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# Optimization of a Series of Mu Opioid Receptor (MOR) Agonists with High G Protein Signaling Bias

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**KEYWORDS:** Benzimidazolone, Bias, Mu Opioid Receptor, G Protein Coupled Receptor,  $\beta$ arrestin2.

**ABSTRACT:** While mu opioid receptor (MOR) agonists are especially effective as broad-spectrum pain relievers, it has been exceptionally difficult to achieve a clear separation of analgesia from many problematic side effects. Recently, many groups have sought MOR agonists that induce minimal  $\beta$ arrestin-mediated signaling, since MOR agonist-treated  $\beta$ arrestin2 knockout mice were found to display enhanced antinociceptive effects with significantly less respiratory depression and tachyphylaxis. Substantial data now exists to support the premise that G protein signaling biased MOR agonists can be effective analgesic agents. We recently showed

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3 that, within a chemical series, the degree of bias correlates linearly with the magnitude of the  
4 respiratory safety index. Herein we describe the synthesis and optimization of piperidine  
5 benzimidazolone MOR agonists that together display a wide range of bias (G/ $\beta$ arr2). We identify  
6 structural features affecting potency and maximizing bias and show that many have desirable  
7 properties, such as long half-lives and high brain penetration.  
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## 18 INTRODUCTION

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20 Agonists of the mu opioid receptor (MOR), such as morphine and fentanyl, are extensively  
21 used for the treatment of moderate to severe pain due to their high efficacy;<sup>1</sup> however, the dose  
22 required to achieve adequate pain relief often elicits multiple unwanted side effects, including  
23 respiratory suppression, constipation, and tolerance. The respiratory suppressive effect of opioids  
24 is especially concerning, as it is the root cause of death by opioid overdose, which claimed more  
25 than 40,000 victims in the USA in 2016.<sup>2</sup> The identification and development of safer analgesic  
26 agents may play an important role in combatting the opioid epidemic.  
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37 The most effective opioid pain relievers are agonists of the MOR.<sup>3</sup> As with all G protein  
38 coupled receptors (GPCRs), agonist binding to MOR initiates the dissociation of heterotrimeric  
39 G protein subunits and the activation of subsequent downstream signaling. The MOR also  
40 interacts with  $\beta$ arrestins, scaffolding and regulatory proteins with multifaceted roles, including  
41 receptor desensitization of the G protein cascades and the facilitation of receptor internalization  
42 and signaling that can be distinct from G protein mediated responses.<sup>4</sup> Studies using  $\beta$ arrestin2  
43 knockout ( $\beta$ arr2-KO) mice strongly suggest that the interaction between MOR and  $\beta$ arrestin2  
44 produces many of morphine's undesirable effects *in vivo*.<sup>5,6,7,8,9,10</sup> In the  $\beta$ arr2-KO mice,  
45 morphine retained its analgesic properties, yet, in comparison to wild-type littermates,  
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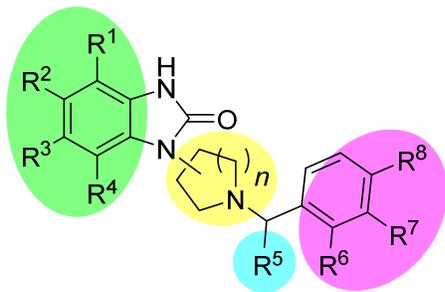
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3 constipation, tolerance, and respiratory suppression were largely attenuated.<sup>5,6,7,8,10</sup> Based upon  
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5 this work, it was hypothesized that a MOR agonist capable of activating G protein signaling  
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7 without prompting the engagement of  $\beta$ arrestins might separate analgesia from many of the  
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9 adverse effects that arise downstream of MOR activation.  
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12 The ability of a compound to stimulate one signaling pathway over another upon engaging the  
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14 receptor is referred to “functional selectivity” or “biased agonism.”<sup>4,11,12,13,14</sup> Compounds that  
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16 preferentially activate G protein signaling over  $\beta$ arrestin2 recruitment in the MOR have been  
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18 developed by a number of groups.<sup>15,16,17,18,19</sup> Two reported clinical trials with TRV130 (aka  
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20 Oliceridine) demonstrate that one such G protein biased MOR agonist has analgesic efficacy  
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22 with a modest improvement in respiratory suppression compared to morphine.<sup>20,21</sup> These clinical  
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24 results suggest that imparting G coupling bias in MOR agonists may lead to the discovery and  
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26 eventual availability of safer opioid analgesic agents, especially if the magnitude of signaling  
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28 bias is augmented so that more than a modest improvement in therapeutic index may be  
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30 achieved.  
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35 We recently disclosed members of a family of substituted piperidine benzimidazolone MOR  
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37 agonists with high affinity for the MOR (0.3-14 nM), high G protein signaling bias (up to 100  
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39 fold bias relative to DAMGO in some assays), and high selectivity for MOR over the other  
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41 opioid receptors.<sup>22</sup> The modular design of the core scaffold permitted extensive structural  
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43 diversification, an important advantage since we found that only a small subset of potential  
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45 analogs in the series display significant G protein signaling bias. We have optimized these MOR  
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47 agonists for potency, G protein signaling bias, and drug-like properties. Our objective has been  
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49 to identify compounds with morphine-like efficacy *in vivo* (or better) that would not induce  
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51 respiratory suppression at, or even well above, an efficacious dose. Within a set of related  
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3 compounds, we achieved this goal and demonstrated that the degree of signaling bias within the  
4 series linearly correlated to the magnitude of protection from respiratory suppression.<sup>22</sup> Given  
5 that slight structural changes can greatly impact signaling bias, understanding the structural  
6 features favoring alternative MOR signaling pathways could greatly enhance efforts to identify  
7 new safe and efficacious analgesic agents.  
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12 Herein, we report the first comprehensive bias-focused structure-activity relationship (SAR)  
13 study for this class of MOR agonists. We systematically varied the R<sup>1</sup>-R<sup>8</sup> substituents of the  
14 generalized N-benzyl cycloamino benzimidazolone scaffold as well as varied the ring size in the  
15 central saturated ring, which in most cases is a piperidine (Figure 1). Many structural changes  
16 were found to markedly impact the properties of MOR agonists. The iterative design and  
17 evaluation of analogs drove the optimization of G protein signaling potency, the deselection of  
18  $\beta$ arrestin interactions, and the optimization of desirable drug-like properties, such as a suitable  
19 half-life, lack of cytochrome P450 (CYP) inhibition, and high blood-brain barrier (BBB)  
20 permeability. Signaling bias was characterized using separate cell-based assays designed to  
21 measure G protein coupling and  $\beta$ arrestin2 recruitment. Bias was quantitated by applying the  
22 operational model,<sup>23</sup> which measures the affinity, potency, and efficacy of the compound in each  
23 assay relative to the reference full agonist [D-Ala<sup>2</sup>, NMe-Phe<sup>4</sup>, Gly-ol<sup>5</sup>]-enkephalin (DAMGO)  
24 and then allows for comparison across assays.<sup>11,23</sup> For optimized biased analogs, we measured  
25 various *in vitro* parameters, such as liver microsome stability and CYP inhibition, then followed  
26 up with *in vivo* studies for metabolic stability and BBB permeability. Results from these *in vitro*  
27 and *in vivo* studies advance our understanding of the chemical properties that underlie MOR  
28 signaling bias and may aid the development of additional promising leads separating analgesic  
29 activity from unwanted, and potentially deadly, respiratory suppression.  
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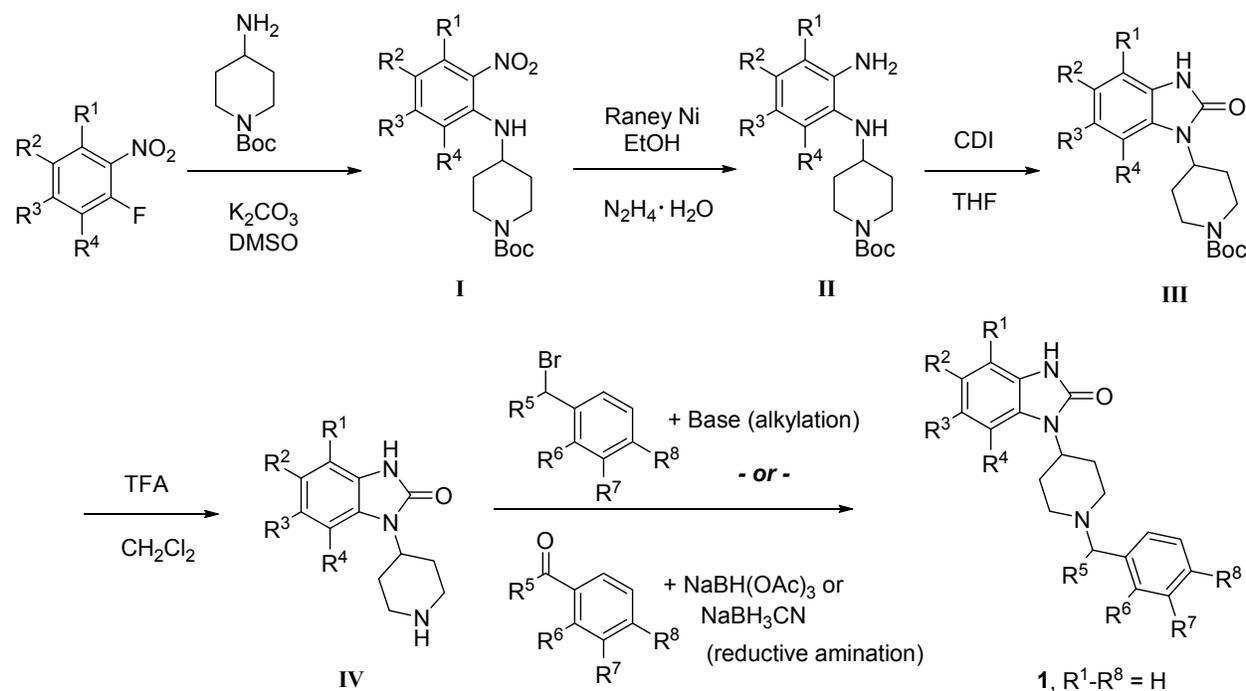


**Figure 1.** General structure of N-benzyl piperidine 4-benzimidazolones. The sites for structure diversification  $R^1$ - $R^8$  and  $n$  are highlighted by the colored circles.

## RESULTS AND DISCUSSION

**Synthesis.** As shown in Scheme 1, a general five-step synthetic route was used to synthesize the majority of the benzimidazolone derivatives<sup>24</sup> (compounds with a central piperidine ring are shown; see Scheme S1, Supporting Information for an alternate route). In the general procedure, nucleophilic aromatic substitution of a fluoronitrobenzene starting material with N-Boc-4-aminopiperidine (or in some cases with a homolog having a 5- or 7-membered ring) was followed by nitro group reduction of **I**, cyclic urea formation of **II**, Boc deprotection of **III**, and finally, either direct alkylation or reductive amination of **IV** produced the desired analogs. Reductive amination used an aldehyde ( $R^5 = H$ ) or ketone ( $R^5 = Me$  or  $Et$ ). The products were isolated and characterized in free base form, unless indicated, and then were evaluated in all biological and pharmacological assays as either the free base or as the mono mesylate salt.

**Scheme 1.** General Five-Step Synthetic Route for the Preparation of Substituted Piperidine 4-Benzimidazolones.



**Structure Activity Relationships.** In these SAR studies, substituents R<sup>1</sup>-R<sup>8</sup> were varied, as was the size of the central ring (Figure 1). Each analog was screened for its ability to activate G protein coupling at MOR using the standard <sup>35</sup>S-GTPγS binding assay in membranes from CHO-K1 cells expressing the human MOR. βarrestin2 recruitment profiles to the human MOR were determined using a cell-based commercially available enzyme-fragment complementation assay (EFC). In both assays, full dose response curves for the test compound were run in parallel to the reference full agonist, DAMGO, as previously described.<sup>22</sup> To quantitatively compare the differences observed between the two signaling assays, the operational model was used to calculate  $\Delta\Delta\log(\tau/K_A)$  values, with confidence intervals and bias factors (the anti-log of

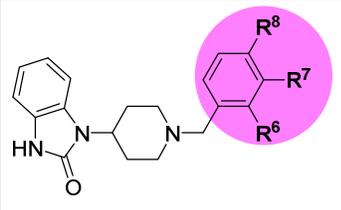
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3  $\Delta\Delta\log(\tau/K_A)$ .<sup>22,23</sup> A bias factor of 1 ( $\Delta\Delta\log(\tau/K_A)$  value of 0) indicates that the compound is  
4 unbiased relative to DAMGO. A bias factor less than 1 ( $\Delta\Delta\log(\tau/K_A)$  value less than 0) indicates  
5 bias towards  $\beta$ arrestin2 recruitment over GTP $\gamma$ S binding, while a bias factor greater than 1  
6 ( $\Delta\Delta\log(\tau/K_A)$  value greater than 0) indicates bias towards GTP $\gamma$ S binding over  $\beta$ arrestin2  
7 recruitment. Additionally, we used morphine as a clinically relevant opioid analgesic for  
8 comparison purposes. In these assays, we found morphine, with a bias factor of 1.8, to show a  
9 slight bias towards G protein coupling over  $\beta$ arrestin2 recruitment compared to DAMGO. Safer  
10 analgesic agents would be expected to have significantly higher levels of G protein coupling  
11 bias.<sup>22</sup>

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24 As shown in Table 1, the parent N-benzyl piperidine 4-benzimidazolone (**1**), with all  
25 substituents R<sup>1</sup>-R<sup>8</sup> = H, had modest potency in the GTP $\gamma$ S assay ( $EC_{50}$  = 2.2  $\mu$ M) but was a full  
26 agonist ( $E_{MAX}$  = 78% of DAMGO). Compound **1** performed similar to DAMGO in both assays  
27 (GTP $\gamma$ S/ $\beta$ arr2 bias factor = 0.9). After exploring the impact of various R<sup>6</sup>-R<sup>8</sup> substituents on the  
28 N-benzyl ring, we found that the addition of an *ortho*-Cl (**2**) or *para*-Cl substituent (**4**)<sup>22</sup>  
29 improved potency. The *ortho*-Cl substituent also imparted a modest G protein signaling bias  
30 (GTP $\gamma$ S/ $\beta$ arr2 bias factor = 2.5).

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Many *para*-substituted analogs (**4-7**) were similar in potency and efficacy in the GTP $\gamma$ S assay  
( $EC_{50}$  = 367-591 nM,  $E_{MAX}$  = 68-88%) and were similarly unbiased (GTP $\gamma$ S/ $\beta$ arr2 bias factors =  
0.9-1.2). *Para*-substituted analogs (**8-10**) were less potent and/or efficacious in the GTP $\gamma$ S assay  
compared to **4-7**, with **10** showing no significant activity until the 10  $\mu$ M concentration. The  
*meta*-substituted compounds **3**, **11**, and **12** were also less potent and less efficacious in the  
GTP $\gamma$ S assay in comparison to their *para*-substituted counterparts, with compound **11** acting as a  
partial GTP $\gamma$ S agonist ( $E_{MAX}$  = 33% of DAMGO). When a second Cl substituent was added to

the *para* position (**13**), the GTP $\gamma$ S potency and efficacy was reduced further and the potency at  $\beta$ arrestin2 was outside the experimental range of the assay. Thus, *ortho* and *para* substituted N-benzyl analogs were of greatest interest in this series from the perspective of obtaining biased agonists of useful potency. The potential for combining the *ortho* and *para* effects was demonstrated with compounds **14** and **15**, in which **14** is a potent full agonist showing some bias for G protein signaling ( $EC_{50} = 152$  nM,  $E_{MAX} = 93\%$ , GTP $\gamma$ S/ $\beta$ arr2 bias factor = 3.8).

**Table 1.** GTP $\gamma$ S Binding and  $\beta$ arrestin2 Recruitment Activity of N-Benzyl Piperidine 4-Benzimidazolone Derivatives with N-Benzyl Substituents R<sup>6</sup>-R<sup>8</sup>.<sup>a</sup>

				<sup>35</sup> S-GTP $\gamma$ S Binding <sup>b</sup>			$\beta$ arrestin2 Recruitment <sup>b</sup>			Bias (GTP $\gamma$ S/ $\beta$ arr2)	
Agonist	R <sup>6</sup>	R <sup>7</sup>	R <sup>8</sup>	EC <sub>50</sub> (nM)	E <sub>MAX</sub> (%) <sup>c</sup>	$\Delta\log(\tau/K_A)$	EC <sub>50</sub> (nM) <sup>c</sup>	E <sub>MAX</sub> (%) <sup>c</sup>	$\Delta\log(\tau/K_A)$	$\Delta\log(\tau/K_A)$ (95% CI) <sup>d</sup>	Bias Factor
DAMGO				33 ± 1	100		220 ± 8.3	100		0	1.0
Morphine				64 ± 3	83 ± 1	-0.3 ± 0.03	379 ± 17	24 ± 1	-0.6 ± 0.03	0.25 (0.17 to 0.33) <sup>e</sup>	1.8
<b>1</b>				2183 ± 290	78 ± 3	-1.8 ± 0.06	8255 ± 1676	40 ± 2	-1.8 ± 0.07	-0.04 (-0.25 to 0.16)	0.9
<b>2</b>	Cl			200 ± 19	92 ± 2	-0.8 ± 0.07	4487 ± 606	96 ± 9	-1.2 ± 0.05	0.4 (0.21 to 0.59) <sup>f</sup>	2.5
<b>3</b>		Cl		1354 ± 195	71 ± 1	-2 ± 0.06	>10,000 <sup>c</sup>	10 ± 0.4 <sup>c</sup>	-2.0 ± 0.09	0.22 (-0.08 to 0.52)	1.6
<b>4<sup>h</sup></b>			Cl	551 ± 58	68 ± 4	-1.4 ± 0.09	7656 ± 1469	67 ± 10	-1.5 ± 0.08	0.07 (-0.25 to 0.39)	1.2
<b>5</b>			Me	433 ± 47	86 ± 2	-1.0 ± 0.06	2400 ± 497	63 ± 3	-1.1 ± 0.08	0.01 (-0.27 to 0.29)	1.0
<b>6</b>			Br	367 ± 37	76 ± 4	-1.2 ± 0.1	6611 ± 1076	77 ± 6	-1.3 ± 0.06	0.05 (-0.22 to 0.31)	1.1
<b>7</b>			OMe	591 ± 49	88 ± 3	-1.2 ± 0.05	2254 ± 303	41 ± 3	-1.2 ± 0.01	-0.04 (-0.45 to 0.36)	0.91

8		NHC(O)Me	760 ± 75	89 ± 6	-1.4 ± 0.04	3435 ± 889	29 ± 3	-1.4 ± 0.1	0.06 (-0.36 to 0.47)	1.1	
9		CN	3424 ± 693	48 ± 2	-2.2 ± 0.1	NC <sup>c</sup>	1 ± 0.5 <sup>c</sup>	/	/	/	
10		C(O)NH <sub>2</sub>	NC	7.7 ± 1 <sup>c</sup>	/	NC <sup>c</sup>	0.03 ± 0.1 <sup>c</sup>	/	/	/	
11		OMe	1158 ± 160	33 ± 1	-1.9 ± 0.02	>10,000 <sup>c</sup>	8 ± 1 <sup>c</sup>	-2.2 ± 0.2	0.30 (-0.03 to 0.62)	2.0	
12		OCF <sub>3</sub>	1621 ± 183	64 ± 4	-1.8 ± 0.05	9326 ± 1738	29 ± 4	-1.8 ± 0.09	0.03 (-0.19 to 0.24)	1.1	
13		Cl	Cl	6423 ± 565	27 ± 2	-2.8 ± 0.2	NC <sup>c</sup>	4 ± 3 <sup>c</sup>	/	/	
14	Me		Cl	152 ± 10	93 ± 1	-0.6 ± 0.03	2880 ± 411	75 ± 5	-1.2 ± 0.1	0.58 (0.30 to 0.87) <sup>g</sup>	3.8
15	F		Br	110 ± 16	96 ± 0.1	-0.7 ± 0.04	3357 ± 60	94 ± 6	-1.1 ± 0.04	0.39 (0.22 to 0.56) <sup>g</sup>	2.5

<sup>a</sup>If blank or not shown, R<sup>x</sup> = H. “/” = not determined. <sup>b</sup>Within each assay, potency (EC<sub>50</sub>) and efficacy (E<sub>MAX</sub>) values were calculated by non-linear regression and Δlog(τ/K<sub>A</sub>) values were determined by the operational model, relative to DAMGO. Data are presented as mean ± SEM of three or more assays run in duplicate or triplicate. <sup>c</sup>In instances where the data did not converge to a nonlinear regression curve fitting due to not reaching plateau at the highest concentrations tested (10<sup>-4.5</sup>M in the βarrestin assay, 10<sup>-5</sup>M in the G protein assay) the EC<sub>50</sub> is noted as >10,000 nM (if some stimulation could be observed) or NC (if the maximum concentration produced no effect) and the percentage of maximum stimulation at 10,000 nM is provided rather than an E<sub>MAX</sub> value where indicated. <sup>d</sup>For ΔΔlog(τ/K<sub>A</sub>) values, 95% confidence intervals (95% CI) are provided. Significant bias compared to DAMGO was determined by an unpaired, two-tailed t test: <sup>e</sup>p<0.0001; <sup>f</sup>p<0.001; <sup>g</sup>p<0.01, otherwise p>0.05. <sup>h</sup>Compound **4** was previously reported as SR-20382<sup>22</sup>

As shown in Table 2, adding an N-benzylic methyl group (R<sup>5</sup> = Me) substantially increased potency in the parent racemic compound (**16**, EC<sub>50</sub> = 102 nM)<sup>22</sup> as compared to **2**, but had little effect on GTPγS/βarr2 bias. Analogous to the Table 1 series, the addition of an *ortho*- or *para*-Cl substituent further augmented potency (EC<sub>50</sub> = 31 and 16 nM, respectively). In this case, the *para*-Cl compound **18**<sup>22</sup> was more biased than was the *ortho*-Cl compound **17**. The boost in GTPγS potency and efficacy eroded when an ethyl rather than a methyl group was present (**19**).

Replacement of the *para*-Cl substituent with either *para*-F substituent (**20**) or *para*-OMe group (**22**) gave potent compounds but with reduced bias; however, replacement with either a *para*-Br substituent (**21**) or *para*-OCF<sub>3</sub> group (**26**), increased GTPγS/βarr2 bias to 4.1 and 8.3, respectively. Increasing the size of the alkoxy group to ethoxy (**23**), *isopropoxy* (**24**), or ethylene

dioxy (**25**)<sup>22</sup> eroded potency in the GTP $\gamma$ S assay; however, the GTP $\gamma$ S/ $\beta$ arr2 bias factor of **24** was modestly increased to 3.6. Compounds **20** and **25**, interestingly, promoted bias towards  $\beta$ arrestin2 recruitment, opposite of what is expected to provide analgesia with reduced side effects (GTP $\gamma$ S/ $\beta$ arr2 bias factors = 0.56 and 0.47, respectively). We previously observed  $\beta$ arrestin2 bias for fentanyl vs. DAMGO at the human MOR when comparing GTP $\gamma$ S binding and the  $\beta$ arrestin2 recruitment using the enzyme fragment complementation assays (as used in this current study) (GTP $\gamma$ S/ $\beta$ arr2 bias factor = 0.18, measured relative to DAMGO),<sup>22</sup> and drew correlations with a reduced therapeutic index comparing mouse hot plate responses to changes in arterial oxygen saturation. Compound **25**, aka SR-11501, shared similar profiles with fentanyl in both the *in vivo* and *in vitro* assays<sup>22</sup>; and thus, it is attractive to speculate that preference for  $\beta$ arrestin2 recruitment over G protein coupling *in vitro* will consistently indicate a very narrow respiratory safety index *in vivo*. Compounds, such as **20** and additional compounds across a range of bias factors, will allow further testing of this hypothesis. Finally, compounds **27** and **28** showed that the addition of an *ortho*-F substituent had little effect on the potency, efficacy, and bias of compounds having *para*-Cl (**18**) and *para*-Br (**21**) substituents.

**Table 2.** GTP $\gamma$ S Binding and  $\beta$ arrestin2 Recruitment Activity of N-Benzyl Piperidine 4-Benzimidazolone Derivatives with N-Benzyl Substituents R<sup>5</sup>-R<sup>8</sup>.<sup>a</sup>

					<sup>35</sup> S-GTP $\gamma$ S Binding <sup>b</sup>			$\beta$ arrestin2 Recruitment <sup>b</sup>			Bias (GTP $\gamma$ S/ $\beta$ arr2)	
Agonist	R <sup>5</sup>	R <sup>6</sup>	R <sup>7</sup>	R <sup>8</sup>	EC <sub>50</sub>	E <sub>MAX</sub>	$\Delta\log(\tau/K_A)$	EC <sub>50</sub>	E <sub>MAX</sub>	$\Delta\log(\tau/K_A)$	$\Delta\log(\tau/K_A)$	Bias

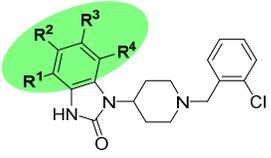
				(nM)	(%)		(nM)	(%)		(95% CI) <sup>c</sup>	Factor
<b>DAMGO</b>				33 ± 1	100		220 ± 8.3	100		0	1.0
<b>Morphine</b>				64 ± 3	83 ± 1	-0.3 ± 0.03	379 ± 17	24 ± 1	-0.6 ± 0.03	0.25 (0.17 to 0.33) <sup>d</sup>	1.8
<b>16<sup>e</sup></b>	Me			102 ± 10	89 ± 7	-0.5 ± 0.04	447 ± 52	67 ± 4	-0.3 ± 0.07	-0.18 (-0.45 to 0.08)	0.7
<b>17</b>	Me	Cl		31 ± 0.21	96 ± 1	0.05 ± 0.05	199 ± 19	79 ± 15	0.1 ± 0.06	-0.01 (-0.2 to 0.18)	1.0
<b>18<sup>e</sup></b>	Me		Cl	16 ± 1.2	97 ± 7	0.3 ± 0.07	184 ± 24	95 ± 4	0.02 ± 0.06	0.32 (0.09 to 0.56) <sup>f</sup>	2.1
<b>19</b>	Et		Cl	1593 ± 194	58 ± 1	-1.9 ± 0.08	6050 ± 1233	32 ± 4	-2 ± 0.08	-0.09 (-0.36 to 0.17)	0.81
<b>20</b>	Me		F	75 ± 8.3	93 ± 2	-0.4 ± 0.05	377 ± 76	78 ± 5	-0.2 ± 0.1	-0.26 (-0.53 to 0.02)	0.56
<b>21</b>	Me		Br	4.8 ± 0.41	91 ± 2	0.8 ± 0.02	182 ± 42	92 ± 5	0.2 ± 0.1	0.61 (0.11 to 1.11) <sup>f</sup>	4.1
<b>22</b>	Me		OMe	13 ± 0.64	95 ± 2	0.4 ± 0.03	87 ± 18	75 ± 8	0.4 ± 0.1	-0.06 (-0.50 to 0.37)	0.86
<b>23</b>	Me		OEt	189 ± 25	94 ± 2	-0.7 ± 0.06	1074 ± 165	50 ± 3	-0.6 ± 0.1	-0.04 (-0.33 to 0.24)	0.90
<b>24</b>	Me		OiPr	399 ± 31	83 ± 2	-1.1 ± 0.06	5413 ± 373	33 ± 1	-1.6 ± 0.1	0.56 (0.22 to 0.90) <sup>f</sup>	3.6
<b>25<sup>e</sup></b>	Me		-OCH <sub>2</sub> CH <sub>2</sub> O-	98 ± 8.5	77 ± 3.9	-0.5 ± 0.02	407 ± 50	59 ± 2.2	-0.2 ± 0.06	-0.32 (-0.45 to -0.2) <sup>d</sup>	0.47
<b>26</b>	Me		OCF <sub>3</sub>	31 ± 4.7	96 ± 1	0.1 ± 0.08	1678 ± 328	100 ± 3	-0.8 ± 0.06	0.92 (0.64 to 1.20) <sup>d</sup>	8.3
<b>27</b>	Me	F	Br	18 ± 2.4	93 ± 2	0.2 ± 0.04	523 ± 67	82 ± 7	-0.4 ± 0.05	0.52 (0.39 to 0.66) <sup>d</sup>	3.3
<b>28</b>	Me	F	Cl	22 ± 2.1	98 ± 3	0.2 ± 0.04	484 ± 94	82 ± 6	-0.3 ± 0.09	0.46 (0.10 to 0.82) <sup>f</sup>	2.9

<sup>a</sup>If blank or not shown, R<sup>x</sup> = H. All compounds were made and tested as racemates. <sup>b</sup>Within each assay, potency (EC<sub>50</sub>) and efficacy (E<sub>MAX</sub>) values were calculated by non-linear regression and  $\Delta\log(\tau/K_A)$  values were determined by the operational model, relative to DAMGO. Data are presented as mean ± SEM of three or more assays run in duplicate or triplicate. <sup>c</sup>For  $\Delta\Delta\log(\tau/K_A)$  values, 95% confidence intervals (95% CI) are provided. Significant bias compared to DAMGO was determined by an unpaired, two-tailed t test: <sup>d</sup>p < 0.0001; <sup>e</sup>Compounds **16**, **18**, and **25** were previously reported as (±)SR-8595, (±)SR-11065, and (±)SR-11501, respectively.<sup>22</sup>

We then investigated the effect of varying benzimidazolone substituents R<sup>1</sup>-R<sup>4</sup> (see Table 3). In this study, for consistency in interpretation, we held the distal N-benzyl substituent constant as an *ortho*-Cl (R<sup>6</sup> = Cl). Compounds with a Cl at R<sup>1</sup> (**29**), R<sup>2</sup> (**30**), or R<sup>3</sup> (**31**) were full agonists with increased GTPγS potency, GTPγS efficacy, and GTPγS/βarr2 bias relative to the des-Cl

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2  
3 analog **2**. Compound **32**, with  $R^4 = \text{Cl}$ , however, was far less active. The near-complete lack of  
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5 potency for compounds **30** and **31** in the  $\beta$ arrestin2 recruitment assay ( $\text{EC}_{50} > 10 \mu\text{M}$ ) was  
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7 encouraging and led us to explore whether groups other than Cl might also de-select  $\beta$ arrestin2  
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9 recruitment. Thus, we studied compounds with various  $R^2$  substituents (**33-39**) and found that the  
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11 groups shown erode  $\text{GTP}\gamma$  potency and/or  $\text{GTP}\gamma\text{S}/\beta$ arr2 bias, except for compound **33**,  $R^2 = \text{Me}$ ,  
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13 which was similar in potency and efficacy in the  $\text{GTP}\gamma$  assay but was slightly less biased due to  
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15 the higher potency in the  $\beta$ arrestin2 recruitment assay ( $\text{EC}_{50} = 6.8 \mu\text{M}$ ). We concluded that a  
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17 chloro substituent in the benzimidazolone ring system ( $R^1$ ,  $R^2$ , or  $R^3 = \text{Cl}$ ) favors higher potency  
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19 and efficacy in the  $\text{GTP}\gamma\text{S}$  assay while giving substantial bias (i.e., minimal  $\beta$ arrestin2  
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21 recruitment).  
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31 **Table 3.**  $\text{GTP}\gamma\text{S}$  Binding and  $\beta$ arrestin2 Recruitment Activity of N-Benzyl Piperidine 4-  
32  
33 Benzimidazolone Derivatives with Benzimidazolone Substituents  $R^1$ - $R^4$ .<sup>a</sup>  
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					$^{35}\text{S}$ - $\text{GTP}\gamma\text{S}$ Binding <sup>b</sup>			$\beta$ arrestin2 Recruitment <sup>b</sup>			Bias ( $\text{GTP}\gamma\text{S}/\beta$ arr2)	
Agonist	$R^1$	$R^2$	$R^3$	$R^4$	$\text{EC}_{50}$ (nM)	$E_{\text{MAX}}$ (%) <sup>c</sup>	$\Delta\log(\tau/K_A)$	$\text{EC}_{50}$ (nM)	$E_{\text{MAX}}$ (%) <sup>c</sup>	$\Delta\log(\tau/K_A)$	$\Delta\Delta\log(\tau/K_A)$ (95% CI) <sup>d</sup>	Bias Factor
DAMGO					33 ± 1	100		220 ± 8.3	100		0	1.0
Morphine					64 ± 3	83 ± 1	-0.3 ± 0.03	379 ± 17	24 ± 1	-0.6 ± 0.03	0.25 (0.17 to 0.33)	1.8
<b>2</b>					200 ± 19	92 ± 2	-0.8 ± 0.07	4487 ± 606	96 ± 9	-1.2 ± 0.05	0.4 (0.21 to 0.59)	2.5
<b>29</b>	Cl				48 ± 6.7	99 ± 1	-0.1 ± 0.09	1962 ± 326	69 ± 10	-0.9 ± 0.08	0.76 (0.51 to 1.01)	5.8
<b>30</b>		Cl			184 ± 23	93 ± 2	-0.7 ± 0.09	>10,000	47 ± 4 <sup>e</sup>	-1.6 ± 0.06	0.85 (0.60 to 1.11)	7.1
<b>31</b>			Cl		148 ± 16	99 ± 2	-0.6 ± 0.08	>10,000	40 ± 4 <sup>e</sup>	-1.5 ± 0.2	0.84 (0.46 to 1.22)	6.9

32			Cl	3109 ± 747	66 ± 16	-2.2 ± 0.07	NC	1 ± 1 <sup>c</sup>	/	/	/
33		Me		224 ± 9.6	99 ± 2	-0.8 ± 0.03	6769 ± 1337	79 ± 4	-1.5 ± 0.05	0.65 (0.49 to 0.81)	4.5
34		Br		57 ± 5	97 ± 2	-0.4 ± 0.04	843 ± 31	80 ± 3	-0.5 ± 0.03	0.12 (-0.03 to 0.27)	1.3
35		F		111 ± 16	96 ± 1	-0.7 ± 0.1	1644 ± 315	78 ± 6	-0.8 ± 0.09	0.11 (-0.26 to 0.47)	1.3
36		OMe		2123 ± 76	103 ± 3	-1.9 ± 0.01	>10,000	18 ± 2 <sup>c</sup>	-2.2 ± 0.1	0.28 (-0.17 to 0.73)	1.9
37		OCF <sub>3</sub>		1236 ± 111	57 ± 1	-1.9 ± 0.03	NC	3 ± 1 <sup>c</sup>	/	/	/
38		SO <sub>2</sub> Me		7618 ± 737	75 ± 7	-2.5 ± 0.07	>10,000	6 ± 1 <sup>c</sup>	-2.5 ± 0.04	0.01 (-0.15 to 0.17)	1.0
39		CN		>10,000	17 ± 2 <sup>c</sup>	-2.8 ± 0.06	NC	-0.2 ± 0.2 <sup>c</sup>	/	/	/

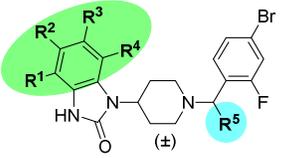
<sup>a</sup>If blank or not shown, R<sup>x</sup> = H. “/” = not determined. <sup>b</sup>Within each assay, potency (EC<sub>50</sub>) and efficacy (E<sub>MAX</sub>) values were calculated by non-linear regression and  $\Delta\log(\tau/K_A)$  values were determined by the operational model, relative to DAMGO. Data are presented as mean ± SEM of three or more assays run in duplicate or triplicate. <sup>c</sup>In instances where the data did not converge (NC) and therefore no potency value could be calculated, or produced a value outside the experimental range (EC<sub>50</sub> > 10,000 nM), the percentage of maximum stimulation at 10,000 nM is provided. <sup>d</sup>For  $\Delta\Delta\log(\tau/K_A)$  values, 95% confidence intervals (95% CI) are provided. Significant bias compared to DAMGO was determined by an unpaired, two-tailed t test: <sup>e</sup>p < 0.0001; <sup>f</sup>p < 0.001; <sup>g</sup>p < 0.01, otherwise p > 0.05.

To determine if the benzimidazolone chloro effect was compatible with substituents other than *ortho*-Cl on the distal N-benzyl ring, as well as to evaluate compatibility with a benzylic methyl at R<sup>5</sup> (which was expected to increase potency, as was shown in Table 2), another diverse series of analogs was investigated (Table 4). This set of compounds contained, in the N-benzyl ring, *ortho*-F and *para*-Br substituents at R<sup>6</sup> and R<sup>8</sup>, respectively, since this combination had given advantages in potency and bias (see Table 2, compare **17** and **27**).

Compared to compound **15**, with R<sup>1</sup>-R<sup>5</sup> = H, the presence of Cl at R<sup>1</sup> (**40**), R<sup>2</sup> (**41**), or R<sup>3</sup> (**42**) had little effect on potency in the GTP $\gamma$ S assay, but bias was more pronounced, especially in compound **41** (GTP $\gamma$ / $\beta$ arr2 bias factor = 23, Table 4). Potency in the GTP $\gamma$ S assay was greatly augmented, as noted earlier (Table 2), with the addition of a benzylic methyl group at R<sup>5</sup>: see

compounds **15** and **27**, while bias was relatively unchanged. Di and trichlorinated analogs were also prepared. A Cl present at both R<sup>1</sup> and R<sup>3</sup> (**43**) had no advantage over the reference compound **15** with regard to potency in G protein signaling or in bias disfavoring  $\beta$ arrestin2 recruitment. On the other hand, a Cl at both R<sup>2</sup> and R<sup>3</sup> (**44**), while similar in potency to compound **15** in the GTP $\gamma$ S signaling assay (EC<sub>50</sub> = 91 nM), was essentially devoid of all  $\beta$ arrestin2 recruitment activity (EC<sub>50</sub> > 10  $\mu$ M, E<sub>MAX</sub> = 12% of DAMGO, GTP $\gamma$ / $\beta$ arr2 bias factor = 56). As expected based upon earlier data for compound **32** (Table 3), a Cl at R<sup>4</sup> is not tolerated; thus, the trichloro compound **45** lost potency in the GTP $\gamma$ S assay.

**Table 4.** GTP $\gamma$ S Binding and  $\beta$ arrestin2 Recruitment Activity of N-Benzyl Piperidine 4-Benzimidazolone Derivatives with Benzimidazolone Substituents R<sup>1</sup>-R<sup>5</sup>.<sup>a</sup>

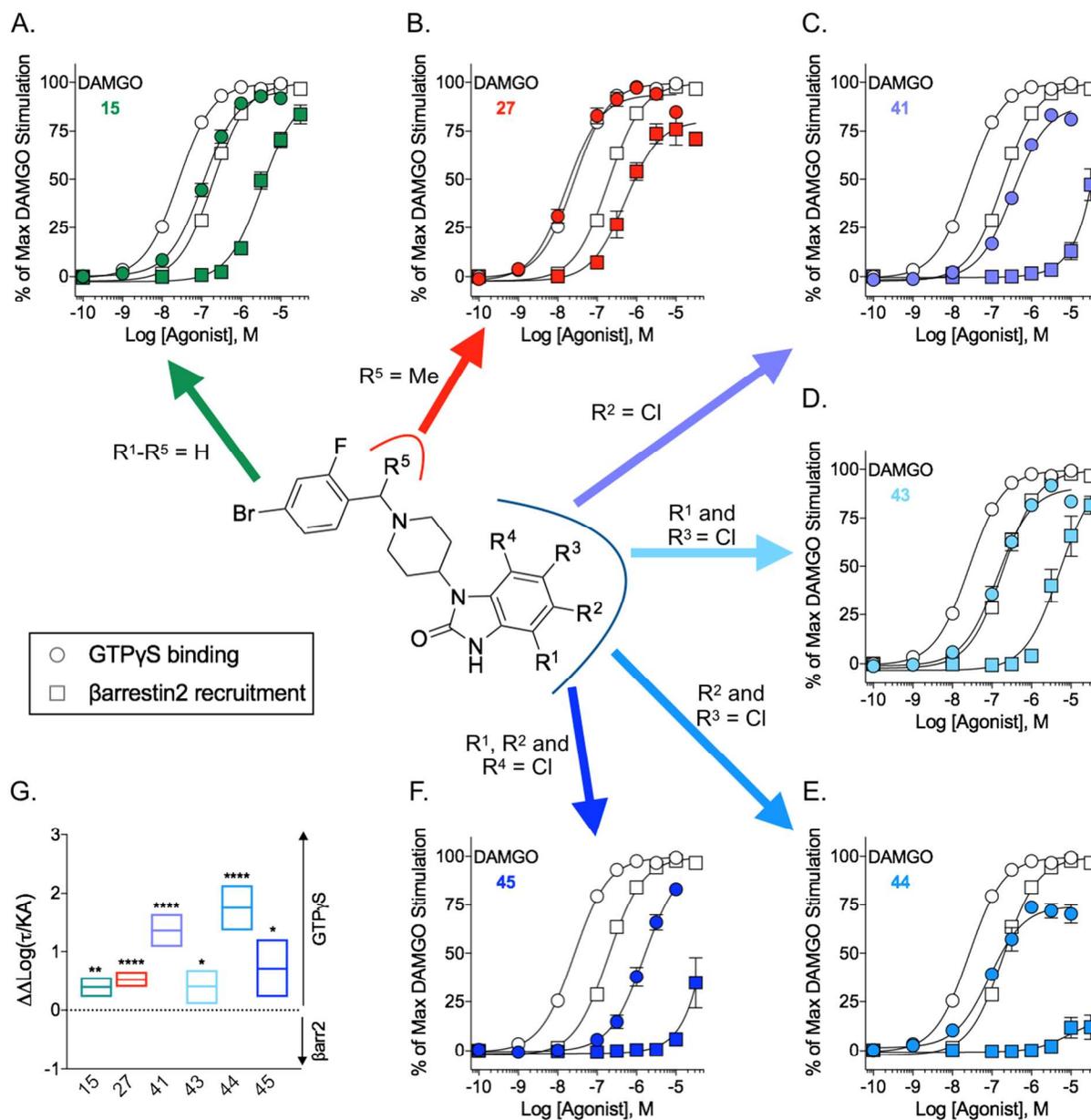
						<sup>35</sup> S-GTP $\gamma$ S Binding <sup>c</sup>			$\beta$ arrestin2 Recruitment <sup>c</sup>			Bias (GTP $\gamma$ S/ $\beta$ arr2)	
Agonist	R <sup>1</sup>	R <sup>2</sup>	R <sup>3</sup>	R <sup>4</sup>	R <sup>5</sup>	EC <sub>50</sub> (nM)	E <sub>MAX</sub> (%) <sup>d</sup>	$\Delta$ log( $\tau$ /K <sub>A</sub> )	EC <sub>50</sub> (nM)	E <sub>MAX</sub> (%) <sup>d</sup>	$\Delta$ log( $\tau$ /K <sub>A</sub> )	$\Delta$ log( $\tau$ /K <sub>A</sub> ) (95% CI) <sup>e</sup>	Bias Factor
DAMGO						33 ± 1	100		220 ± 8.3	100		0	1.0
Morphine						64 ± 3	83 ± 1	-0.3 ± 0.03	379 ± 17	24 ± 1	-0.6 ± 0.03	0.25 (0.17 to 0.33) <sup>f</sup>	1.8
<b>15</b>						110 ± 16	96 ± 0.06	-0.7 ± 0.04	3357 ± 60	94 ± 6	-1.1 ± 0.04	0.39 (0.22 to 0.56) <sup>g</sup>	2.5
<b>27</b>					Me <sup>b</sup>	18 ± 2.4	93 ± 2	0.2 ± 0.04	523 ± 67	82 ± 7	-0.4 ± 0.05	0.52 (0.39 to 0.66) <sup>f</sup>	3.3
<b>40</b>	Cl					55 ± 8.1	93 ± 2	-0.5 ± 0.1	2912 ± 189	71 ± 6	-1.1 ± 0.1	0.59 (0.21 to 0.98) <sup>h</sup>	3.9
<b>41</b>		Cl				355 ± 26	89 ± 2	-1.1 ± 0.04	>10,000	14 ± 4 <sup>d</sup>	-2.5 ± 0.2	1.36 (1.08 to 1.64) <sup>f</sup>	<b>23</b>
<b>42</b>			Cl			127 ± 11	88 ± 7	-0.6 ± 0.07	3459 ± 634	49 ± 9	-1.3 ± 0.1	0.62 (0.31 to 0.94) <sup>g</sup>	4.2
<b>43</b>	Cl		Cl			153 ± 32	91 ± 2	-0.9 ± 0.1	>10,000	66 ± 10 <sup>d</sup>	-1.3 ± 0.02	0.40 (0.10 to 0.69) <sup>h</sup>	2.5

<b>44</b>		Cl	Cl			91 ± 5.5	74 ± 4	-0.6 ± 0.06	>10,000	12 ± 5 <sup>d</sup>	-2.4 ± 0.1	1.75 (1.36 to 2.14) <sup>f</sup>	<b>56</b>
<b>45</b>	Cl	Cl		Cl		1764 ± 384	99 ± 3	-1.8 ± 0.1	>10,000	5.9 ± 3 <sup>d</sup>	-2.5 ± 0.06	0.71 (0.22 to 1.21) <sup>h</sup>	5.2

<sup>a</sup>If blank or not shown, R<sup>x</sup> = H. <sup>b</sup>Compounds were made and tested as racemates. <sup>c</sup>Within each assay, potency (EC<sub>50</sub>) and efficacy (E<sub>MAX</sub>) values were calculated by non-linear regression and Δlog(τ/K<sub>A</sub>) values were determined by the operational model, relative to DAMGO. Data are presented as mean ± SEM of three or more assays run in duplicate or triplicate. <sup>d</sup>In instances where the data produced a value outside the experimental range (EC<sub>50</sub> >10,000 nM), the percentage of maximum stimulation at 10,000 nM is provided. <sup>e</sup>For ΔΔlog(τ/K<sub>A</sub>) values, 95% confidence intervals (95% CI) are provided. Significant bias compared to DAMGO was determined by an unpaired, two-tailed t test: <sup>f</sup>p<0.0001; <sup>g</sup>p<0.01; <sup>h</sup>p<0.05.

The potency and bias effects for the compound series in Table 4 are made even more apparent when comparing full dose-response curves, as shown in Figure 2. Moving clockwise within the Figure, panel A shows curves for the GTPγS binding assay (circles) and the βarrestin2 recruitment assay (squares) for the modestly biased reference compound **15** (green) compared with DAMGO (white). Panel B shows the potency enhancement seen for **27**, which has a benzylic methyl group: note the left-shifted curve (red circles). Panel C shows data for the mono-chloro substituted compound **41**, which is less potent since it lacks a benzylic methyl group but is still highly biased. Panel D shows data for compound **43**, the less biased and less potent dichloro benzimidazolone analog. Panel E shows the enhanced signaling bias of compound **44**: note the flattened βarrestin2 curve (blue squares). The right-shifted curve (dark blue circles) in panel F depicts the significantly less potent compound **45**. It is worth noting that at the highest concentrations tested, in some cases we see an increase in response; however, we are unable to determine if the response would plateau, as solubility limitations preclude higher concentrations in the assay. Therefore, in the tables, % maximum stimulation at 10,000 nM is provided when potency cannot be calculated. Finally, panel G graphically compares the analogs and depicts the

substantial signaling bias seen for compounds **41** and **44** (GTP $\gamma$ / $\beta$ arr2 bias factors = 23 and 56, respectively).

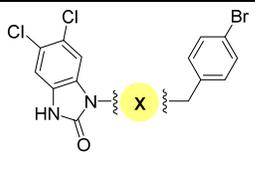
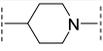
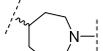
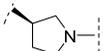
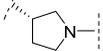
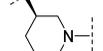
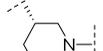


**Figure 2.** SAR increasing activation of G protein binding with differential  $\beta$ arrestin 2 signaling profiles. Four analogs were synthesized to determine how the addition of chlorine substituents to the benzimidazolone ring affects bias (**41**, **43-45**). Analogs were compared to compound **15**, which has an unsubstituted benzimidazolone ring.

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3 Compound **27** was made to determine the effect that the addition of a benzylic methyl group had on bias compared  
4 to **15**. Concentration response curves in the GTP $\gamma$ S binding (circles) and  $\beta$ arrestin2-EFC assays (squares) are shown  
5 for the test compounds (solid symbols) versus DAMGO (open symbols) and are presented as mean  $\pm$  S.E.M.; curves  
6 are the result of 3 parameter nonlinear regression analysis.  $\Delta\Delta\text{Log}(\tau/K_A)$  values are plotted to demonstrate relative  
7 bias, with 95% confidence intervals. Significant bias compared to DAMGO was determined by an unpaired, two-  
8 tailed t test: \*\*\*\*p<0.0001; \*\*p<0.01; \*p<0.05.

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20 With the finding that compound **44**, with  $R^2 = R^3 = \text{Cl}$ , has the greatest bias among Table 4  
21 analogs, we then held this portion of the structure constant and also held the substitution pattern  
22 in the distal N-benzyl ring constant, with a *para*-Br substituent, which we had shown to confer  
23  $\beta$ arrestin2 bias (see compound **15** in Table 1 and compound **21** in Table 2). The additional *ortho*-  
24 F substituent in Table 4 analogs had only a small effect: compare compound **44** (Table 4) to  
25 compound **46** (Table 5). With both ends of the molecule held constant, we probed the effect of  
26 changing the size of the central ring, as well as altering where the benzimidazolone is attached  
27 (Table 5). The 7-membered (**47**) and 5-membered ring analogs (**48** and **49**) were less potent in  
28 the GTP $\gamma$ S assay and were less biased relative to the corresponding 4-substituted piperidine (**46**,  
29  $\text{EC}_{50} = 149$  nM and GTP $\gamma$ S/ $\beta$ arr2 bias factor = 45).<sup>22</sup> Furthermore, the 3-substituted piperidines  
30 (**50** and **51**) were essentially inactive as MOR agonists, with no significant efficacy until tested at  
31 the 10  $\mu\text{M}$  concentration.

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53 **Table 5.** GTP $\gamma$ S Binding and  $\beta$ arrestin2 Recruitment Activity of N-Benzyl Benzimidazolone  
54 Derivatives.  
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		$^{35}\text{S}$ -GTP $\gamma$ S Binding <sup>a</sup>			$\beta$ arrestin2 Recruitment <sup>a</sup>			Bias (GTP $\gamma$ S/ $\beta$ arr2)	
Agonist	X	EC <sub>50</sub> (nM)	E <sub>MAX</sub> (%) <sup>b</sup>	$\Delta\log(\tau/K_A)$	EC <sub>50</sub> (nM)	E <sub>MAX</sub> (%) <sup>b</sup>	$\Delta\log(\tau/K_A)$	$\Delta\Delta\log(\tau/K_A)$ (95% CI) <sup>c</sup>	Bias Factor
DAMGO		33 ± 1	100		220 ± 8.3	100		0	1.0
Morphine		64 ± 3	83 ± 1	-0.3 ± 0.03	379 ± 17	24 ± 1	-0.6 ± 0.03	0.25 (0.17 to 0.33) <sup>d</sup>	1.8
46 <sup>e</sup>		149 ± 9.1	83 ± 4	-0.8 ± 0.04	>10,000	3.6 ± 1 <sup>b</sup>	-2.4 ± 0.04	1.66 (1.53 to 1.79) <sup>d</sup>	45
47		1403 ± 225	90 ± 9	-1.7 ± 0.05	>10,000	6.7 ± 4 <sup>b</sup>	-2.6 ± 0.2	0.93 (0.31 to 1.55) <sup>f</sup>	8.5
48		2975 ± 400	87 ± 6	-1.9 ± 0.07	>10,000	7.5 ± 3 <sup>b</sup>	-2.7 ± 0.09	0.79 (0.51 to 1.07) <sup>d</sup>	6.2
49		373 ± 31	90 ± 4	-1.1 ± 0.08	>10,000	29 ± 6 <sup>b</sup>	-1.8 ± 0.2	0.73 (0.34 to 1.11) <sup>g</sup>	5.3
50		>10,000	24 ± 6 <sup>b</sup>	-2.7 ± 0.4	NC	-0.3 ± 0.1 <sup>b</sup>	/	/	/
51		>10,000	21 ± 10 <sup>b</sup>	-2.8 ± 0.08	NC	-0.3 ± 0.2	/	/	/

<sup>a</sup>Within each assay, potency (EC<sub>50</sub>) and efficacy (E<sub>MAX</sub>) values were calculated by non-linear regression and  $\Delta\log(\tau/K_A)$  values were determined by the operational model, relative to DAMGO. Data are presented as mean ± SEM of three or more assays run in duplicate or triplicate. <sup>b</sup>In instances where the data did not converge (NC) or produced a value outside the experimental range (EC<sub>50</sub> >10,000 nM), the percentage of maximum stimulation at 10,000 nM is provided. <sup>c</sup>For  $\Delta\Delta\log(\tau/K_A)$  values, 95% confidence intervals (95% CI) are provided. Significant bias compared to DAMGO was determined by an unpaired, two-tailed t test: <sup>d</sup>p<0.0001; <sup>e</sup>Compound 46 was previously reported as SR-15099. <sup>22</sup> <sup>f</sup>p<0.05; <sup>g</sup>p<0.01. “/” = not determined.

**Drug Metabolism and Pharmacokinetics (DMPK) Properties.** In order for a compound to advance into further development, it must be metabolically stable and must have suitable tissue distribution (including, in this case, high brain exposure). Furthermore, compounds must be non-toxic at efficacious doses and must not prompt drug-drug interactions. As a preliminary gauge of

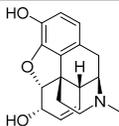
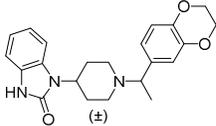
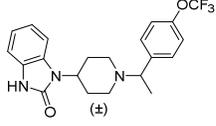
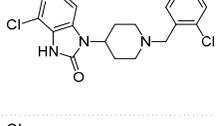
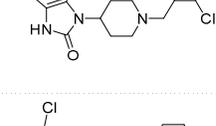
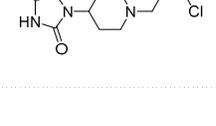
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3 compound metabolic stability, we assessed the stability of promising compounds in the presence  
4 of mouse and human liver microsomes. To determine the potential for drug-drug interactions, we  
5 studied their ability to inhibit four of the major metabolizing cytochrome P450 (CYP) isoforms.  
6  
7 This *in vitro* DMPK data was used, along with the GTP $\gamma$ S potency and GTP $\gamma$ S/ $\beta$ arr2 bias data, to  
8 select compounds for further PK evaluation *in vivo*.  
9

10  
11 Table 6 shows selected *in vitro* and *in vivo* properties of many compounds in the series that  
12 were chosen for follow-up due to their significant GTP $\gamma$ S potency ( $EC_{50} < 400$  nM) and high  
13 bias (GTP $\gamma$ S/ $\beta$ arr2 bias factor  $> 5$ ). An exception is compound **25**, which was used as a  
14 comparator. This compound, like fentanyl,<sup>22</sup> is  $\beta$ arrestin2 biased (relative to DAMGO) and  
15 thus, is expected to have enhanced respiratory suppressive effects that could limit its usefulness.  
16 In this series, the inhibition of CYP isoforms was not a major concern: among the compounds  
17 tested, only compound **29** gave  $> 50\%$  inhibition of any CYP isoform at 10  $\mu$ M. The liver  
18 microsome stability of these compounds was more widely variable. Fortunately, human liver  
19 microsome stability exceeding 1 hour was seen in some of the compounds (**26**, **41**, **44**, and **46**).  
20  
21 Though these compounds were generally less stable to mouse liver microsomes compared to the  
22 human, stability was sufficient for *in vivo* PK evaluations in mice. Of note, we previously  
23 reported that the G protein and  $\beta$ arrestin2 signaling profiles for two compounds in this series (**25**  
24 and **46**) are consistent between the mouse and human MOR.<sup>22</sup>  
25  
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27  
28 To determine if our MOR agonists could penetrate the BBB, which is required in an opioid  
29 analgesic agent, compound **25** and the compounds most highly biased towards GTP $\gamma$ S over  $\beta$ arr2  
30 (**41**, **44**, and **46**) were administered intraperitoneally (*i.p.*) at 6 mg/kg<sup>22</sup> and brain levels were  
31 determined after 1 hour. As shown in Table 6, these compounds were present in the brain 1 hour  
32 following systemic injection at levels exceeding that seen with a systemic injection of morphine  
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at the same dose. High brain levels at this time point are consistent with enhanced compound stability as indicated by liver microsome stability data, suggesting the value of using *in vitro* DMPK assessments to drive the selection of compounds for *in vivo* evaluation.

**Table 6.** *In Vitro* and *In Vivo* Evaluation of Select N-Benzyl Piperidine 4-Benzimidazolone Derivatives.

Agonist	Structure <sup>a</sup>	<sup>35</sup> S-GTPγS EC50 (nM) <sup>b</sup>	Bias Factor	CP450 Inhibition %				Microsome Stability		Brain Level (μM) <sup>c</sup>
				Inhibition at 10 μM				T <sub>1/2</sub> life (minutes)		
				1A2	2C9	2D6	3A4	Human	Mouse	
Morphine		64	1.8							0.39 ± 0.07
25 <sup>d</sup>		98	0.47	6	-30	-3	3	16	1	0.70 ± 0.16
26		31	8.3	-40	1	-34	-46	120	14	
29		48	5.8	40	55	50	13	8	3	
30		184	7.1	-23	20	38	-2	6	3	
31		148	6.9	12	34	41	0	17	4	

41		355	23	4	41	18	17	84	17	17 ± 0.35
44		91	56	-10	-1	-16	-16	73	31	4.6 ± 0.28
46 <sup>d</sup>		149	45	5	14	-1	-3	233	35	11 ± 2.2

Microsomal stability assays were conducted *in vitro*. Brain levels of compound were determined in mice 1 hour following a 6 mg/kg, *i.p.* injection of the compound indicated. <sup>a</sup>Compounds **25** and **26** were made and tested as racemates. <sup>b</sup>Data are presented as mean ± SEM of three or more assays run in duplicate or triplicate. <sup>d</sup>Compounds **25** and **46** were previously reported as (±)SR-11501 and SR-15099, respectively.<sup>22</sup>

## CONCLUSIONS

In analyzing data for the set of 51 related compounds shown in Tables 1-5, as well as DMPK and efficacy properties (Table 6 and Figure 2), we have established a pharmacophore for G protein biased MOR agonism. Certain hydrophobic substituents, especially halogen atoms in the distal benzimidazolone and the N-benzyl ring, are essential for extreme G protein coupling bias. The presence of a central piperidine ring is also preferred. In many cases, a benzylic methyl group augments potency and has a small but typically beneficial impact upon bias. The structural basis for these effects is currently under investigation. Additional ongoing studies include: more extensive target selectivity profiling of top compounds, safety studies, antinociceptive studies in multiple species, and the separation and/or the stereoselective asymmetric synthesis of individual isomers of analogs reported here as racemates (e.g., Table 2 compounds). Because the degree of

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3 respiratory safety correlates linearly with the magnitude of the GTP $\gamma$ S/ $\beta$ arr2 bias factor as  
4  
5 calculated herein,<sup>22</sup> we hope that the trends noted in this SAR study may guide the design of  
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7 substantially safer opioid analgesic agents.  
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## 14 EXPERIMENTAL SECTION

16  
17 **General Procedures.** Materials were purchased from commercial vendors and used without  
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19 purification. All moisture-sensitive reactions were performed under argon. Experiments were  
20  
21 monitored by LCMS or TLC and visualized using an ultraviolet lamp (254 nm) or staining with  
22  
23 KMnO<sub>4</sub>. Flash column chromatography was performed using a Teledyne ISCO Combiflash®  
24  
25 Rf+ and Luknova silica gel cartridges. All NMR data was collected at room temperature on a  
26  
27 Brüker Ultrashield 400 MHz nuclear magnetic resonance spectrometer. Chemical shifts for <sup>1</sup>H  
28  
29 NMR spectra are reported in parts per million (ppm) relative to residual solvent signal as an  
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31 internal standard: DMSO ( $\delta$  2.50), CHCl<sub>3</sub> ( $\delta$  7.26), or MeOH ( $\delta$  3.31). Multiplicities are given as:  
32  
33 s (singlet), d (doublet), t (triplet), q (quartet), or m (multiplet). Coupling constants are reported as  
34  
35 a *J* value in Hertz (Hz). Mass spectra were recorded on a Thermo Scientific 3000 LCQ Fleet  
36  
37 system (ESI) using a Discovery® HS C18 HPLC column (10 cm x 2.1 mm, 5  $\mu$ m) at 35 °C with  
38  
39 UV detection at 210, 254, and 280 nm. Flow rate was 0.7 mL/min using a solvent gradient of 5-  
40  
41 95% B over 4 min (total run time = 6 min), where A = 0.1% formic acid in water and B = 0.1%  
42  
43 formic acid in acetonitrile. Analytical HPLC was performed on an Agilent 1100 series using an  
44  
45 Agilent Eclipse XDB-C18 HPLC column (4.6 x 150 mm, 5  $\mu$ m) with UV detection at 254 nm.  
46  
47 Flow rate was 1.75 mL/min using a solvent gradient of 10-90% B over 8 min (total run time = 10  
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49 min), where A = 0.1% TFA and 1% acetonitrile in water and B = acetonitrile. The purity of all  
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3 compounds used in the bioassays was determined to be  $\geq 95\%$  by analytical HPLC. For the *in*  
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5 *vitro* studies, DAMGO (Tocris) and morphine sulfate pentahydrate (NIDA Drug Supply  
6  
7 Program) were dissolved in water as 10 mM stocks. All other compounds were dissolved in  
8  
9 100% DMSO as 10 mM stocks. For all assays, the final DMSO concentration was 1%.

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13 ***tert*-Butyl 4-((2-nitrophenyl)amino)piperidine-1-carboxylate, (I)**<sup>22</sup>  
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16 A mixture of 1-fluoro-2-nitrobenzene (0.5 mL, 5.0 mmol), N-boc-4-aminopiperidine (1.0 g, 5.0  
17  
18 mmol), K<sub>2</sub>CO<sub>3</sub> (0.8 g, 5.9 mmol), and DMSO (5 mL) was stirred at room temperature overnight  
19  
20 under argon. The reaction mixture was quenched with water and the aqueous layer was extracted  
21  
22 with CH<sub>2</sub>Cl<sub>2</sub>; the combined organic layers were dried over Na<sub>2</sub>SO<sub>4</sub> and concentrated to dryness.  
23  
24 Purification afforded pure product **I** (1.5 g, 93% yield). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  8.17 (dd,  
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26  $J = 8.6, 1.8$  Hz, 1H), 8.08 (d,  $J = 1.6$  Hz, 1H), 7.42 (td,  $J = 7.8, 1.6$  Hz, 1H), 6.86 (d,  $J = 8.4$  Hz,  
27  
28 1H), 6.64 (td,  $J = 7.8, 1.2$  Hz, 1H), 4.01 (dt,  $J = 13.6, 3.8$  Hz, 1H), 3.70-3.64 (m, 1H), 3.04 (td,  $J$   
29  
30 = 12.2, 3.0 Hz, 2H).  
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35 ***tert*-Butyl 4-((2-aminophenyl)amino)piperidine-1-carboxylate, (II)**<sup>22</sup>  
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38 A 50% aqueous suspension of Raney nickel (11.9 mL) was added to a mixture of **I** (1.5 g, 4.7  
39  
40 mmol) in absolute EtOH (95 mL). Hydrazine hydrate (2.3 mL, 47 mmol) was then added  
41  
42 dropwise. The mixture was heated to 45 °C, stirred for 10 min, and then filtered through a pad of  
43  
44 Celite<sup>®</sup>. The pad was washed with MeOH and the filtrate was concentrated to dryness.  
45  
46 Purification afforded pure product **II** (1.0 g, 74% yield). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  6.79 (td,  
47  
48  $J = 7.2, 2.2$  Hz, 1H), 6.72-6.66 (m, 3H), 4.14-4.02 (m, 1H), 3.41-3.34 (m, 4H), 2.93 (t,  $J = 11.8$   
49  
50 Hz, 2H), 2.00 (dd,  $J = 13.0, 3.0$  Hz, 2H), 1.48 (s, 9H), 1.36 (qd,  $J = 12.2, 4.0$  Hz, 2H); MS(*m/z*):  
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52 [M+H] calc'd for C<sub>16</sub>H<sub>26</sub>N<sub>3</sub>O<sub>2</sub> is 292.19, found 291.76.  
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***tert*-Butyl 4-(2-oxo-2,3-dihydro-1*H*-benzo[*d*]imidazol-1-yl)piperidine-1-carboxylate, (III)<sup>22</sup>**

CDI (780 mg, 4.8 mmol) was slowly added to a solution of **II** (1.0 g, 3.4 mmol) in THF (30 mL). The reaction mixture was stirred overnight at room temperature under argon and then quenched with 10% HCl. The aqueous layer was extracted with EtOAc. The combined organic layers were dried over Na<sub>2</sub>SO<sub>4</sub> and concentrated to dryness. Purification afforded pure product **III** (0.6 g, 55% yield). <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 9.30 (s, 1H), 7.16-7.06 (m, 4H), 4.48 (tt, *J* = 12.6, 4.0 Hz, 1H), 4.33 (dd, *J* = 11.6, 2.0 Hz, 2H), 2.88 (td, *J* = 13.0, 2.4 Hz, 2H), 2.34 (qd, *J* = 12.8, 4.4 Hz, 2H), 1.84 (dd, *J* = 12.0, 2.4 Hz, 2H), 1.51 (s, 9H); MS(*m/z*): [M+H] calc'd for C<sub>17</sub>H<sub>24</sub>N<sub>3</sub>O<sub>3</sub> is 318.17, found 317.71.

**1-(Piperidin-4-yl)-1*H*-benzo[*d*]imidazol-2(3*H*)-one TFA salt, (IV)<sup>22</sup>**

Compound **III** (0.6 g, 1.9 mmol) was dissolved in a 33% solution of TFA in CH<sub>2</sub>Cl<sub>2</sub> (6 mL). The reaction mixture was stirred at room temperature until completion and then was concentrated to dryness. The solid was dissolved in 1:1 water/acetonitrile. The solution was frozen and then subjected to lyophilization overnight giving **IV** in the form of a TFA salt. This material was used without further purification. <sup>1</sup>H NMR (400 MHz, CD<sub>3</sub>OD) δ 7.29-7.26 (m, 1H), 7.11-7.08 (m, 3H), 4.55 (tt, *J* = 12.2, 4.2 Hz, 1H), 3.58 (dt, *J* = 12.8, 2.2 Hz, 2H), 3.22 (td, *J* = 13.2, 2.4 Hz, 2H), 2.74 (qd, *J* = 12.6, 4.4 Hz, 2H), 2.06 (dd, *J* = 12.8, 1.6 Hz, 2H); MS(*m/z*): [M+H] calc'd for C<sub>12</sub>H<sub>16</sub>N<sub>3</sub>O is 218.12, found 217.92.

**1-(1-(Benzyl)piperidin-4-yl)-1*H*-benzo[*d*]imidazol-2(3*H*)-one, (1)<sup>25</sup>**

A mixture of **IV** (free base, 110 mg, 0.5 mmol), benzyl bromide (87 mg, 0.5 mmol), DIPEA (132 μL, 0.8 mmol), and DMF (1 mL) was stirred at room temperature overnight under argon. Upon completion, the solvent was removed under reduced pressure and the residue was

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3 dissolved in EtOAc (20 mL). This reaction mixture was washed with saturated NaHCO<sub>3</sub>  
4 followed by brine. The organic layer was dried over Na<sub>2</sub>SO<sub>4</sub> and concentrated to dryness.  
5  
6 Purification afforded pure product **1** (139 mg, 89% yield). <sup>1</sup>H NMR (400 MHz, (CD<sub>3</sub>)<sub>2</sub>SO) δ  
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8 10.82 (s, 1H), 7.35-7.34 (m, 4H), 7.28-7.21 (m, 1H), 7.01-6.95 (m, 3H), 4.14 (tt, *J* = 12.4, 4.0  
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10 Hz, 1H), 3.53 (s, 2H), 2.94 (d, *J* = 11.6 Hz, 2H), 2.35 (qd, *J* = 12.4, 4.0 Hz, 2H), 2.09 (t, *J* = 11.0  
11  
12 Hz, 2H), 1.64 (d, *J* = 9.2 Hz, 2H); MS(*m/z*): [M+H] calc'd for C<sub>19</sub>H<sub>22</sub>N<sub>3</sub>O is 308.17, found  
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14 308.06; HPLC *t<sub>R</sub>* = 3.39 min.  
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### 19 20 **GTPγS Binding** 21 22

23 MOR-stimulated GTPγS binding was determined in membranes prepared from CHO-hMOR  
24 cells as described.<sup>22</sup> CHO-hMOR cells were serum-starved for 30 minutes and collected via  
25 gentle scraping in EDTA buffer. Membranes were then prepared via dounce homogenization and  
26 centrifugation at 20,000 x *g* for 30 minutes at 4°C. GTPγS binding reactions were performed in  
27  
28 200 μL volumes containing 10 μg CHO-hMOR membranes, 50 μM guanosine-5'-diphosphate  
29  
30 (GDP, Sigma Aldrich G7127), 0.1 nM <sup>35</sup>S-GTPγS (PerkinElmer NEG030H) and concentrations  
31  
32 of the compounds ranging from 0.1 nM to 10 μM were incubated for 1 hour at room temperature.  
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34 Reactions were terminated by rapid filtration through GF/B glass fiber filter plates (PerkinElmer)  
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36 and radioactivity was counted with a TopCount NXT Scintillation Counter (PerkinElmer).  
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### 45 **βarrestin2 Enzyme Fragment Complementation Assay** 46 47

48 USOS-βarrestin-hMOR-PathHunter cells (DiscoverX 93-0213C3) were used to determine  
49  
50 βarrestin2 interactions with the MOR by an enzyme fragment complementation assay.<sup>22</sup> The  
51  
52 cells (4,000 cells/well) were incubated with 0.1 nM to 10 or 31 μM (depending on compound  
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54 solubility) concentrations of the test compounds at 37 °C for 90 minutes and βarrestin2  
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3 translocation was determined according to the manufacturer's instructions (DiscoverX).  
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5 Luminescence was measured using a SpectraMax M5<sup>e</sup> Microplate Reader (Molecular Devices)  
6  
7 with 1 second integration times.  
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### 10 **Pharmacokinetic Parameters**

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14 To determine stability in hepatic microsomes, compound (1  $\mu$ M) was incubated with 1 mg/mL  
15  
16 human or mouse hepatic microsomes at 37°C with continuous shaking.<sup>26</sup> At 0, 5, 10, 20, 40 and  
17  
18 60 minute time-points, aliquots were removed and acetonitrile was added to quench the reactions  
19  
20 and precipitate the proteins. Samples were then centrifuged through 0.45 micron filter plates and  
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22 half-lives were determined by LC-MS/MS.  
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26 To determine cytochrome P450 inhibition, 1  $\mu$ M compound was incubated with human liver  
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28 microsomes and selective marker substrates (1A2, phenacetin demethylation to acetaminophen;  
29  
30 2C9, tolbutamide hydroxylation to hydroxytolbutamide; 2D6, bufuralol hydroxylation to 4'-  
31  
32 hydroxybufuralol; 3A4, midazolam hydroxylation to 1'-hydroxymidazolam). After a 10 minute  
33  
34 incubation, the reaction was terminated and the percent inhibition was determined.<sup>27</sup>  
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39 *Pharmacokinetics in mice:* Male C57BL6/J mice were purchased from Jackson Laboratory  
40  
41 (Bar Harbor, ME) and assessed at 10-12 weeks per age. All mice were used in accordance with  
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43 the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals with  
44  
45 approval by the Scripps Research Institutional Animal Care and Use Committee (IACUC). To  
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47 determine brain penetrance, mice were injected with drug (*i.p.* at mg/kg at 10  $\mu$ L/g volumes  
48  
49 mouse body weight using a vehicle of 1:1:8 DMSO, Tween80, and purified water). Mice were  
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51 sacrificed by cervical dislocation 1 hour following drug treatment and isolated brains were flash  
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3 frozen in liquid nitrogen. Drug levels were determined using a LC-MS operated in positive-ion  
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5 mode, 1 hour after treatment.  
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### 8 **Data and Statistical Analysis**

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11 GraphPad Prism (v. 7.0) was used for data and statistical analysis. All data are presented as  
12 mean  $\pm$  S.E.M or with 95% confidence intervals, as indicated in the figure and table legends. For  
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14 the *in vitro* studies, the assays were run in duplicate, with at least 3 independent replicates and  
15  
16 DAMGO was run as the reference compound in every experiment for normalization. EC<sub>50</sub> and  
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18 E<sub>MAX</sub> values were calculated by nonlinear (three parameter) regression analysis. Due to the limit  
19  
20 of solubility for some compounds, E<sub>MAX</sub> values are reported at 10  $\mu$ M concentrations in instances  
21  
22 where the data did not converge or where potency values were outside the linear experimental  
23  
24 range of the assay.  
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31 *Analysis of bias:* Bias factors were calculated according to the operational model using  
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33 DAMGO as the reference agonist. As in the prior manuscript, the conservative constraint was  
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35 applied to the operational model to fit the KA value to fall between 0 and 10<sup>-15</sup>M to allow for  
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37 curve fitting in the absence of reaching a maximum plateau in one of the responses; the  
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39  $\Delta\log(\tau/KA)$  values, relative to DAMGO were constrained to be less than 10.<sup>22,27</sup>  
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### 46 **ASSOCIATED CONTENT**

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49 The Supporting Information is available free of charge on the ACS Publications website at  
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51 DOI:  
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3 Synthetic procedures and characterization details of additional compounds, plus molecular  
4 strings, plus <sup>1</sup>H NMR spectra of key compounds that were used for *in vivo* studies (PDF).  
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### 24 Author Contributions

25  
26  
27 The manuscript was written through contributions of all authors. All authors have given approval  
28  
29 to the final version of the manuscript. ‡N.M.K and C.L.S have contributed equally.  
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### 32 Notes

33  
34  
35 The authors declare no competing financial interest.  
36  
37

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44  
45 described herein.  
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## 52 ABBREVIATIONS

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3 BBB, blood-brain barrier; Boc, *tert*-Butyloxycarbonyl, CDI, 1,1-carbonyldiimidazole; CH<sub>2</sub>Cl<sub>2</sub>,  
4 methylene chloride; CYP, cytochrome P450; DAMGO, [D-Ala<sup>2</sup>, NMe-Phe<sup>4</sup>, Gly-ol<sup>5</sup>]-  
5 enkephalin; DIPEA, N,N-diisopropylethylamine; DMPK, drug metabolism and  
6 pharmacokinetics; DMSO, dimethylsulfoxide; EC<sub>50</sub>, half maximal effective concentration; E<sub>MAX</sub>,  
7 maximal efficacy; EFC, enzyme fragment complementation; EtOH, ethanol; GPCR, G protein-  
8 coupled receptor; HPLC; high pressure liquid chromatography; *i.p.*, intraperitoneally; H<sub>2</sub>NNH<sub>2</sub>,  
9 hydrazine; K<sub>2</sub>CO<sub>3</sub>, potassium carbonate; kg, kilogram; KMnO<sub>4</sub>, potassium permanganate; KO,  
10 knockout; mg, milligram; LC/MS, liquid chromatography mass spectrum; MOR, mu opioid  
11 receptor; NaBH<sub>3</sub>CN, sodium cyanoborohydride; NaBH(OAc)<sub>3</sub>, sodium triacetoxyborohydride;  
12 Ni, nickel; nm, nanometer; nM, nanomolar; NMR, nuclear magnetic resonance; PK,  
13 pharmacokinetics; <sup>35</sup>S-GTPγS, G protein-coupling assay; SAR, structure activity relationship;  
14 TFA, trifluoroacetic acid; TLC, thin layer chromatography; THF, tetrahydrofuran; μM,  
15 micromolar; μg, microgram.  
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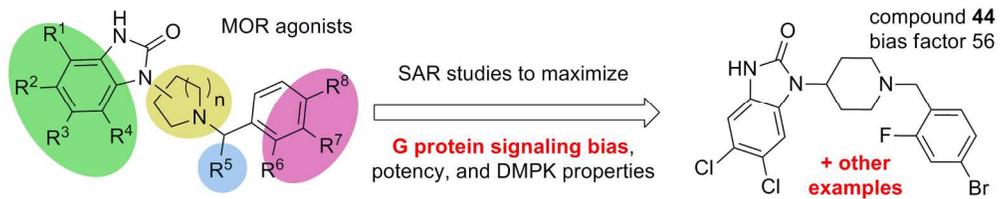
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