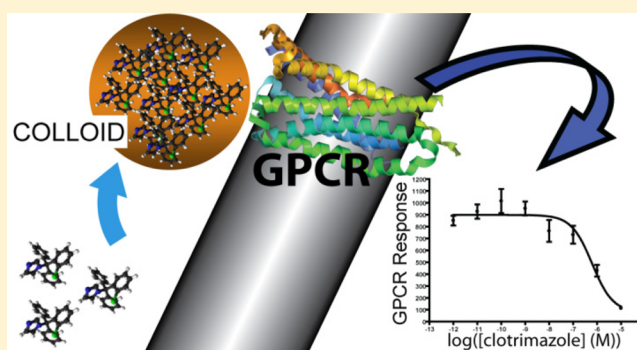


# Colloidal Aggregation Causes Inhibition of G Protein-Coupled Receptors

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## Supporting Information

**ABSTRACT:** Colloidal aggregation is the dominant mechanism for artifactual inhibition of soluble proteins, and controls against it are now widely deployed. Conversely, investigating this mechanism for membrane-bound receptors has proven difficult. Here we investigate the activity of four well-characterized aggregators against three G protein-coupled receptors (GPCRs) recognizing peptide and protein ligands. Each of the aggregators was active at micromolar concentrations against the three GPCRs in cell-based assays. This activity could be attenuated by either centrifugation of the inhibitor stock solution or by addition of Tween-80 detergent. In the absence of agonist, the aggregators acted as inverse agonists, consistent with a direct receptor interaction. Meanwhile, several literature GPCR ligands that resemble aggregators themselves formed colloids, by both physical and enzymological tests. These observations suggest that some GPCRs may be artifactually antagonized by colloidal aggregates, an effect that merits the attention of investigators in this field.



Since the advent of the molecular era, target-based assays have been central to both drug discovery and chemical biology<sup>1</sup> and have been especially prominent in high throughput screening (HTS). A key challenge in these assays is the occurrence of false-positive hits, which can account for more than 95% of the active molecules in a screen. Many mechanisms have been proposed to explain these nuisance hits, including chemical reactivity,<sup>2</sup> assay and reporter-gene interference,<sup>3–6</sup> flexibility,<sup>7</sup> oxidation potential,<sup>8</sup> formal charge,<sup>9</sup> liability to degradation and precipitation,<sup>10</sup> and the chemotypes of “heavy-hitters”.<sup>11</sup> The dominant mechanism of artifactual inhibition, and occasionally of artifactual activation,<sup>12</sup> of soluble proteins, however, is the formation of colloidal aggregates by the organic molecules being screened.<sup>13–17</sup> These colloids have characteristic features: they are several orders of magnitude larger than the proteins that they inhibit, ranging from about 50 to over 500 nm in radius;<sup>18</sup> they experience a critical aggregation concentration (CAC) that is reversed by dilution; they are disrupted by several nonionic detergents; they often exhibit steep dose–response curves;<sup>19</sup> and they may be precipitated by centrifugation.<sup>18,20</sup> Once formed, these aggregates inhibit proteins nonspecifically by partial denaturation.<sup>18,21</sup> No class of organic molecule appears to be free from aggregation, and the phenomenon has been observed among small molecule hits, leads, natural products, and drugs,<sup>22–26</sup>

thereby affecting assays *in vitro*, in cell culture,<sup>26,27</sup> and in simulated gastric and intestinal fluids.<sup>25,28</sup>

Though cell-based assays are similarly prone to false positives by “frequent hitters”,<sup>29–31</sup> inhibition by colloidal aggregates has thus far only been demonstrated for soluble proteins. Since membrane-bound receptors account for over 50% of drug targets, we wondered whether colloids might affect these receptors in typical cell-based assays. Until now, we have struggled to gain traction in these systems, in which our traditional tools of detergent disruption<sup>32</sup> and manipulating protein concentration<sup>33</sup> were hard to deploy. In a recent docking screen against the G protein-coupled receptor (GPCR) CXCR4, however, we found that several of the hits were active as aggregators in enzyme-based counter-screens, and could be precipitated by centrifugation, consistent with their activity via a colloidal mechanism.<sup>34</sup> These observations suggested routes to investigate colloid-based inhibition more broadly among GPCRs.

Here, we ask whether well-described colloidal aggregators, such as tetra-iodophenolphthalein (TIPT), quercetin, clotrimazole, and itraconazole, would inhibit GPCRs, and if so, whether we could develop simple and definitive experiments to identify them. We looked to see if these well-studied colloid formers

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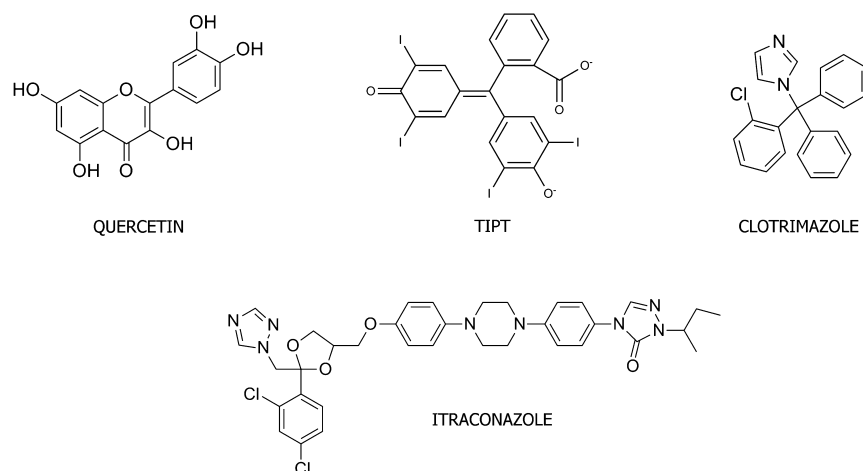


Figure 1. Aggregating small molecules used in this study.

Table 1. Effects of Clotrimazole, Itraconazole, Quercetin, and TIPT on the  $\beta$ -Arrestin Recruitment Assay for CCR4, CX3CR1, and  $V_2R$ <sup>a</sup>

GPCR Compound	CCR4				CX3CR1			V <sub>2</sub> R	
	Centrifugation	Detergent	Centrifugation	Detergent	Centrifugation	Detergent	Centrifugation	Detergent	
	pIC <sub>50</sub> ± SEM (IC <sub>50</sub> (μM))	pIC <sub>50</sub> ± SEM (IC <sub>50</sub> (μM))	pIC <sub>50</sub> ± SEM (IC <sub>50</sub> (μM))	pIC <sub>50</sub> ± SEM (IC <sub>50</sub> (μM))	pIC <sub>50</sub> ± SEM (IC <sub>50</sub> (μM))	pIC <sub>50</sub> ± SEM (IC <sub>50</sub> (μM))	pIC <sub>50</sub> ± SEM (IC <sub>50</sub> (μM))	pIC <sub>50</sub> ± SEM (IC <sub>50</sub> (μM))	pIC <sub>50</sub> ± SEM (IC <sub>50</sub> (μM))
Clotrimazole	NE <sup>b</sup>	NE <sup>b</sup>	NE <sup>b</sup>	5.7 ± 0.3 (2.0)	5.0 ± 0.1 (10.8 μM)	5.1 ± 0.7 (7.9)	5.6 ± 0.1 (2.7)	4.8 ± 0.2 (15.1)	NE <sup>b</sup>
Itraconazole	6.3 ± 0.2 (0.5)	5.1 ± 0.1 (7.2 μM)	4.9 ± 0.4 (11.4)	6.8 ± 0.2 (0.1)	5.3 ± 0.2 (5.0 μM)	4.7 ± 0.3 (20.8)	6.1 ± 0.2 (0.8)	4.9 ± 0.1 (11.9)	5.2 ± 0.2 (6.0)
Quercetin	5.0 ± 0.3 (10.2)	<4.5 (> 100 μM)	<4.5 (>100)	5.3 ± 0.3 (4.5)	NE <sup>b</sup>	<4 (>32)	5.2 ± 0.1 (7.0)	4.4 ± 0.3 (37.8)	4.8 ± 0.3 (15.2)
TIPT	5.4 ± 0.3 (3.5)	NE <sup>b</sup>	5.0 ± 0.4 (9.9)	5.7 ± 0.3 (2.2)	NE <sup>b</sup>	<4 (>32)	6.4 ± 0.1 (0.4)	NE <sup>b</sup>	4.3 ± 0.3 (45.0)

<sup>a</sup> The pIC<sub>50</sub> and corresponding IC<sub>50</sub> values on direct treatment with aggregators, after centrifugation, and with detergent are shown. <sup>b</sup>No effect on receptor activity.

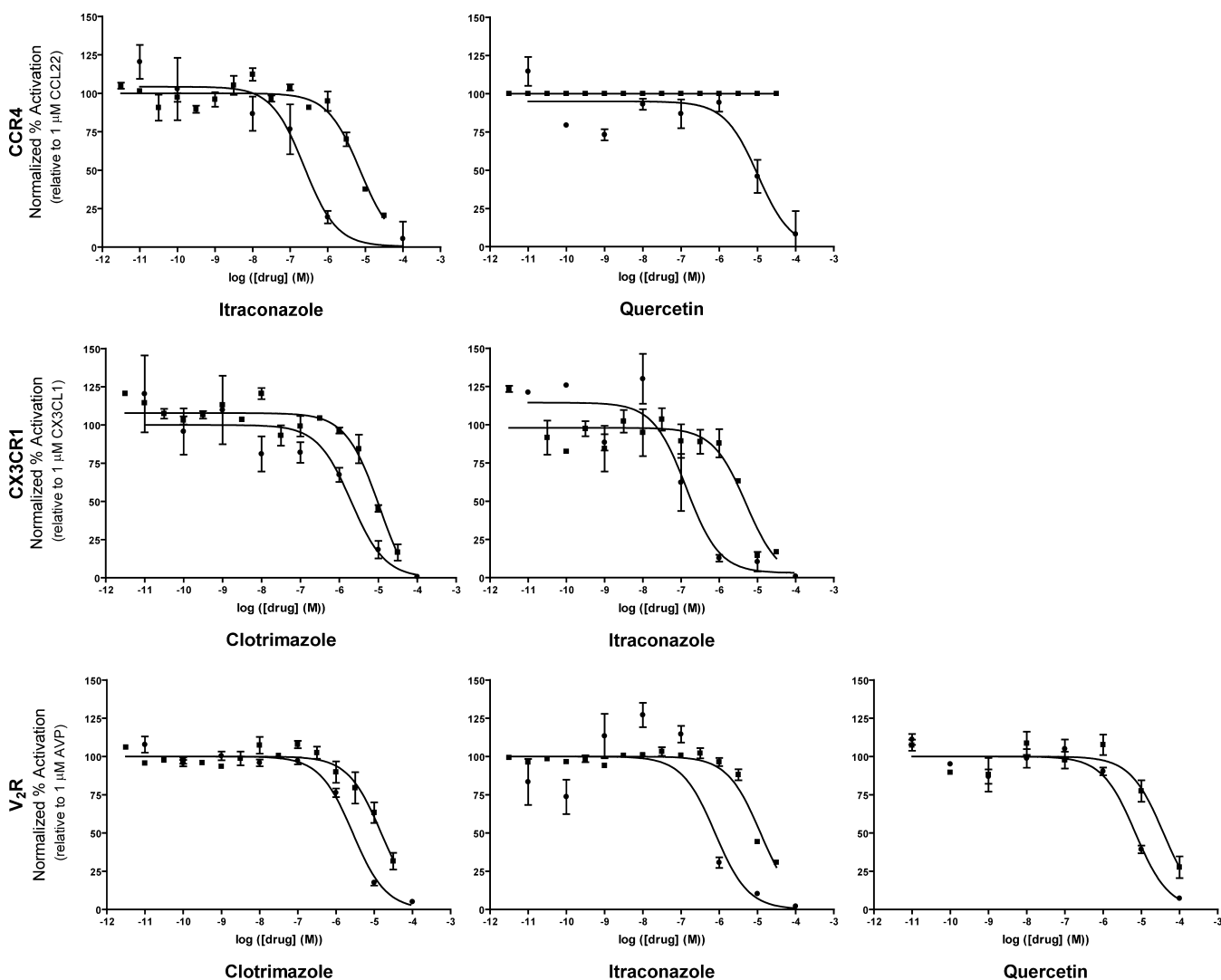
would inhibit the signaling of the peptidergic GPCRs vasopressin-2 receptor (V<sub>2</sub>R), chemokine receptor 4 (CCR4), and C-X-C receptor 3 (CX3CR1), each measured in live cell assays. We further asked if these compounds would inhibit only in the concentration range where they are known to form colloids, and if inhibition could be attenuated by removing or disrupting the colloids, providing rapid counter-screens. We found that these well-known aggregators do antagonize the three GPCRs—this activity is attributed to their colloidal form, and does not appear to be a property of the soluble forms of these organic molecules. Correspondingly, several ligands reported to be active against GPCRs are shown, by light-scattering and detergent-sensitive activity in counter-screens, to form colloids at relevant concentrations. Thus, known colloid formers are active against multiple GPCRs in a manner directly related to aggregation, and several new GPCR inhibitors appear to form colloids. The implications for ligand discovery screens against GPCRs are considered.

## RESULTS

To explore the effects of colloidal aggregation on GPCR activity, we used four molecules well-characterized for colloid formation at micromolar concentrations: clotrimazole, itraconazole, quercetin, and TIPT (Figure 1).<sup>20,22,23,25</sup> We used the chemokine receptors CCR4 and CX3CR1, and the vasopressin 2 receptor (V<sub>2</sub>R) as model peptide-activated GPCRs, measuring activity using the  $\beta$ -arrestin recruitment-mediated luciferase reporter assay that quantifies receptor activation/

inhibition upon ligand binding.<sup>35</sup> As the four organic molecules bear little physical or topological similarity to known ligands of these GPCRs, we expected soluble, monomeric forms of the organic molecules to be relatively inert to these targets. Thus, broad GPCR activity would, in itself, be an indication of colloid formation.

Our first question was thus whether these known aggregators have GPCR activity at concentrations above their known CAC values.<sup>20,25</sup> Quercetin, itraconazole, and TIPT inhibited CCL22-dependent CCR4 activation with IC<sub>50</sub> values of 10.2 μM, 0.5 μM, and 3.5 μM, respectively (Table 1). Similarly, all four of the compounds inhibited CX3CR1 activity after stimulation with CX3CL1; IC<sub>50</sub> values were 2.0 μM for clotrimazole, 0.1 μM for itraconazole, 4.5 μM for quercetin, and 2.2 μM for TIPT (Table 1). Last, clotrimazole, itraconazole, quercetin, and TIPT also inhibited vasopressin-dependent activity of V<sub>2</sub>R, with IC<sub>50</sub> values of 2.7 μM, 0.8 μM, 7.0 μM, and 0.4 μM, respectively (Table 1). These IC<sub>50</sub> values are within the range where colloidal inhibition has been observed for these compounds.<sup>22,23,25</sup> This promiscuous inhibition of various GPCR proteins by molecules unrelated to known target ligands, at concentrations at which these molecules have been observed to form colloids, suggests that the four aggregating compounds may be acting as nonspecific colloidal inhibitors of the three GPCRs. Meanwhile, in nontransfected controls the colloids had no measurable nonspecific effects on cell viability (SI Figure S2).



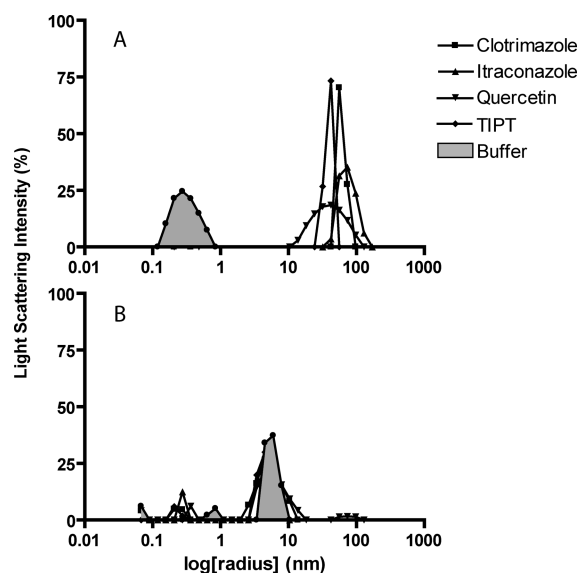
**Figure 2.** Centrifugation treatment. Concentration–response curves are shown for CCR4, CX3CR1, and  $V_2R$  in the  $\beta$ -arrestin recruitment assay. Circles represent data points for aggregators without treatment; squares represent data points after centrifugation ( $\pm$ SEM). Calculated  $pIC_{50}$  and  $IC_{50}$  values are displayed in Table 1.

Of course, showing inhibition does not fully demonstrate a colloidal mechanism. It has been shown that colloids can be removed from solution by centrifugation.<sup>18,20,34</sup> To test whether the colloidal particles were themselves the inhibitory species, we centrifuged aqueous mixtures of the colloids, and treated the cells with the resulting supernatant. If colloids are indeed the inhibitory species, rather than the soluble monomer, the supernatant should show less activity than the original mixture. Alternatively, solutions of well-behaved inhibitors are unaffected by such centrifugation. Consistent with a colloidal mechanism, the inhibitory potency of all four inhibitors was substantially weakened by centrifugation, relative to the noncentrifuged control (Figure 2). Against CCR4, itraconazole showed a 36-fold increase of its  $IC_{50}$ ; changes in inhibitory activity were also pronounced for quercetin (Table 1). Against CX3CR1, clotrimazole showed an increase in  $IC_{50}$ , from 2.0 to 10.8  $\mu$ M, while the shift for itraconazole was much more substantial, increasing from 0.1 to 5.0  $\mu$ M (Table 1). Similarly, clotrimazole, itraconazole, and quercetin all showed substantial  $IC_{50}$  increases against the  $V_2R$  after centrifugation: from 2.7 to 15.1  $\mu$ M, 0.8 to 11.9  $\mu$ M, and 7.0 to 37.8  $\mu$ M, respectively (Table 1, Figure 2). These results are consistent with a

colloidal-based mechanism, given that removal of colloidal particles by centrifugation reduced the inhibition caused by these molecules.

Although the addition of nonionic detergents can disrupt colloid formation,<sup>18</sup> the use of detergents in cell culture often has toxic effects, and this had long been a stumbling block in investigating colloidal effects on cell-based assays. However, recent work has shown that low concentrations of Tween-80 are both well-tolerated in cell culture and disrupt many colloids.<sup>26</sup> To investigate the ability of Tween-80 to disrupt the colloids formed by clotrimazole, itraconazole, quercetin, and TIPT, we measured particle formation in the presence and absence of this detergent by dynamic light scattering (DLS). We found that Tween-80 completely disrupts the colloids formed by these small molecule aggregators (Figure 3).

In live cell-based control experiments, the percentage of Tween-80 tested had little or no measurable effect on the growth of the cells, nor on the activity of the receptors. We note that because the Tween-80 is removed with the rest of the cell media before measuring  $\beta$ -arrestin signaling, via the induction of luciferase, there is no detergent effect on luciferin itself or luciferase activity. Knowing that Tween-80 disperses

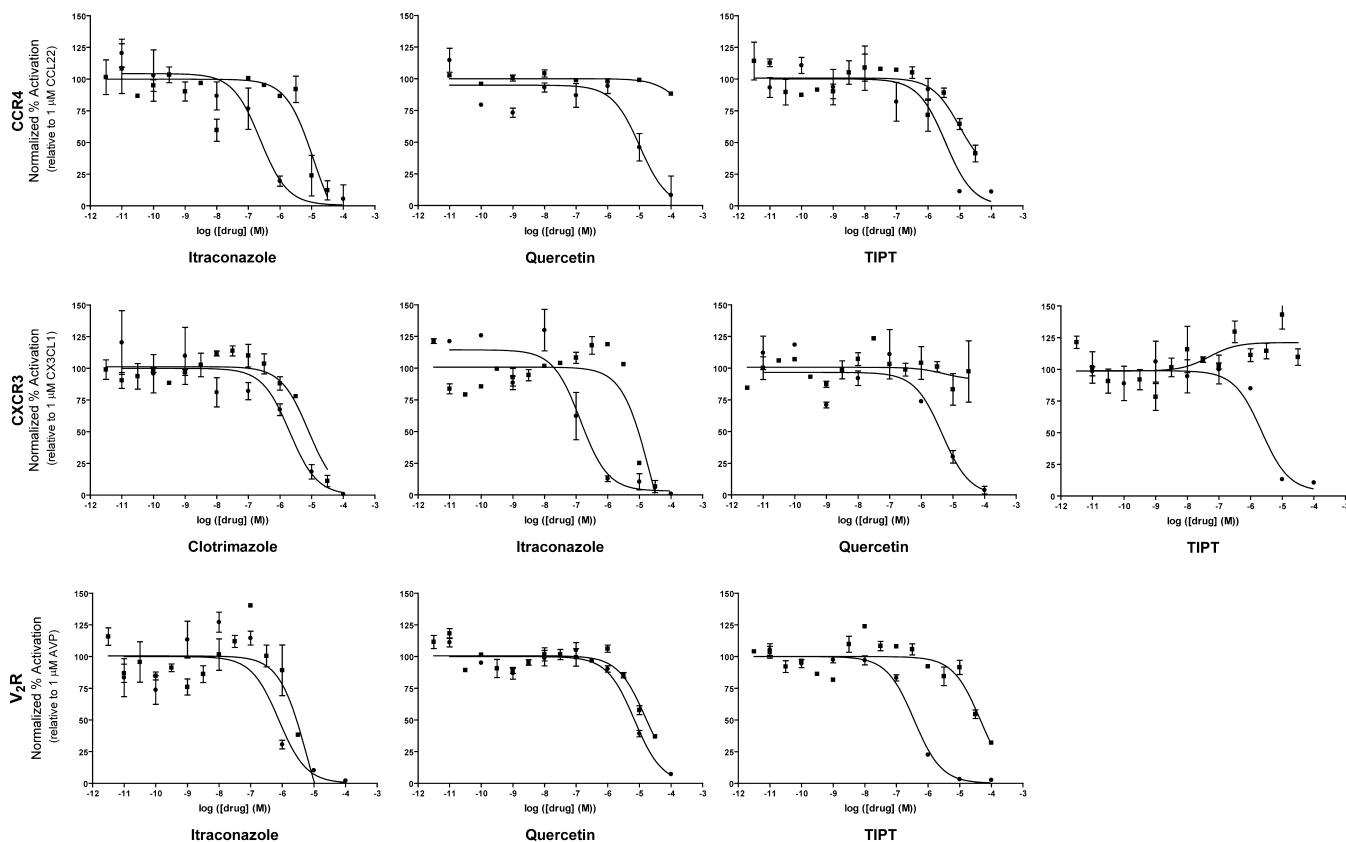


**Figure 3.** Particle formation by colloid-forming molecules measured by DLS in the (A) absence of Tween-80 and (B) presence of Tween-80.

colloids but does not affect the assay itself, we repeated the  $\beta$ -arrestin recruitment assays in the presence of Tween-80. Again, consistent with a colloidal mechanism, the addition of detergent substantially diminished the activity of all four inhibitors against the three receptors, from 3- to 200-fold, when compared to inhibition in the absence of detergent (Figure 4).

Against CCR4, on addition of Tween-80 the  $IC_{50}$  values of itraconazole and quercetin increased from 0.5 to 11.4  $\mu M$  and from 10.2 to >100  $\mu M$ , respectively, while TIPT's shift was more moderate, increasing from 3.5 to 9.9  $\mu M$  (Table 1). Against CX3CR1, all four compounds showed substantial increases in  $IC_{50}$  values on addition of Tween-80: 2.0 to 7.9  $\mu M$  for clotrimazole; 0.1 to 20.8  $\mu M$  for itraconazole; 4.5 to >32  $\mu M$  for quercetin; and from 2.2 to >32  $\mu M$  for TIPT (Table 1). Similarly, the inhibition of  $V_2R$  decreased substantially in the presence of detergent: the  $IC_{50}$  for itraconazole increased from 0.8 to 6.0  $\mu M$ ; TIPT increased from 0.4 to 45.0  $\mu M$ ; and the  $IC_{50}$  for quercetin doubled, from 7.0 to 15.2  $\mu M$  (Table 1). The  $IC_{50}$  values calculated for all four aggregators against the GPCRs measured here are similar to the  $IC_{50}$  values observed for the same molecules against soluble enzymes that have become standard markers for aggregation, such as  $\beta$ -lactamase and cruzain.<sup>22,23,25</sup>

We also investigated the inhibition of  $V_2R$  using a second assay type,  $Ca^{2+}$  mobilization. Similar  $IC_{50}$  values were observed as with the  $\beta$ -arrestin recruitment assays for all four aggregators (SI Table S1, Figure S1). Intriguingly, the dose–response curves were steep in the  $Ca^{2+}$  mobilization assay, with Hill slope coefficients as high as 5 (SI Table S1); both observations are consistent with a colloidal mechanism. We note that, in this  $Ca^{2+}$  mobilization assay, inhibition was unaffected by centrifugation or detergent treatment. This likely reflects the composition of the (proprietary) calcium assay dye kits, which contain fluorescent dyes as well as masking dyes. Many such dyes are well-known aggregators,<sup>26,36–38</sup> and have been shown



**Figure 4.** Detergent treatment. Concentration–response curves are shown for CCR4, CX3CR1, and  $V_2R$  in the  $\beta$ -arrestin recruitment assay. Circles represent data points for aggregators without treatment; squares represent data points after addition of detergent ( $\pm$ SEM). Calculated  $pIC_{50}$  and  $IC_{50}$  values are displayed in Table 1.

to interact with each other and other aggregators in nonadditive ways,<sup>39</sup> substantially altering colloidal characteristics and assay results. Consistent with this view, the assay kit alone formed colloid-like particles by DLS of 166 nm in size, and inhibited two unrelated enzymes, chymotrypsin and malate dehydrogenase (SI Table S2). This suggests that components of the assay kit—likely the dyes contained in it—themselves form colloidal aggregates. As the Fluo-4 Direct kit composition is proprietary, these effects cannot be further disentangled. For the purposes of this study, the key result is that IC<sub>50</sub> values measured with the four aggregators by calcium mobilization are fully consistent with those measured by a  $\beta$ -arrestin recruitment assay, with concentration–response slopes that are commensurate with aggregation-based inhibition of many other targets.<sup>18–20</sup>

Of the four well-studied aggregators used in this study, the flavonoid quercetin is among the most promiscuous, appearing in hundreds of papers with well over 50 reported targets;<sup>40</sup> among these, it has been reported to act against at least 12 different GPCRs, including GLP-1 receptor, SHT<sub>2A</sub> serotonin receptor,  $\alpha_{2A}$  adrenergic receptor, and the D<sub>2</sub> dopamine receptor.<sup>41–45</sup> Whereas quercetin is a well-behaved kinase inhibitor at mid-nanomolar concentrations, at micromolar concentrations its broad promiscuity likely reflects a colloid-based mechanism.<sup>46</sup> Knowing that quercetin acted via a colloid-based mechanism against CCR4, CX3CR1, and V<sub>2</sub>R, we wondered whether related flavonoids that have been reported to act on GPCRs would themselves form colloids. We therefore tested four flavonoids reported to be active on GPCRs for colloid formation. At low to midmicromolar concentrations, two of these, genistein and luteolin, formed particles by DLS and inhibited two counter-screening enzymes,  $\beta$ -lactamase and cruzain, in the same midmicromolar range, as they are observed to be active against multiple GPCRs<sup>41,47–51</sup> (Table 3). Also consistent with colloid formation, enzyme inhibition was eliminated by the addition of 0.01% (v/v) Triton X-100 and 0.025% (v/v) Tween-80.

**Table 2. Colloids Sequester Peptidic Ligands<sup>a</sup>**

sample	radius (nm)	% $\beta$ -lactamase activity
Vasopressin	0.18 ± 0.1	101.7 ± 3.7
TIPT	56.1 ± 3.1	23.0 ± 12.1
TIPT + Vasopressin	97.3 ± 9.4	93.0 ± 2.8

<sup>a</sup>The particle radii measured by DLS and the corresponding effects on  $\beta$ -lactamase activity are listed for vasopressin and TIPT alone and in combination.

Colloidal inhibition, certainly of soluble proteins, arises from the sequestration of the protein target itself, in a partly denatured form, by the colloidal particle.<sup>20,21</sup> Since CCR4, CX3CR1, and V<sub>2</sub>R are all peptidic GPCRs, we wondered if their antagonism arose from association of the colloids with the receptors themselves or with their peptide agonists. To test for an interaction between colloid and peptide, we used DLS to measure the size of TIPT colloids alone and mixed with vasopressin. The colloids formed by TIPT substantially increase in size when mixed with vasopressin, from 56.1 to 97.3 nm in radius, suggesting that the peptide monomers are being sequestered onto the colloidal surfaces (Table 2). We also measured the inhibition of  $\beta$ -lactamase by TIPT colloids with and without preincubation with vasopressin; proteins—and perhaps peptides—can prophylactically coat colloids, preventing inhibition of a second enzyme. TIPT at 10  $\mu$ M, above the

CAC where colloids appear, leads to 77% inhibition of  $\beta$ -lactamase; preincubating the colloidal mixture with vasopressin almost fully blocks this inhibition (7% inhibition), suggesting that the peptide binds the colloidal surface and competes with  $\beta$ -lactamase binding. These observations support the idea that the colloids can directly associate with the peptide ligands, which could contribute to inhibition by sequestering and depleting agonist.

While peptide sequestration can thus contribute to apparent GPCR antagonism, the colloids may also associate with the receptors themselves, as they do with soluble enzymes. To investigate this, we measured the effect of the four aggregators on basal GPCR signaling, which occurs in the absence of agonist. Three of the four aggregators, clotrimazole, itraconazole, and TIPT, inhibited basal receptor activity, acting as apparent inverse agonists, with IC<sub>50</sub> values of 1.6 ± 3.2, 0.2 ± 2.0, and 1.6 ± 6.3  $\mu$ M, respectively (Figure 5). These observations support a mechanism where the colloids directly associate with the GPCRs themselves, acting as inverse agonists. This mechanism is sufficient to explain their inhibitory activity, though peptide-agonist sequestration may also play a role.

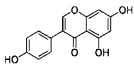
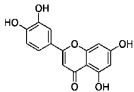
## DISCUSSION

The point of this study is uncomplicated: colloidal aggregation, well-appreciated as a major artifact in ligand discovery against soluble proteins, can also affect GPCRs. This conclusion is supported by two lines of evidence. First, well-studied colloid-formers inhibit a peptide- and two protein-activated GPCRs in a colloid-dependent manner: inhibition only occurs in the concentration range where these molecules aggregate and, in well-controlled  $\beta$ -arrestin recruitment assays, inhibition is disrupted by removing the colloidal particles from solution, either by centrifugation or by addition of detergent. In a second, complementary line of investigation, flavonoids reported as active against other GPCRs are shown to form colloids directly, by dynamic light scattering (DLS) and by detergent-dependent inhibition of classic counter-screen enzymes like  $\beta$ -lactamase. Taken together, these results suggest that colloid-based activity may contribute to artifactual ligand discovery against G protein-coupled receptors, which remain crucial targets for probe and drug discovery.

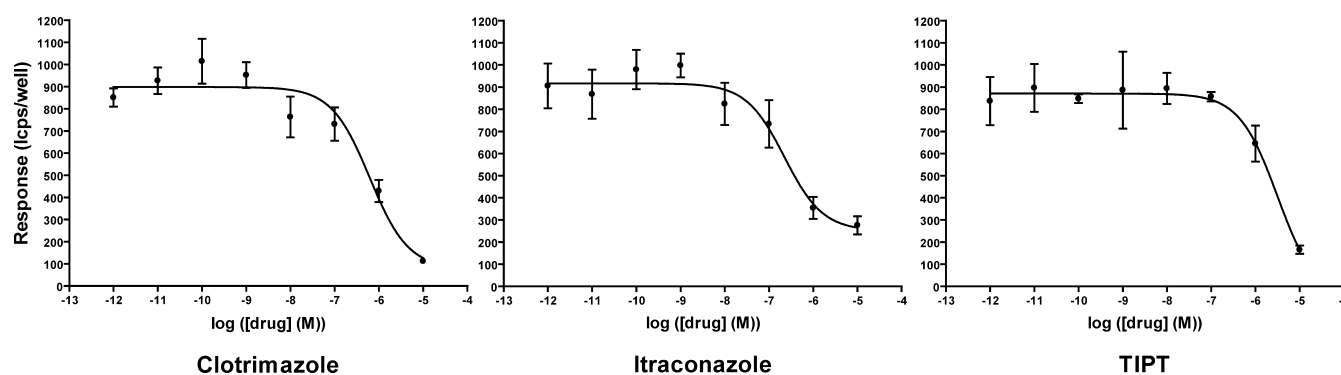
If the key point of this paper is simple, it nevertheless took substantial effort to learn how to perturb the assays in such a way as to demonstrate a colloid-based mechanism. Whereas it is straightforward to show activity of a colloid in a GPCR assay, mechanistically attributing this to colloids demanded techniques to disrupt them or remove them from solution. The twin techniques of Tween-80 addition and of centrifugation—which may be accomplished on a benchtop microcentrifuge—were key to enabling this study, and are brought together here for the first time. Both have the virtue of simplicity, and may be deployed on a large scale. Other investigators may find them useful as controls for ligand discovery against GPCRs.

Several caveats merit mentioning. Whereas the mechanism of colloidal inhibition of soluble proteins is relatively well-understood—sequestration of the protein by the colloid in a partly denatured form<sup>18,20,21</sup>—their mechanism of action on GPCRs is only partly illuminated by this study. The observation that they act as inverse agonists, in the absence of activating agonist, supports a direct role on the GPCRs themselves. However, unless the colloids actually strip the GPCRs from the membrane, this cannot be by exactly the same mechanism as

Table 3. Colloid Formation by Flavonoids<sup>a</sup>

Compound	IC <sub>50</sub> vs $\beta$ -lactamase ( $\mu$ M)		IC <sub>50</sub> vs cruzain ( $\mu$ M)		Concn ( $\mu$ M)	Colloid radius (nm)
	No Detergent	+ 0.01% Triton	No Detergent	+ 0.025% Tween-80		
 Genistein	16.7 $\pm$ 0.1	> 100	82.7 $\pm$ 0.03	> 400	75	37.4 $\pm$ 5.2
 Luteolin	21.9 $\pm$ 0.2	> 100	27.3 $\pm$ 0.03	155.7 $\pm$ 0.6	50	108.3 $\pm$ 17.9

<sup>a</sup>The colloid radii measured by DLS and the effects on enzyme activity in the presence and absence of detergent are shown.



**Figure 5.** Concentration–response curves for CCR4 in the  $\beta$ -arrestin recruitment assay in the *absence* of the CCR4 agonist, CCL22 ( $\pm$ SEM). The depression of basal activity by the colloid-formers in the absence of the agonist suggests inverse agonism.

for sequestration of soluble proteins. Indeed, activity via membrane perturbation cannot be discounted, though in control experiments showed no gross effect on cell morphology or viability. Nor can activity via the agonist itself be discounted—indeed, our results suggest that at least peptide agonists will be sequestered by the colloids, possibly contributing to inhibition. In some assay formats, aggregating compound will behave differently. Even here, in the calcium-mobilization assay, though the aggregating compounds were just as effective as in the  $\beta$ -arrestin recruitment assay, reversibility was harder to see. We suspect this owes to the presence of dyes in the kit used, but this is hard to deconvolute owing to the proprietary nature of this kit. More broadly, cell-based assays that include a large amount of protein, such as are found in serum-related media, will be less susceptible to colloidal-aggregation; though the colloids themselves often persist in these and related media,<sup>25,26,52</sup> their activity is often reduced, if not fully eliminated.<sup>27</sup> The assays used in this study, however, remove the serum-supplemented media and replace it with serum-free media before treatment with compound and therefore remain quite sensitive to aggregation-based effects. Finally, it is uncertain what range of GPCRs will be affected by colloidal aggregation—we have only observed such inhibition against peptidergic and protein–ligand GPCRs, though the activity of promiscuous flavonoids on other families of GPCRs suggests that their effects might be more broad.

These caveats should not obscure the key observation of this study: colloidal aggregation can lead to artifactual activity

against GPCRs. Given the prevalence of aggregators in screening libraries,<sup>13–16</sup> the artifactual activity of colloidal aggregators against GPCRs could not only waste much effort and time, but may mask the activities of weaker but better behaved compounds in screening campaigns. More positively, the detergent and spin-down techniques described here may be readily deployed on a large scale and can rapidly eliminate most artifacts acting by this mechanism. Given the importance of these targets in biology and medicine, this mechanism of action against GPCRs merits close attention.

## METHODS

**Materials.** CCL22 and CX3CL1 were purchased from R&D Biosystems. Bright-Glo luciferase reagent was obtained from Promega. Clotrimazole was purchased from MP Biomolecules; itraconazole was purchased from AK Scientific; Arg-vasopressin, quercetin, and TIPT were purchased from Sigma. All compounds tested had purity of  $\geq$ 95%.

**Cell Culture.** HTLA cells were cultured in DMEM, supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, 100 mg/mL streptomycin, 5 mg/L puromycin, and 100 mg/L hygromycin (HTLA media). This cell line was kindly provided by Gilad Barnea in the Axel laboratory.

**$\beta$ -Arrestin Recruitment Assay.** Recruitment of  $\beta$ -arrestin via agonist-stimulated receptors (CCR4, CX3CR1, and V<sub>2</sub>R) was quantified using the previously described  $\beta$ -arrestin recruitment assay.<sup>53</sup> Briefly, HTLA cells stably expressing a  $\beta$ -arrestin2-TEV protease fusion protein and a tTA-dependent firefly luciferase reporter gene were plated in HTLA medium. Using a CaPO<sub>4</sub> method reported

earlier,<sup>54</sup> cells were transfected with 18  $\mu\text{g}$  of the receptor- $V_2$ -tTA construct and incubated overnight at 37 °C, 5%  $\text{CO}_2$  (day 1). The following day, cells were plated (20 000 cells/well, 50  $\mu\text{L}$ /well) in white, clear-bottom, PLK-coated, 384-well plates in DMEM containing 1% dialyzed FBS and antibiotics and incubated overnight at 37 °C, 5%  $\text{CO}_2$  (day 2). On day 3, the media was replaced by serum-free DMEM containing antibiotics, and cells were allowed to recuperate for 2–3 h. Serial dilutions of the reference and test ligands (0.001 nM to 100  $\mu\text{M}$ , final concentrations, prepared as 6 $\times$ ) were made in sterile drug buffer (1 $\times$  HBSS, 20 mM HEPES, 6% DMSO, pH 7.4). For the centrifugation treatment, concentrated solutions of the ligands were spun at 13 000g for 20 min on a benchtop microfuge at room temperature. For the detergent treatment, Tween-80 was added to concentrated solutions of the ligands for a final concentration of 0.025% (v/v). Cells were stimulated with 10  $\mu\text{L}$ /well of reference or test ligand, treated or untreated, and incubated for 20 min. Cells were stimulated a second time with reference ligand at 1  $\mu\text{M}$  and incubated overnight at 37 °C, 5%  $\text{CO}_2$ . On day 4, the media was removed from the wells and 20  $\mu\text{L}$  of 10 $\times$  Brite-Glo reagent (Promega) was added. Cells were incubated for 30 min at RT and luminescence was read using a Trilux plate-reader (1 s/well). Data were normalized to vehicle (0%) and reference ligands (100%) and regressed using the sigmoidal dose–response function built into GraphPad Prism 5.0.

**Dynamic Light Scattering.** To measure the effect of Tween-80 on colloid formation, compounds were diluted from concentrated stocks in DMSO into filtered 1 $\times$  HBSS containing 20 mM HEPES, pH 7.4, with and without 0.025% (v/v) Tween-80. The final DMSO concentration was 1%, and compound concentrations were as follows: 30  $\mu\text{M}$  TIPT and clotrimazole, 1  $\mu\text{M}$  itraconazole, 100  $\mu\text{M}$  quercetin. Samples were run in triplicate. Figure 3 shows one representative histogram for each sample. TIPT, genistein, and luteolin samples were measured in filtered 50 mM potassium phosphate, pH 7.0. To measure the effect of vasopressin on the size of TIPT colloids, light scattering was measured for 10  $\mu\text{M}$  TIPT with and without 1  $\mu\text{M}$  vasopressin; 1  $\mu\text{M}$  vasopressin was measured alone in phosphate buffer as well. Genistein was measured at 50  $\mu\text{M}$  and luteolin was measured at 75  $\mu\text{M}$ . Samples were run in quadruplicate. All measurements were made at room temperature using a DynaPro MS/X (Wyatt Technology) with a 55 mW laser at 826.6 nm. The laser power was 100%, and the detector angle was 90°.

**Enzyme Inhibition Assays.** AmpC  $\beta$ -lactamase inhibition was measured in 50 mM potassium phosphate, pH 7.0, at room temperature. TIPT (10  $\mu\text{M}$ , final concentration) was diluted from a 10 mM stock in DMSO.  $\beta$ -Lactamase was measured with and without a five minute preincubation with 1  $\mu\text{M}$  vasopressin.  $\beta$ -Lactamase (1 nM) was added to the samples and the mixture was incubated for 5 min; the reaction was initiated by adding 69  $\mu\text{M}$  CENTA substrate (Tydock Pharma; Modena, Italy). Genistein and luteolin were diluted from concentration DMSO stocks to obtain full dose–response curves against  $\beta$ -lactamase with final concentrations of DMSO of 1% (v/v). Compound was incubated with 0.2 nM  $\beta$ -lactamase for 5 min, with and without 0.01% (v/v) Triton X-100, and the reaction was initiated by adding 46  $\mu\text{M}$  CENTA. The final reaction volumes were 1 mL. Change in absorbance was monitored at 405 nm for 2.5 min using an HP 8453 UV–vis spectrophotometer (Agilent, Technologies, Santa Clara, CA). The assay was performed in quadruplicate in methacrylate cuvettes. Cruzain inhibition was measured in 0.1 M sodium acetate, pH 5.5, containing 5 mM DTT. Genistein and luteolin were incubated with 0.8 nM cruzain (with and without 0.025% (v/v) Tween-80) for 5 min until reactions were initiated by adding the fluorogenic substrate Z-Phe-Arg-aminomethylcoumarin (Z-FR-AMC). The final reaction volume was 200  $\mu\text{L}$ . Final concentrations were 0.4 nM cruzain, 2.5  $\mu\text{M}$  Z-FR-AMC, and 1% DMSO. Enzyme inhibition was measured by monitoring the increase in fluorescence for 5 min (excitation wavelength of 355 nm, emission wavelength of 460 nm) in a microtiter plate spectrofluorimeter (FlexStation, Molecular Devices). Assays were performed in duplicate in 96-well plates and were repeated in two separate experiments. Dose–response curves were plotted, and  $\text{IC}_{50}$  values were calculated using GraphPad Prism 4

(GraphPad, San Diego, CA) using a sigmoidal dose–response curve analysis with variable slope.

## ■ ASSOCIATED CONTENT

### § Supporting Information

Results from calcium mobilization and cell viability assays, along with aggregation characteristics of the calcium assay kit, are included. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS USED

GPCR, G protein-coupled receptor; HTS, high-throughput screening; CAC, critical aggregation concentration; TIPT, tetra-iodophenolphthalein;  $V_2R$ , vasopressin-2 receptor; CCR4, chemokine receptor 4; CX3CR1, C-X-C receptor 3; DLS, dynamic light scattering

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