the C2 stereochemistry in **16** (**17**) decreased activity 7-fold at $\mu$ receptors ($K_e$ = 1500 nM vs $K_e$ = 210 nM) and 2-fold at $\delta$ receptors ($K_e$ = 1700 nM vs $K_e$ = 950 nM) compared to **15**. This change also decreased activity 9-fold at $\kappa$ receptors ($K_e$ = 1900 nM vs $K_e$ = 220 nM). These results indicate that the addition of a galloyl group to the 3-hydroxyl group may increase activity at opioid receptors. However, it is unclear whether the

gallate group is essential for activity or if other structural modifications to this group will also lead to increased activity.

One final modification studied was the addition of a hydroxyl group to the 2-position catechol ring of (−)-epicatechin (**14**) to afford (−)-epigallocatechin (**18**), a flavonoid found in green tea. A previous report indicated that **18** had modest affinity for the opiate receptor.[40] Up to 10 $\mu$M, **18** had no intrinsic activity at any of the opioid receptors. However, **18** had antagonist activity at $\mu$ receptors ($K_e$ = 300 nM) and $\delta$ receptors ($K_e$ = 1990 nM). To our delight, **18** also had antagonist activity at $\kappa$ receptors ($K_e$ = 250 nM) similar to **16**.

In conclusion, several flavonoids have been evaluated for opioid receptor activity. We have shown that amentoflavone (**4**) and hyperoside (**6**), flavonoids present in *H. perforatum*, have $\kappa$ antagonist activity in vitro. In addition, preliminary SAR investigations have identified that the stereochemistry of the C2 and C3 positions is important for antagonist activity and selectivity. Further exploration of these findings is underway and will be reported in due course.

## Experimental Section

**General Experimental Procedures.** Unless otherwise indicated, all compounds were purchased from ChromaDex (Irvine, CA) or Sigma (St. Louis, MO). Their identity was verified by $^1$H and $^{13}$C NMR and melting point and is in agreement with previously published data.[41−52] DAMGO, DPDPE, and U69,593 were obtained via the Research Technology Branch, NIDA, and were prepared by Multiple Peptide Systems (San Diego, CA). [$^{35}$S]GTP-$\gamma$-S was obtained from Perkin-Elmer Inc. (Boston, MA), and GTP-$\gamma$-S and GDP were obtained from Sigma Chemical Company (St. Louis, MO).

**Intrinsic Activity at Human Opioid Receptors.** Test compounds were first assayed at 10 $\mu$M for intrinsic activity (agonist or inverse agonist) using the [$^{35}$S]GTP-$\gamma$-S binding assay and CHO cell membrane homogenates that express the human $\kappa$OR, $\mu$OR, or $\delta$OR. The subtype selective agonists (D-Ala$^2$,MePhe$^4$,Gly-ol$^5$)enkephalin (DAMGO, $\mu$OR), (D-Pen$^2$,D-Pen$^5$)enkephalin (DPDPE, $\delta$OR), or U69,593 ($\kappa$OR) were run as positive controls as appropriate. The CHO membranes were incubated in duplicate in 1.4 mL polypropylene tubes (Matrix Technologies, Hudson, NH) with positive control or test compound, 0.1 nM [$^{35}$S]GTP-$\gamma$-S, and 1 $\mu$M GDP in 50 mM HEPES buffer (pH 7.4) at room temperature for 1 h, after which bound radioligand was separated from free via rapid vacuum filtration over GF-B filters with a Brandel Scientific (Gaithersburg, MD) 96-well harvester. Bound radioactivity was determined using a TopCount 12-detector instrument (Packard Instruments) using standard scintillation counting techniques. The data were normalized to samples containing vehicle (basal binding). Any compound with intrinsic activity had its $EC_{50}$ (agonist) or $IC_{50}$ (inverse agonist) determined using an 8-point concentration−response curve, and the results were compared to a concentration−response curve of the appropriate subtype-selective agonist assayed in parallel. Compounds without intrinsic activity were assayed for antagonist activity.

**Apparent Affinity ($K_e$) at Human Opioid Receptors**. The ability of a single concentration of test compound to shift the agonist dose−response curve to the right was used to determine its $K_e$. Assay conditions were identical to those for the determination of intrinsic activity except that the final GDP concentration was 10 $\mu$M. Agonist concentration−response curves were run in the presence or absence of a single concentration of test compound, and the $EC_{50}$ values determined from a three-parameter logistic curve fit to the data with Prism (version 4.0, GraphPad Software, Inc., San Diego, CA). The $K_e$ values were calculated using the formula $K_e = [L]/[(A'/A) - 1)]$, where [L] is the concentration of antagonist and $A'$ and $A$ are the agonist $EC_{50}$ values in the presence or absence of antagonist, respectively.

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