

# Pepducin targeting the C-X-C chemokine receptor type 4 acts as a biased agonist favoring activation of the inhibitory G protein

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Short lipidated peptide sequences derived from various intracellular loop regions of G protein-coupled receptors (GPCRs) are named pepducins and act as allosteric modulators of a number of GPCRs. Recently, a pepducin selectively targeting the C-X-C chemokine receptor type 4 (CXCR4) was found to be an allosteric agonist, active in both cell-based assays and in vivo. However, the precise mechanism of action of this class of ligands remains poorly understood. In particular, given the diversity of signaling effectors that can be engaged by a given receptor, it is not clear whether pepducins can show biased signaling leading to functional selectivity. To explore the ligand-biased potential of pepducins, we assessed the effect of the CXCR4 selective pepducin, ATI-2341, on the ability of the receptor to engage the inhibitory G proteins (Gi1, Gi2 and Gi3), G13, and  $\beta$ -arrestins. Using bioluminescence resonance energy transfer-based biosensors, we found that, in contrast to the natural CXCR4 ligand, stromal cell-derived factor-1 $\alpha$ , which promotes the engagement of the three Gi subtypes, G13 and the two  $\beta$ -arrestins, ATI-2341 leads to the engagement of the Gi subtypes but not G13 or the  $\beta$ -arrestins. Calculation of the transduction ratio for each pathway revealed a strong negative bias of ATI-2341 toward G13 and  $\beta$ -arrestins, revealing functional selectivity for the Gi pathways. The negative bias toward  $\beta$ -arrestins results from the reduced ability of the pepducin to promote GPCR kinase-mediated phosphorylation of the receptor. In addition to revealing ligand-biased signaling of pepducins, these findings shed some light on the mechanism of action of a unique class of allosteric regulators.

BRET | lipid-anchored peptide | beta-arrestin | protein-protein interaction | cell signaling

Pepducins represent a class of molecules that regulate the activity of G protein-coupled receptors (GPCRs). These lipid-modified peptides are derived from the amino acid sequences of one of the four intracellular loops of a target GPCR (1). Although the precise mode of action is not completely understood, it is believed that pepducins bind to their target receptors and allosterically modulate their signaling activity (2, 3). Pepducins have been identified for several GPCRs, including the protease-activated receptors PAR1 (1, 4–10), PAR2 (1, 11–13), and PAR4 (6, 9), the formyl peptide receptor-2 (14), the melanocortin type 4 receptor (1), the sphingosine 1-phosphate receptor 3 (15), and the C-X-C chemokine receptor type 1, 2 (CXCR1, CXCR2) (16), and 4 (CXCR4) (2, 17, 18). They have been found to act as allosteric agonists as well as negative or positive allosteric modulators. However, in most cases, their activity was assessed for only one or a few signaling pathways engaged by the receptors.

One of the receptors for which pepducins were developed is CXCR4. This receptor, expressed in many tissues including hematopoietic and circulating cells, is a coreceptor for the entry of HIV (19, 20) and controls many physiological functions, including cell migration, mobilization, and retention of polymorphonuclear neutrophils (PMNs) and hematopoietic stem

cells, as well as progenitor cells (HSPCs) in the bone marrow niche (21–23). It has also been found to play an important role in tumor progression, angiogenesis, and metastasis of a variety of cancers (24–26). A CXCR4 antagonist, AMD-3100 (Mozobil), is used to mobilize HSPCs from the bone marrow for transplantation of leukemic patients (27).

A pepducin derived from the first intracellular loop of CXCR4 (ATI-2341) was found to be an allosteric agonist on the chemotactic response elicited on a human T-lymphoblastic leukemia cell line (CCRF-CEM cells) that endogenously expresses CXCR4 (18). This activity of ATI-2341 was confirmed in fresh human PMNs as well as in vivo for their ability to promote the mobilization of PMNs and HSPCs in the peripheral circulation of both mice and monkeys (18). Of note, unlike the clinically used CXCR4 antagonist AMD-3100 and stromal cell-derived factor-1 $\alpha$  (SDF-1; also known as CXCL12) that promote the mobilization of lymphocytes in addition to PMNs and HSPCs (18, 28–30), ATI-2341 was without effect on the mobilization of lymphocytes (18), indicating that ATI-2341 may display functional selectivity.

## Significance

Pepducins are a class of biologics that allosterically control G protein-coupled receptor (GPCR) activity, but very little is known about their mode of action. Here, we report that ATI-2341, a pepducin targeting the C-X-C chemokine receptor type 4 (CXCR4), functions as a biased ligand, favoring  $G_{\alpha 13}$  activation over  $G_{\alpha 13}$ . Moreover, contrary to the natural CXCR4 agonist, stromal cell-derived factor-1 $\alpha$ , ATI-2341 does not promote  $\beta$ -arrestin recruitment. In addition to revealing the selective signaling underlying ATI-2341 effects on hematopoietic cell mobilization, the study shows that pepducins are powerful tools offering perspectives for studying GPCR functional selectivity that could impact the development of drugs with fewer side effects.

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Conflict of interest statement: This study was performed as a collaboration between the academic laboratories of M.B. and J.L.B. and scientists at Anchor Therapeutics. Anchor Therapeutics is a biotechnology company that designs and develops pepducins as modulators of G protein-coupled receptors (GPCRs) for therapeutic purposes. Neither M.B., J.L.B., or members of their laboratories have financial interests related to Anchor Therapeutics. The study was partly supported by a grant from Anchor Therapeutics.

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When assessing the effect of ATI-2341 on the signaling activity of CXCR4, the peptidic agonist was found to be an allosteric agonist, activating the inhibitory heterotrimeric G protein (Gi) to promote inhibition of cAMP production and induce calcium mobilization (18, 31). In recent years, many GPCRs have been shown to engage in promiscuous signaling activities involving more than one G protein subtype as well as G protein-independent signaling. More importantly, it was found that different ligands can selectively couple to a subset of the signaling pathways that can be engaged by a receptor. In some cases, a given ligand can even have opposite efficacies on two different signaling pathways: a concept known as “ligand-biased signaling” or “functional selectivity” (32–36). In addition to its coupling to Gi, CXCR4 has also been found to signal through the engagement and activation of G13 (37, 38) and  $\beta$ -arrestin2 (39, 40), both pathways being proposed to contribute to the chemotactic responses. These observations raise the question of whether the CXCR4-selective peptidic agonist, ATI-2341, is an allosteric agonist on all of the signaling pathways identified for the chemokine receptor or whether it could show bias toward selective signaling pathways and thus be functionally selective.

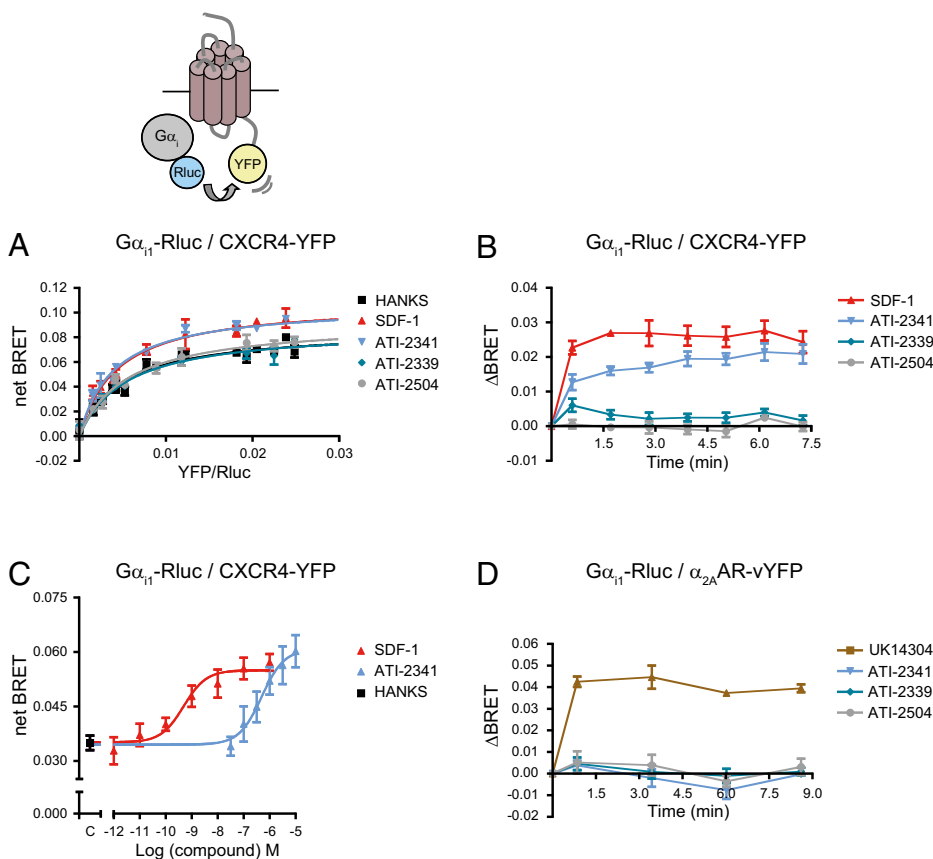
To determine whether peptidic agonists can display functional selectivity on CXCR4 signaling at the molecular level, we took advantage of bioluminescence resonance energy transfer (BRET)-based assays that allow the direct monitoring of the engagement and activation of proximal signaling effectors. More specifically, we compared the ability of ATI-2341 and the natural agonist of the receptor, SDF-1, to promote the engagement/activation of three Gi family members ( $G_{i1}$ ,  $G_{i2}$ , and  $G_{i3}$ ), G13,  $\beta$ -arrestin1, and  $\beta$ -arrestin2. We found that, whereas SDF-1 promotes the engagement of all of the signaling effectors, ATI-2341 selectively led to the functional engagement of the Gi family members and had no effect on G13 or the two  $\beta$ -arrestins. The lack of recruitment of  $\beta$ -arrestins results from the poor recruitment of G protein-coupled

receptor kinases (GRKs) to the receptor because, in contrast to SDF-1 that stimulates the phosphorylation of CXCR4 by protein kinase C (PKC), GRK2/3, and GRK6, ATI-2341 promotes effective PKC-dependent phosphorylation of the receptor but minimal GRK2/3 recruitment, as well as minimal GRK6-dependent phosphorylation.

Taken together, our results demonstrate that the peptidic agonist ATI-2341 is a functionally selective allosteric regulator of CXCR4 that activates Gi-dependent pathways without modulating G13 and  $\beta$ -arrestin pathways. These data indicate that ATI-2341 could have physiological actions that may differ from the natural ligand SDF-1 and the clinically used AMD-3100, raising the intriguing possibility that ATI-2341 may have distinct clinical properties. These findings also shed some light on the mechanism of action of peptidic agonists and indicates that, similar to orthosteric ligands, these allosteric regulators can be functionally selective.

## Results

**CXCR4-Derived Peptidic Agonist ATI-2341 Promotes the Engagement and Activation of  $G_{i1}$  but Not  $G_{i3}$ .** To dissect the signaling pathways engaged by ATI-2341, we first assessed the ability of this peptidic agonist to promote the engagement and activation of its cognate G protein, Gi. For this purpose, the engagement of  $G_{i1}$  by CXCR4 was first determined by measuring BRET between  $G_{i1}$ -Renilla luciferase (Rluc) and CXCR4-Yellow Fluorescent Protein (YFP). Similar to the endogenous ligand SDF-1, ATI-2341 induced a significant increase in the maximal BRET<sub>480-YFP</sub> signal as derived from BRET titration curves between  $G_{i1}$  and CXCR4 (Fig. 1A). In contrast, two negative control compounds, ATI-2339 [a related peptidic agonist with a modified sequence missing the last three amino acids of ATI-2341 that is inactive in CXCR4-stimulated second messenger assays (18)] and ATI-2504 [a nonlipidated analog of ATI-2341 (18)] (both sequences are shown in Table S1), had no effect (Fig. 1A). The BRET increase



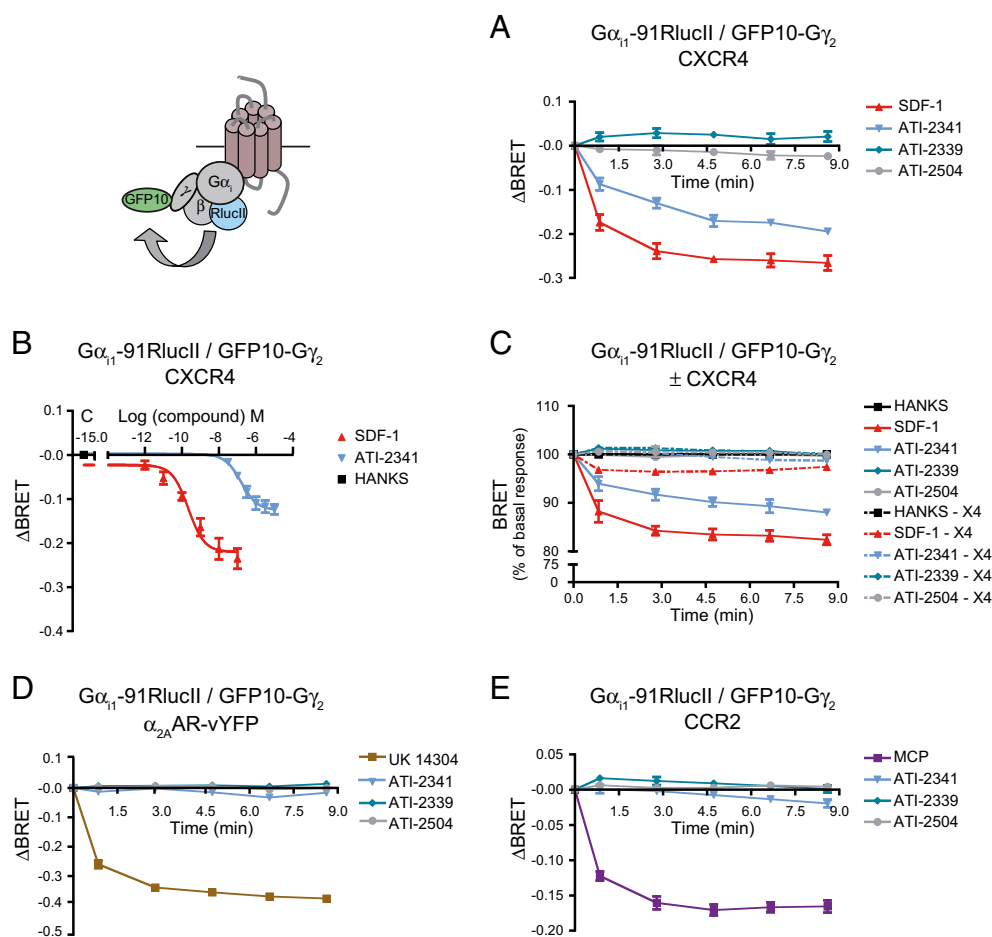
**Fig. 1.** ATI-2341 promotes the engagement of  $G_{i1}$ . (A) BRET titration curves were performed in cells cotransfected with a constant amount of  $G_{i1}$ -Rluc and increasing amounts of CXCR4-YFP. Cells were stimulated with 200 nM SDF-1, 1  $\mu$ M peptidic agonists (ATI-2341, ATI-2339, ATI-2504), or vehicle (Hanks) for 5 min. BRET was measured following coel-h addition using the BRET<sub>480-YFP</sub> filter set. The curves shown are derived from individual titration curves that are representative of four independent experiments. The error bars represent SD from duplicate wells. (B) Kinetics of the ligand-promoted BRET between  $G_{i1}$ -Rluc and CXCR4-YFP. BRET was measured at the indicated times following the addition of 200 nM SDF-1 or 1  $\mu$ M peptidic agonists. Data shown are the mean  $\pm$  SEM of four independent experiments. (C) Effect of increasing concentrations of SDF-1 or ATI-2341 on the BRET between  $G_{i1}$ -Rluc and CXCR4-YFP. BRET was measured 5 min following the addition of the ligands ( $EC_{50}$ : ATI-2341,  $533 \pm 91$  nM vs. SDF-1,  $0.53 \pm 0.19$  nM). Data shown are the mean  $\pm$  SEM of five independent experiments. (D) Kinetics of the ligand-promoted BRET between  $G_{i1}$ -Rluc and  $\alpha_{2A}$  AR-vYFP. BRET was measured at the indicated times following the addition of 100 nM UK14304 or 1  $\mu$ M peptidic agonist. Data shown are the mean  $\pm$  SEM of three independent experiments.

was rapid, reaching its maximum at the earliest time point measured (1 min) and remaining constant for at least 7.5 min, a kinetic profile very similar to the one observed for SDF-1 (Fig. 1*B*). Similar results were observed for  $G\alpha_{i1}$ ,  $G\alpha_{i2}$ , and  $G\alpha_{i3}$  when assessing BRET<sub>400-GFP2</sub> between CXCR4-green fluorescent protein (GFP) 2 and the  $G\alpha_{i1/2/3}$ -RlucII constructs (Fig. S1), indicating that the results are independent of the BRET configuration used (BRET<sub>480-YFP</sub> vs. BRET<sub>400-GFP2</sub>) and that the three  $G\alpha_i$  subunits can be engaged by the pepducin-activated CXCR4. Although the efficacy reached is similar for both compounds (Fig. 1*C*), the potency of ATI-2341 was significantly lower than that of SDF-1 ( $EC_{50}$  values: ATI-2341,  $533 \pm 91$  nM vs. SDF-1,  $0.53 \pm 0.19$  nM), in agreement with the potencies previously observed in second messenger assays (18). The selectivity of ATI-2341 for CXCR4 was confirmed using BRET partners derived from a different GPCR signaling system,  $\alpha_2$ -adrenergic engagement of  $G_i$  (41). In contrast to the known  $\alpha_2$ -adrenergic agonist, UK14304, ATI-2341 did not promote an increase in BRET between  $\alpha_{2A}$ AR-venusYFP ( $\nu$ YFP) and  $G\alpha_{i1}$ -Rluc (Fig. 1*D*).

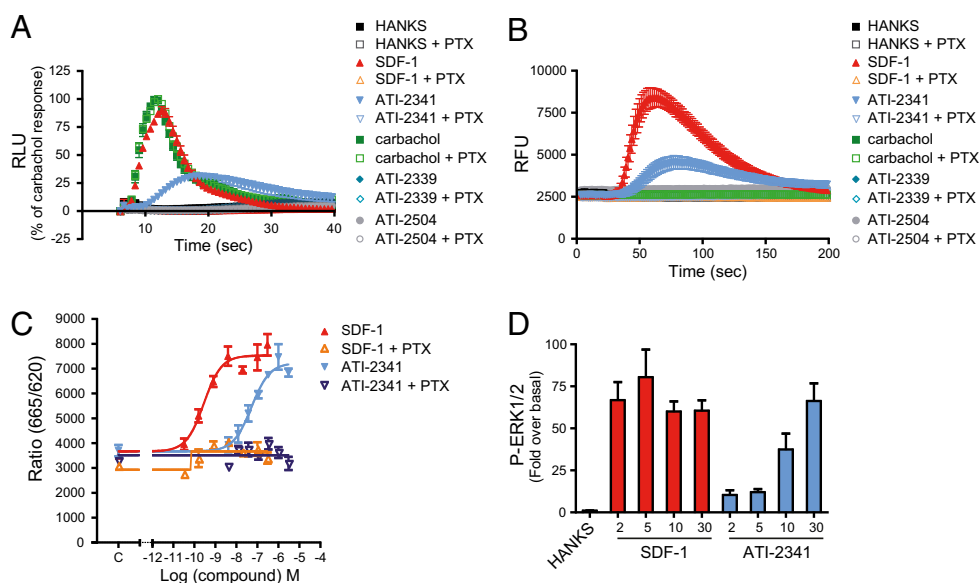
To determine whether this pepducin-promoted engagement of  $G\alpha_i$  by CXCR4 results in the activation of the G protein, we used a BRET-based assay that monitors the separation of  $G\alpha_{i1}$  and  $G\gamma_2$  that is promoted by agonist stimulation (41). Similarly to SDF-1, ATI-2341 promoted a rapid decrease in BRET<sub>400-GFP10</sub> between  $G\alpha_{i1}$ -RlucII and GFP10- $G\gamma_2$  in cells coexpressing the untagged CXCR4 and  $G\beta_1$  subunit (Fig. 2*A* and *B*), confirming that the pepducin-promoted engagement of  $G\alpha_{i1}$  by CXCR4 results in its activation. The time required to reach the maximal efficacy was slightly increased for ATI-2341 relative to that of

SDF-1 ( $t_{1/2}$ : ATI-2341,  $1.40 \pm 0.23$  min vs. SDF-1,  $0.54 \pm 0.15$  min, respectively;  $*P < 0.05$ ). Consistent with the engagement assay, two negative controls, ATI-2339 and ATI-2504, had no effect on the BRET between  $G\alpha_{i1}$ -RlucII and GFP10- $G\gamma_2$ . Further, ATI-2341 was without effect on the BRET signal in cells lacking CXCR4 (Fig. 2*C*), or in cells expressing the  $\alpha_{2A}$ AR or another chemokine receptor, the CCR2 (Fig. 2*D* and *E*), confirming that the activation of  $G\alpha_{i1}$  by ATI-2341 necessitates the presence of CXCR4, thus establishing the selectivity of action of the pepducin. The ATI-2341-activated CXCR4 also promoted the activation of  $G_{i2}$  and  $G_{i3}$  (Fig. S2). Consistent with what was observed for the pepducin-promoted engagement of  $G\alpha_i$  by CXCR4, the activation of  $G_i$  by ATI-2341 was found to be concentration-dependent and confirmed the lower potency of ATI-2341 compared with SDF-1 ( $EC_{50}$  values: ATI-2341,  $208 \pm 69$  nM vs. SDF-1,  $0.25 \pm 0.06$  nM). It should also be noted that the maximal amplitude of the pepducin-promoted BRET decrease is lower than that obtained with SDF-1 (Fig. 2*B*), indicating that ATI-2341 acts as a partial agonist on this pathway. Interestingly, this difference was not observed when considering the engagement of  $G\alpha_{i1}$ , suggesting that the partial agonism results from an incomplete activation and not a reduced binding of  $G_i$ . To corroborate the pepducin-promoted  $G_i$  activation detected with the BRET-based biosensors in nonengineered cells, calcium measurements were performed in a HEK293T cell clone endogenously expressing CXCR4 as well as in the human lymphoblast-T cell line that endogenously expresses CXCR4, SUP-T1 cells (Fig. 3*A* and *B*). In both cases, ATI-2341 and SDF-1 acted as agonists. Both the SDF-1 and pepducin responses were

**Fig. 2.** ATI-2341-promoted  $G\alpha_i$  engagement results in  $G_i$  activation. (A) Kinetics of the ligand-promoted BRET between  $G\alpha_{i1}$ -RlucII and GFP10- $G\gamma_2$ . BRET was measured after coel-400a addition, using the BRET<sub>400-GFP2/10</sub> filter set, at the indicated times following the addition of 500 nM SDF-1 or 1  $\mu$ M pepducins (ATI-2341, ATI-2339, and ATI-2504). Data shown are the mean  $\pm$  SEM of three independent experiments. (B) Effect of increasing concentrations of SDF-1 or ATI-2341 on the BRET between  $G\alpha_{i1}$ -RlucII and GFP10- $G\gamma_2$  in cells cotransfected with the untagged  $G\beta$  subunit and CXCR4. BRET was measured 5 min following the addition of the ligands ( $EC_{50}$  values: ATI-2341,  $208 \pm 69$  nM vs. SDF-1,  $0.25 \pm 0.06$  nM). Data shown are the mean  $\pm$  SEM of at least four independent experiments. (C) Kinetics of the ligand-promoted BRET between  $G\alpha_{i1}$ -RlucII and GFP10- $G\gamma_2$  in cells cotransfected with (solid lines) or without (dotted lines) CXCR4 and with the untagged  $G\beta$  subunit. BRET was measured after coel-400a addition, using the BRET<sub>400-GFP2/10</sub> filter set, at the indicated times following the addition of 500 nM SDF-1 or 1  $\mu$ M pepducins. Data shown are the mean  $\pm$  SEM of three independent experiments. (D and E) Kinetics of the ligand-promoted BRET between  $G\alpha_{i1}$ -RlucII and GFP10- $G\gamma_2$  in cells cotransfected with the untagged  $G\beta$  subunit and  $\alpha_{2A}$ AR (D) or CCR2 (E). BRET was measured after coel-400a addition, using the BRET<sub>400-GFP2/10</sub> filter set, at the indicated times following the addition of 1  $\mu$ M pepducins and 100 nM UK14304 (D) or 200 nM MCP (E). Data shown are the mean  $\pm$  SEM of three to four independent experiments.



**Fig. 3.** ATI-2341 promotes second messengers activation in a Gi-dependent manner. (A) HEK293T cells stably expressing obelin and preincubated overnight in the presence (open symbols) or absence (filled symbols) of 100 ng/mL PTX were injected into wells containing 500 nM SDF-1, 3  $\mu$ M pepducins (ATI-2341, ATI-2339, ATI-2504), 100  $\mu$ M carbachol, or vehicle (Hanks). Luminescence was measured each 600 ms for 34 s after injection. Data were normalized to carbachol-stimulated maximal calcium response. Data are the mean  $\pm$  SEM of three independent experiments. (B) SUP-T1 cells, preincubated overnight in the presence (open symbols) or absence (filled symbols) of 100 ng/mL PTX, were loaded with calcium 4 dye and incubated with 500 nM SDF-1, 3  $\mu$ M pepducins, 100  $\mu$ M carbachol, or vehicle (Hanks). Fluorescence intensity was measured each 1.5 s for 170 s after injection. Data are the mean  $\pm$  SEM of four independent experiments. (C) Effect of increasing concentrations of SDF-1 or ATI-2341 on ERK1/2 activation, in the absence (filled triangles) or presence (open triangles) of 100 ng/mL PTX in cells stably expressing human CXCR4. Data shown are the mean  $\pm$  SEM of three independent experiments. (D) Kinetics of the ligand-promoted ERK1/2 phosphorylation in HEK293T cells stimulated with 500 nM SDF-1 or 3  $\mu$ M ATI-2341 at the indicated times (0–30 min). Data were expressed as fold over basal. Data shown are the mean  $\pm$  SEM of at least three independent experiments.

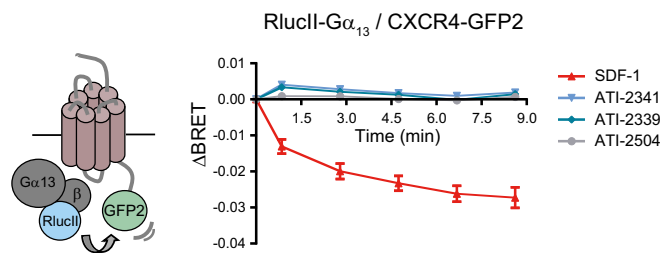


blocked by pretreatment with pertussis toxin (PTX), confirming the activation of the Gi pathway by ATI-2341 in cells natively expressing the receptor. Neither of the two control pepducins ATI-2339 and ATI-2504 promoted calcium mobilization. The ability of ATI-2341 to activate Gi was also confirmed by the complete inhibition of pepducin-stimulated ERK1/2 activity following inactivation of  $G\alpha_i$  with PTX (Fig. 3C). ATI-2341 was also found to activate ERK1/2 in the HEK293T cells endogenously expressing CXCR4 (Fig. 3D).

Because several studies indicate that CXCR4 mediates some of its actions through  $G\alpha_{13}$  (37, 38), we also assessed the ability of ATI-2341 to promote CXCR4 engagement of  $G\alpha_{13}$  by measuring BRET<sub>400-GFP2</sub> between RlucII- $G\alpha_{13}$  and CXCR4-GFP2. Using this biosensor, we previously showed that RlucII- $G\alpha_{13}$  interacts specifically with CXCR4-GFP2 and that SDF-1 promotes a decrease in the BRET signal, reflecting the functional engagement of  $G\alpha_{13}$  by the receptor (38). In contrast to the BRET decrease promoted by SDF-1, ATI-2341 was without effect on the RlucII- $G\alpha_{13}$ /CXCR4-GFP2 BRET signal (Fig. 4). This result suggests that the pepducin cannot promote the engagement of  $G\alpha_{13}$ . Taken together, these data indicate that ATI-2341 acts as a biased ligand, favoring  $G\alpha_i$  proteins over  $G\alpha_{13}$ .

**ATI-2341 Is a Weak Partial Agonist of  $\beta$ -Arrestin Recruitment.** Given the key role of  $\beta$ -arrestins in G protein signaling arrest and initiation of G protein-independent signaling, we next monitored the effect of pepducins on the recruitment of  $\beta$ -arrestin2 to CXCR4. In the absence of ligand, BRET titration curves revealed a very weak BRET signal between CXCR4-RlucII and GFP2- $\beta$ -arrestin2, characteristic of the very weak affinity of  $\beta$ -arrestins for inactive receptors (Fig. 5A). Stimulation with SDF-1 led to a large and saturable BRET increase representing the progressive association of CXCR4-RlucII with GFP2- $\beta$ -arrestin2. In contrast, ATI-2341 promoted only a very small increase in the BRET signal whereas the two control pepducins, ATI-2339 and ATI-2504, had no effect. The SDF-1-promoted recruitment of  $\beta$ -arrestin2 to CXCR4 was very rapid, with a half time of  $2.2 \pm 0.7$  min (Fig. 5B). In contrast, the very weak increase in BRET between CXCR4-RlucII and GFP2- $\beta$ -arrestin2 promoted by ATI-2341 was detectable only after a stimulation of 7.5 min and yielded a half time of  $9.4 \pm 1.2$  min. The recruitment of  $\beta$ -arrestin2 to CXCR4 induced by SDF-1 was dose-dependent,

with an  $EC_{50}$  of  $1.8 \pm 0.3$  nM (Fig. 5C) whereas the weak ATI-2341-promoted  $\beta$ -arrestin2 recruitment revealed an  $EC_{50}$  of  $273.5 \pm 78.6$  nM (Fig. 5C, *Inset*), relatively similar to the values determined for the ATI-2341-promoted Gi engagement (Fig. 1C) and activation (Fig. 2B). To verify that the weak recruitment of  $\beta$ -arrestin2 induced by ATI-2341 to CXCR4 was not due to the BRET configuration used, we monitored  $\beta$ -arrestin2 recruitment to the receptor in the reverse orientation (i.e.,  $\beta$ -arrestin2-RlucII and CXCR4-GFP2) or with BRET<sub>480-YFP</sub> using  $\beta$ -arrestin2-RlucII and CXCR4-YFP. As shown in Fig. S3, although a robust time-dependent  $\beta$ -arrestin2 recruitment could be observed for these two BRET configurations upon SDF-1 stimulation, no ATI-2341-promoted BRET could be detected, confirming that the pepducin is, at best, a very weak activator of the  $\beta$ -arrestin2 pathway. Similar results were obtained for  $\beta$ -arrestin1 (Fig. S4). Given the poor ATI-2341-promoted  $\beta$ -arrestin recruitment, the ability of the pepducin to induce receptor endocytosis was verified in HEK293T cells heterologously expressing an engineered receptor that allows monitoring both the cell surface and the total number of receptor. The receptor is tagged at its N terminus to an HA epitope and at its C terminus with vYFP. Dual FACS analysis allows one to assess the cell-surface receptor reflected by the HA immunoreactivity as a function of the total receptor number reflected by the vYFP fluorescence. As shown in Fig. 6A, ATI-2341 did not promote any significant endocytosis of the receptor compared with the 50% endocytosis promoted by SDF-1, consistent with the very weak recruitment of  $\beta$ -arrestin promoted by ATI-2341 compared with SDF-1. This lack of endocytosis is also consistent with the marginal ATI-2341-promoted BRET observed between  $\beta$ -arrestin2-RlucII and  $\beta$ 2adaptin-YFP (Fig. 6B) used as a sensor of the  $\beta$ -arrestin- and clathrin-dependent endocytosis (42). The ligand-promoted loss of cell surface receptor has also been tested in the SUP-T1 cells endogenously expressing CXCR4 (Fig. 6C). FACS analysis using a monoclonal antibody directed against the extracellular domain of the human CXCR4 (Clone 12G5; BioLegend) revealed that ATI-2341 promoted a significant loss of cell-surface CXCR4 ( $49 \pm 6\%$ ) that was much smaller than the loss promoted by SDF-1 ( $87 \pm 2\%$ ). Although it is difficult to determine the fraction of the loss in cell-surface expression that results from  $\beta$ -arrestin-dependent endocytosis ( $\beta$ -arrestin independent down-regulation could also contribute), the smaller loss observed

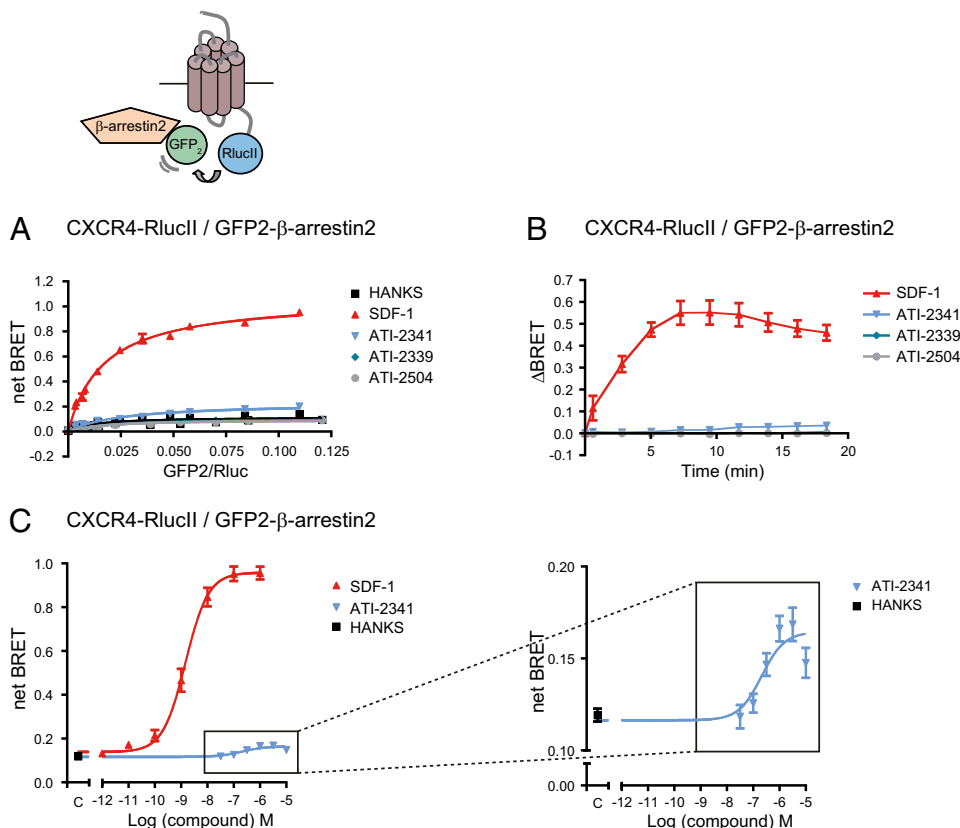


**Fig. 4.** ATI-2341 acts as a biased ligand favoring  $G_{\alpha_i}$  over  $G_{\alpha_{13}}$ . Kinetics of ligand-promoted BRET between RluclI- $G_{\alpha_{13}}$  and CXCR4-GFP2. BRET was measured after coel-400a addition, using the BRET<sub>400-GFP2/10</sub> filter set, at the indicated times following the addition of 200 nM SDF-1, 1  $\mu$ M ATI-2341, 1  $\mu$ M ATI-2339, or 1  $\mu$ M ATI-2504. Data shown are the mean  $\pm$  SEM of three independent experiments.

following ATI-2341 treatment is consistent with the reduced  $\beta$ -arrestin engagement promoted by the pepducin.

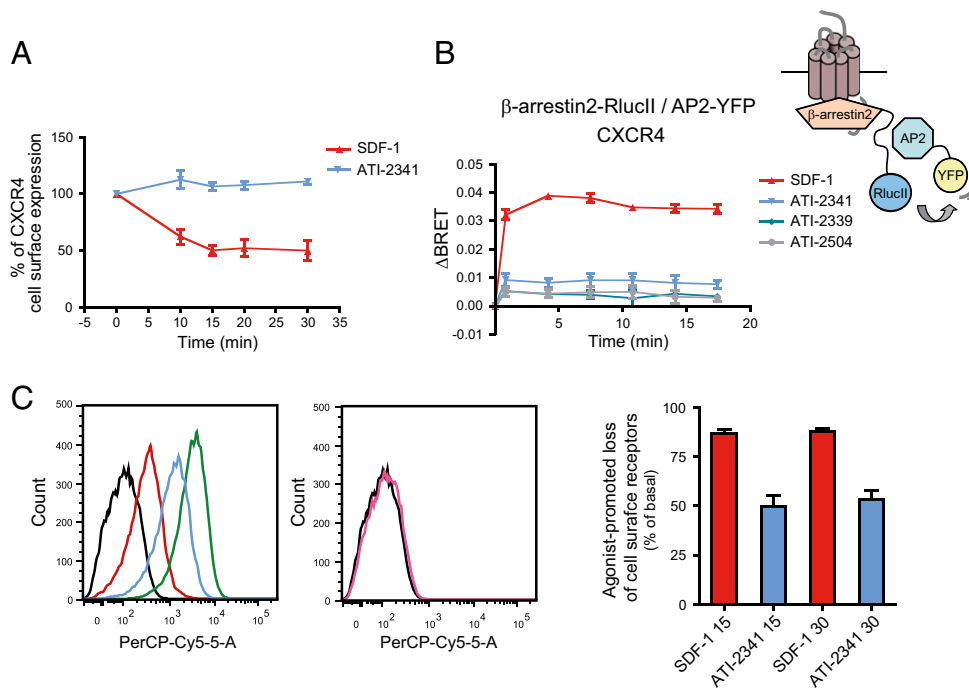
Taken together with the results obtained for  $G_{\alpha_i}$  engagement and Gi activation, these data suggest a strong bias of ATI-2341 toward the Gi pathway vs.  $\beta$ -arrestins. To quantify this bias, we used a method (43) based on the Black and Leff operational model (44). We first determined  $\tau$  and  $K_A$  values using this model.  $\tau$  ( $\tau = [Rt]/K_E$ ), which describes the ligand's efficacy toward specific pathways, is defined by the ratio of the total receptor density ([Rt]) over the general equilibrium dissociation constant ( $K_E$ ).  $K_A$  is defined as the ligand equilibrium dissociation constant of the ligand-receptor complex. Using these parameters, the transduction ratios ( $\tau/K_A$ ) and the transduction coefficients [ $\text{Log}(\tau/K_A)$ ] for SDF-1 and ATI-2341 were deduced for each signaling pathway. To determine the relative ability of SDF-1 and ATI-2341 to activate a given pathway, we calculated the  $\Delta\text{Log}(\tau/K_A)$ ,  $\Delta\text{Log}(\tau/K_A) = \text{Log}(\tau/K_A)_{\text{LIGAND}} - \text{Log}(\tau/K_A)_{\text{SDF-1}}$ , for each of the pathways considered ( $G_{\alpha_i}$  engagement and  $\beta$ -arrestin2 recruitment to CXCR4). Finally, to quantify the ATI-2341 bias for  $G_{\alpha_i}$  engagement vs.  $\beta$ -arrestin2 recruitment, we calculated the  $\Delta\Delta\text{Log}(\tau/K_A)$  as a difference between the  $\Delta\text{Log}(\tau/K_A)$  values obtained for ATI-2341 for both signaling pathways,  $\Delta\Delta\text{Log}(\tau/K_A) = \Delta\text{Log}(\tau/K_A)_{\text{SDF-1}/\text{ATI-2341}(G_{\alpha_i})} - \Delta\text{Log}(\tau/K_A)_{\text{SDF-1}/\text{ATI-2341}(\beta\text{-arrestin2})}$ . The bias factor is defined as the anti-Log value of the  $\Delta\Delta\text{Log}(\tau/K_A)$ . The calculation of a bias factor allows a direct comparison of the relative effectiveness of ATI-2341 for each pathway in a system-independent manner, thus allowing one to distinguish a real bias from a difference that could result from partial agonism toward a less amplified pathway. As shown in Table 1, the  $\Delta\Delta\text{Log}(\tau/K_A)$  comparing  $G_{\alpha_{11}}$  vs.  $\beta$ -arrestin2 engagement of 1.38 corresponds to a bias factor of 24 (i.e., anti-Log of 1.38) that indicates that ATI-2341 promotes the engagement of Gi 24 times more effectively than  $\beta$ -arrestin recruitment. The  $\Delta\text{Log}(\tau/K_A)$  calculated for ATI-2341 pepducin toward  $\beta$ -arrestin2 is significantly different from the  $\Delta\text{Log}(\tau/K_A)$  obtained for the pepducin toward the engagement of  $G_{\alpha_i}$ , indicating a significant bias of the pepducin toward  $G_{\alpha_i}$  vs. the  $\beta$ -arrestin pathway.

**ATI-2341 Promotes PKC- but Not GRK6-Promoted Phosphorylation of CXCR4.** To assess the molecular mechanism underlying the negative bias toward  $\beta$ -arrestin recruitment, and given the role of receptor phosphorylation in the ligand-promoted recruitment of  $\beta$ -arrestin, we examined the SDF-1- and ATI-2341-promoted phosphorylation of the receptor. CXCR4 is phosphorylated by PKC and GRK6 on Ser-324 and -325 (Ser-324/5) in response to SDF-1 (45). To assess the relative contribution of these kinases to the SDF-1- and ATI-2341-promoted phosphorylation, the extent of phosphorylation was determined using an anti-phospho-Ser-324/5-specific antibody in the presence and absence of the broad-spectrum PKC inhibitor bisindolylmaleimide I (Bis I). In a previous study (45), we demonstrated that, in HEK293



**Fig. 5.** ATI-2341 promotes a weak recruitment of  $\beta$ -arrestin to CXCR4. (A) BRET titration curves were performed in cells cotransfected with a constant amount of CXCR4-RluclI and increasing amounts of GFP2- $\beta$ -arrestin2. Cells were stimulated with 500 nM SDF-1, 1  $\mu$ M pepducins (ATI-2341, ATI-2339, or ATI-2504), or vehicle (Hanks) for 10 min before BRET was measured following coel-400a addition using the BRET<sub>480-YFP</sub> filter set. The curves shown are derived from individual titration curves that are representative of three independent experiments. The error bars represent the mean  $\pm$  SD from duplicate wells. (B) Kinetics of the ligand-promoted BRET between CXCR4-RluclI and GFP2- $\beta$ -arrestin2. BRET was measured at the indicated times following the addition of 500 nM SDF-1 or 1  $\mu$ M pepducins. Data shown are the mean  $\pm$  SEM of four independent experiments. (C) Effect of increasing concentrations of SDF-1 or ATI-2341 on the BRET between CXCR4-RluclI and GFP2- $\beta$ -arrestin2. BRET was measured 10 min following the addition of ligands ( $EC_{50}$ : ATI-2341,  $273.5 \pm 78.6$  nM vs. SDF-1,  $1.8 \pm 0.3$  nM). (Inset) The y axis scale enlargement for better visibility. Data shown are the mean  $\pm$  SEM of seven independent experiments.

**Fig. 6.** ATI-2341 promotes a weak CXCR4 internalization. (A) Cell-surface expression of HA-CXCR4-vYFP is measured by flow-cytometry. vYFP emission represents CXCR4 total expression, and HA-Alexa Fluor 647 emission represents CXCR4 plasma membrane expression. The relative cell-surface expression (ratio Alexa/vYFP median emissions) is calculated for the selected cell population and is expressed as a percentage of the untreated condition. Data shown are the mean  $\pm$  SEM of at least three independent experiments. (B) Kinetics of the ligand-promoted BRET between  $\beta$ -arrestin2-RlucII and  $\beta$ 2-adaptin-eYFP in cells stably expressing  $\beta$ 2-adaptin-eYFP transfected with  $\beta$ -arrestin2-RlucII and the untagged CXCR4. BRET was measured after coel-h addition, using the BRET<sub>480-YFP</sub> filter set, at the indicated times following the addition of 500 nM SDF-1 or 1  $\mu$ M pepducins. Data shown are the mean  $\pm$  SEM of three independent experiments. (C) Cell-surface expression of endogenous CXCR4 in SUP-T1 cells was assessed by flow-cytometry analysis. Cells were incubated with Hanks alone (green line) or stimulated with 500 nM SDF-1 (red line) or 1  $\mu$ M ATI-2341 (blue line) for 30 min at 37 °C and stained with PerCP-Cy5.5-conjugated 12G5. The black line and the pink line in histogram plots represent unstained and isotype control-stained cells (PerCP/Cy5.5 Mouse IgG2a, k isotype control antibody), respectively (Left and Center). Data shown are representative of five independent experiments. The agonist-promoted loss of cell surface receptors (15 and 30 min stimulation) was calculated for the selected cell population and is expressed as a percentage of loss of CXCR4 cell-surface expression compared with the untreated condition (Right). Data shown are the mean  $\pm$  SEM of at least three independent experiments.



cells, ~60% of the SDF-1-promoted phosphorylation of Ser-324/5 was mediated by PKC (as determined by Bis I inhibition) and that PKC $\delta$  but not PKC $\alpha$  contributed to some but not all of this phosphorylation (using RNAi). This study also demonstrated that GRK6, but not GRK2, -3, or -5, mediated the remaining ~40% of the SDF-promoted phosphorylation of Ser-324/5. Taken together, these data demonstrated that Bis I can be used to inhibit PKC-mediated phosphorylation of Ser-324/5 while having no effect on GRK6-mediated phosphorylation of Ser-324/5 or Ser-330. As previously described (45), we found that, in the absence of Bis I, SDF-1 promoted a rapid increase in Ser-324/5 phosphorylation, peaking at ~5 min and returning to basal levels within 60 min (Fig. 7A). ATI-2341 also promoted the transient phosphorylation of Ser-324/5, albeit to a lower extent than SDF-1.

The two control pepducins (ATI-2339 and 2504) were without effect on CXCR4 phosphorylation. In the presence of Bis I, the relative ATI-2341-promoted phosphorylation was considerably blunted compared with the SDF-1-stimulated phosphorylation (Fig. 7B), indicating that the ATI-2341-stimulated phosphorylation was predominantly PKC-dependent whereas GRK6 is the predominant kinase involved in the SDF-1-promoted phosphorylation of these residues. Consistent with this notion, SDF-1, but not ATI-2341, stimulated the phosphorylation of Ser-330 (Fig. 7C), a site that is solely phosphorylated by GRK6 in HEK293 cells as previously shown using Bis I and GRKs RNAis (45).

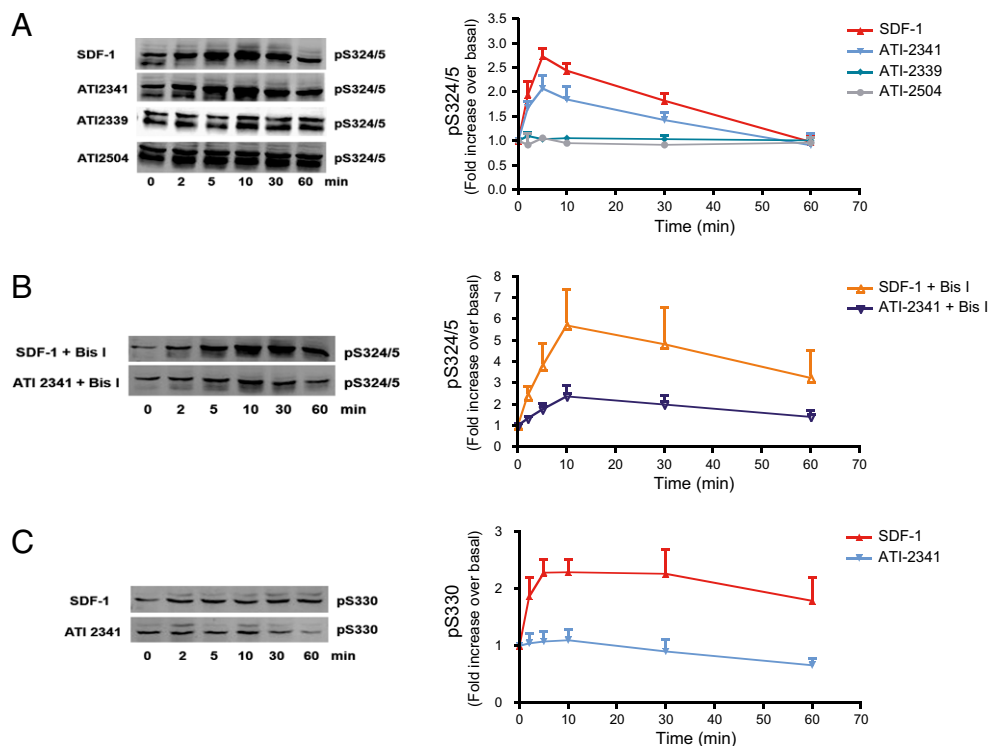
**ATI-2341 Is a Weak Partial Agonist of GRK2 and GRK3 Engagement.** To assess the engagement of two other important kinases, GRK2

**Table 1.** Data describing bias of ATI-2341 for  $G\alpha_i$  engagement and  $\beta$ -arrestin2, GRK2, or GRK3 recruitment to CXCR4

Engagement/recruitment	$\tau$	$K_A$	$\text{Log}(\tau/K_A) \pm \text{SEM}$	$\Delta\text{Log}(\tau/K_A)^\dagger \pm \text{SEM}$	$\Delta\Delta\text{Log}(\tau/K_A) \pm \text{SEM}$	Bias
<b><math>G\alpha_i</math> engagement</b>						
SDF-1	83.09	9.14E-08	8.90 $\pm$ 0.13	0.00 $\pm$ 0.18		
ATI-2341	25.03	1.75E-05	6.20 $\pm$ 0.14	-2.70 $\pm$ 0.19		
<b><math>\beta</math>-arrestin2 recruitment</b>						
SDF-1	358.90	7.12E-07	8.70 $\pm$ 0.02	0.00 $\pm$ 0.03	0.00 $\pm$ 0.19	1.0
ATI-2341	0.04	8.42E-07	4.62 $\pm$ 0.37	-4.08* $\pm$ 0.37	1.38 $\pm$ 0.42	24.0
<b>GRK2 recruitment</b>						
SDF-1	716.60	1.43E-06	8.71 $\pm$ 0.07	0.00 $\pm$ 0.09	0.00 $\pm$ 0.21	1.0
ATI-2341	0.04	1.10E-06	4.62 $\pm$ 0.32	-4.09** $\pm$ 0.32	1.40 $\pm$ 0.38	24.9
<b>GRK3 recruitment</b>						
SDF-1	697.00	2.13E-06	8.51 $\pm$ 0.05	0.00 $\pm$ 0.06	0.00 $\pm$ 0.20	1.0
ATI-2341	0.12	5.57E-06	4.33 $\pm$ 0.27	-4.19** $\pm$ 0.28	1.49 $\pm$ 0.34	30.8

Log( $\tau/K_A$ ),  $\Delta\text{Log}(\tau/K_A)$ , and bias factor values were calculated as described in *Materials and Methods* for  $G\alpha_i$  engagement and  $\beta$ -arrestin2, GRK2, or GRK3 recruitment to CXCR4. \* $P < 0.05$  and \*\* $P < 0.005$  in comparison with the  $\Delta\text{Log}(\tau/K_A)$  obtained for  $G\alpha_i$  engagement.

$^\dagger$ The reference agonist was SDF-1.



**Fig. 7.** ATI-2341 promotes PKC- but not GRK6-dependent phosphorylation of CXCR4. HEK293 cells stably expressing FLAG-tagged CXCR4 were serum-starved for 6 h and then incubated without (A and C) or with (B) 2.5  $\mu$ M of the PKC inhibitor Bis I for 30 min before stimulation with 100 nM SDF-1 or 3  $\mu$ M ATI-2341 for 0–60 min. CXCR4 phosphorylation sites were detected using specific antibodies raised against either Ser-324/5 (A and B) or Ser-330 (C) in CXCR4. (Left) A representative immunoblot visualized using the ODYSSEY infrared imaging system from three to five individual experiments. (Right) Quantitative analysis of the fold increase  $\pm$  SEM with SDF-1 or ATI-2341 from three to five independent experiments.

and -3, which play an important role in the recruitment of  $\beta$ -arrestin, we took advantage of BRET-based biosensors that directly monitor the recruitment of these kinases to the receptor. When monitoring BRET<sub>400-GFP2/10</sub> between CXCR4-RlucII and either GRK2-GFP2 or GRK3-GFP10, a robust and time-dependent SDF-1-promoted increase in BRET was observed for the two kinases (Fig. 8 A–D). In contrast, ATI-2341 promoted only a very modest increase whereas the two control pepducins had no effect. Recruitment of GRK2 and GRK3 to CXCR4 were dose-dependent for both SDF-1 and ATI-2341, with EC<sub>50</sub> values of  $1.9 \pm 0.3$  nM and  $2.0 \pm 0.4$  nM for SDF-1 and  $154 \pm 25$  nM and  $420 \pm 43$  nM for ATI-2341 for GRK2 and GRK3, respectively (Fig. 8 E and F).

These data suggest a negative bias of ATI-2341 toward the GRK recruitment and GRK-mediated phosphorylation of the receptor relative to the Gi engagement and activation. To quantify this bias, we calculated the transduction ratio for ATI-2341- and SDF-1-promoted GRK2 and GRK3 recruitment and compared it with the G $\alpha_i$  engagement to determine the bias factor. As shown in Table 1, the calculated bias factors indicate that ATI-2341 promotes the engagement of G $\alpha_{i1}$  24.9 and 30.8 times more effectively than the recruitment of GRK2 and GRK3, respectively. The  $\Delta\text{Log}(\tau/K_a)$  calculated for ATI-2341 pepducin toward GRK2 and GRK3 were significantly different from the  $\Delta\text{Log}(\tau/K_a)$  obtained for the pepducin toward the engagement of G $\alpha_i$ , indicating a significant bias of the pepducin toward G $\alpha_i$  vs. GRK2 and GRK3 pathways.

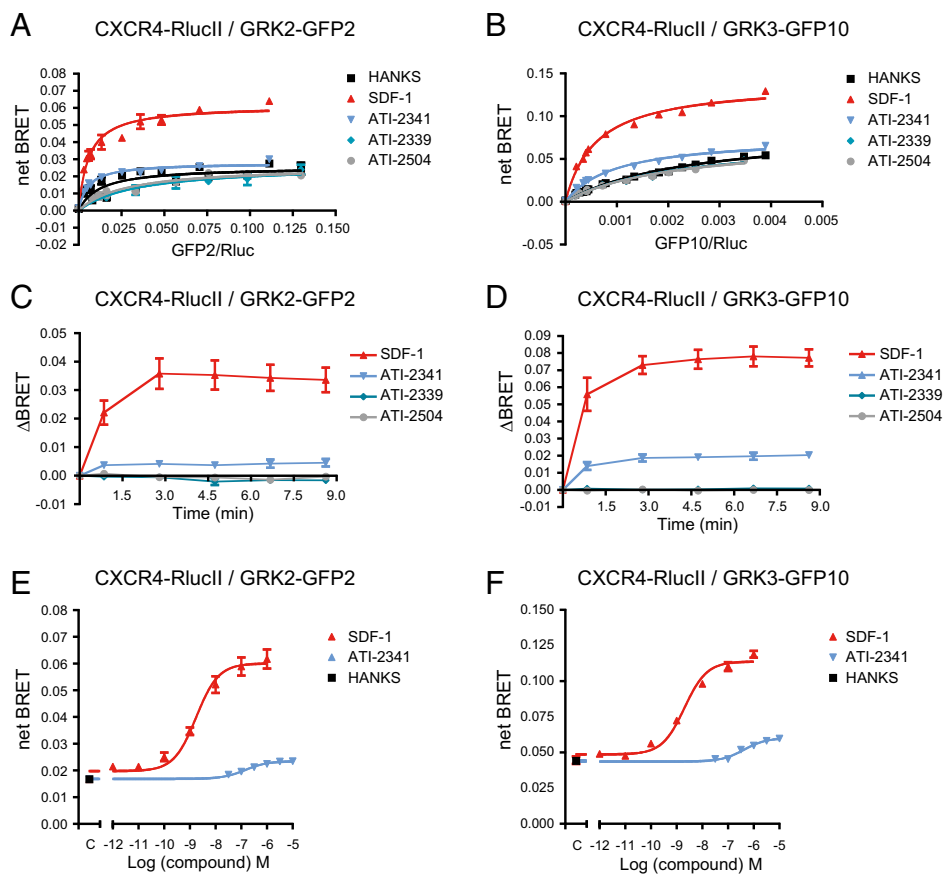
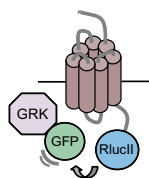
**GRK2 and GRK3 Are Involved in the Recruitment of  $\beta$ -Arrestin to CXCR4.** To confirm that the blunted ATI-2341-promoted recruitment of GRK2 and -3 could explain the negative bias of ATI-2341 toward  $\beta$ -arrestin vs. Gi, we assessed the ability of a mutant form of CXCR4 lacking the major GRK2 and -3 phosphorylation sites, CXCR4 5A tail (45), to recruit  $\beta$ -arrestin2 and activate Gi in response to SDF-1 and ATI-2341. By measuring BRET<sub>400-GFP2</sub> between  $\beta$ -arrestin2-GFP2 and either CXCR4 5A tail-RlucII or wild-type CXCR4-RlucII, we observed that mutation of the GRK2/3 phosphorylation sites greatly inhibited both the robust SDF-1- and modest ATI-2341-promoted recruitment of  $\beta$ -arrestin2 (Fig. 9A), confirming that GRK2 and -3 play a central role in

$\beta$ -arrestin2 recruitment. In contrast, mutation of the GRK2/3 phosphorylation sites did not significantly affect the ability of either SDF-1 or ATI-2341 to activate Gi, as assessed by monitoring BRET<sub>400-GFP10</sub> between G $\alpha_{i1}$ -RlucII and GFP10-G $\gamma_2$  (Fig. 9B).

## Discussion

Although pepducins were already known to allosterically modulate the function of GPCRs, both in cells (1, 2, 5, 9, 17) and in vivo (6, 18, 46), whether pepducins equally modulate all signaling pathways engaged by a receptor or can display functional selectivity by modulating only a subset of these pathways was unknown. The present study shows that the CXCR4-selective pepducin ATI-2341 displays clear functional selectivity, being biased in favor of Gi over  $\beta$ -arrestin and G13, two of the other downstream effectors being engaged by the receptor upon stimulation by its endogenous ligand SDF-1. The study also shows that the lack of engagement of  $\beta$ -arrestin by CXCR4 upon ATI-2341 stimulation results from an agonist-promoted phosphorylation pattern of the receptor that differs between pepducin and SDF-1.

In recent years, the notion that ligands can differentially modulate the activity of the various signaling pathways engaged by a single receptor has gained wide acceptance and is now known as functional selectivity or ligand-biased signaling (32, 33, 47–49). In many instances, ligands that are agonists for one pathway were found to be neutral antagonists or even inverse agonists for another pathway. Such functional selectivity is believed to result from the ability of different ligands to promote or stabilize distinct receptor conformations that in turn have different affinities for the various downstream effectors. Although this concept was first introduced for orthosteric ligands, examples of allosteric ligands acting as functionally selective modulators are emerging. In a recent study, a peptidomimetic derived from the second extracellular loop of the prostaglandin FP receptor was found to be a negative allosteric modulator of the G12/13-Rho signaling pathway while being a positive allosteric modulator of the Gq-PKC pathway (50). When characterizing pepducins that target the protease activated receptor 1 (PAR1), Cisowski et al. (5) found that a pepducin derived from the third intracellular loop fully inhibited cell motility, calcium mobilization, and ERK1/2 activation pro-



**Fig. 8.** ATI-2341 promotes a weak recruitment of GRK2 and GRK3 to CXCR4. BRET titration curves were performed in cells cotransfected with a constant amount of CXCR4-RlucII and increasing amounts of GRK2-GFP2 (A) or GRK3-GFP10 (B). Cells were stimulated with 500 nM SDF-1, 1  $\mu$ M pepducins (ATI-2341, ATI-2339, and ATI-2504), or vehicle (Hanks) for 5 min. BRET was measured following coel-400a addition using the BRET<sub>400-GFP2/10</sub> filter set. The curves shown are derived from individual titration curves that are representative of three (A) or four (B) independent experiments. The error bars represent the mean  $\pm$  SD from duplicate wells. Kinetics of the ligand-promoted BRET between CXCR4-RlucII and GRK2-GFP2 (C) or GRK3-GFP10 (D). BRET was measured at the indicated times following the addition of 500 nM SDF-1 or 1  $\mu$ M pepducins. Data shown are the mean  $\pm$  SEM of five (C) or three (D) independent experiments. Effect of increasing concentrations of SDF-1 or ATI-2341 on the BRET between CXCR4-RlucII and GRK2-GFP2 (E) or GRK3-GFP10 (F). BRET was measured 5 min following the addition of the ligands ( $EC_{50}$  of  $1.9 \pm 0.3$  nM and  $2.0 \pm 0.4$  nM for SDF-1 and  $154 \pm 25$  nM and  $420 \pm 43$  nM for ATI-2341 for GRK2 and GRK3, respectively). Data shown are the mean  $\pm$  SEM of three independent experiments.

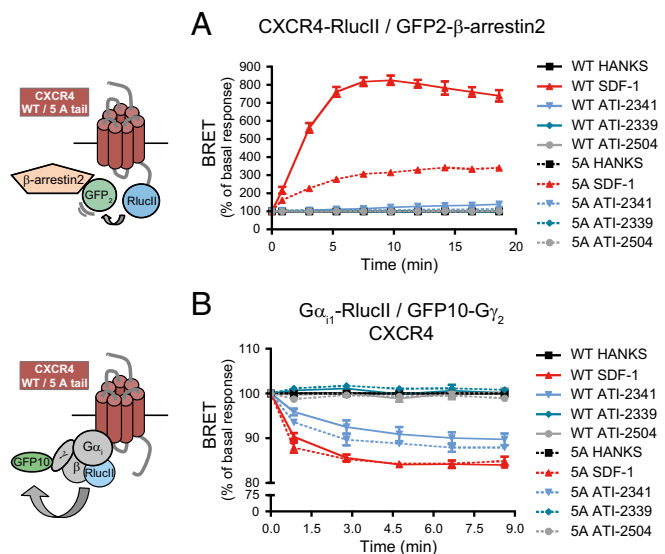
moted by thrombin. In contrast, an intracellular loop 1 pepducin fully blocked cell motility but only partially inhibited calcium mobilization and had no effect on ERK1/2 activation. However, no study to date had assessed the ability of allosteric agonists such as ATI-2341 to display functional selectivity.

Using BRET-based biosensors that directly monitor the engagement of CXCR4 effectors by the receptor, we clearly showed a bias of the allosteric agonist ATI-2341 toward the engagement and the activation of  $G_i$  vs. either the engagement of  $G\alpha_{13}$  or the recruitment of  $\beta$ -arrestins. To exclude the possibility that the apparent bias could result from partial agonism that would be differentially detected by the different assays, the relative transduction ratios ( $\tau/K_A$ ) and bias factors were calculated using a method based on the operational model of receptor activation (43). This method was selected over other approaches that use the affinities derived from radioligand binding assays to determine the  $K_A$  (51) because it has been found impractical to measure accurate binding affinities using pepducins (2). The bias factor calculated for  $G_i$  vs.  $\beta$ -arrestin2 recruitment, using SDF-1 as the reference compound, indicates that ATI-2341 is 24.0 times more efficient in promoting  $G_i$  engagement over  $\beta$ -arrestin2 recruitment, revealing a strong bias. The observation that SDF-1 had very similar relative transduction ratios for  $G_i$  and  $\beta$ -arrestin2 engagement indicates that it does not have a strong bias for one pathway over the other and justifies its use as the reference compound to calculate the bias of the pepducin. No formal bias factor could be calculated for the  $G_{13}$  pathway because the very

weak signal generated by the pepducin prevented the calculation of a  $K_A$ . However, the absence of  $G\alpha_{13}$  engagement strongly suggest a negative bias toward this pathway for the pepducin.

The lower ability of ATI-2341 vs. SDF-1 to promote the recruitment of  $\beta$ -arrestins to CXCR4 was observed for both  $\beta$ -arrestin2 and  $\beta$ -arrestin1 as well as a truncated version of  $\beta$ -arrestin1 that has an increased affinity for the receptor (Fig. S4B). Such negative bias toward the  $\beta$ -arrestin pathway may be due to the different phosphorylation profile promoted by the pepducin vs. SDF-1. Indeed, whereas SDF-1-promoted phosphorylation involves both PKC and GRKs, the preponderant CXCR4 phosphorylation promoted by ATI-2341 was due to PKC. In addition, the pepducin promoted only a very modest GRK2 and GRK3 recruitment compared with SDF-1, the calculated bias factor indicating that ATI-2341 is 24.9- and 30.8-fold more efficient at promoting  $G\alpha_i$  engagement than GRK2 and GRK3 recruitment, respectively, whereas SDF-1 had similar relative transduction ratios for GRK2/3 and  $G_i$ . Although no antibody exists that selectively recognizes the CXCR4 sites phosphorylated by GRK2/GRK3, an antibody detecting the phosphorylation of a GRK6 site, Ser-330, further showed that, in contrast to SDF-1, ATI-2341 does not promote GRK6-mediated phosphorylation of CXCR4. The results presented herein and previous studies clearly showed that phosphorylation of CXCR4 by GRK2 and GRK3 promotes  $\beta$ -arrestin recruitment (45, 52). It can thus be suggested that the decreased propensity





**Fig. 9.**  $\beta$ -arrestin recruitment to CXCR4 is dependent on GRK2 and GRK3. (A) Kinetics of the ligand-promoted BRET between wild type (WT, solid lines) or 5A tail mutant (5A, dotted lines) form of CXCR4-RlucII and GFP2- $\beta$ -arrestin2. BRET was measured after coel-400a addition, using the BRET<sub>480-YFP</sub> filter set, at the indicated times following the addition of 500 nM SDF-1, 1  $\mu$ M pepducins, or vehicle (Hanks). Data shown are the mean  $\pm$  SEM of three independent experiments. BRET values are expressed as the percentage of basal response (Hanks buffer alone). (B) Kinetics of the ligand-promoted BRET between G $\alpha_{i1}$ -RlucII and GFP10-G $\gamma_2$  in cells cotransfected with the untagged G $\beta$  subunit, and WT or 5A tail mutant form of CXCR4. BRET was measured after coel-400a addition, using the BRET<sub>400-GFP2/10</sub> filter set, at the indicated times following the addition of 500 nM SDF-1 or 1  $\mu$ M pepducins. Data shown are the mean  $\pm$  SEM of three independent experiments. BRET values are expressed as the percentage of basal response (Hanks).

of ATI-2341 to favor GRK-mediated recruitment underlies the negative bias toward  $\beta$ -arrestins recruitment.

Given that pepducins are believed to act intracellularly by binding directly to the inner face of targeted GPCRs (1, 53), it may not be surprising that different subsets of downstream effectors than those promoted by the binding of SDF-1 are engaged. However, the precise mechanism by which ATI-2341 can selectively activate G $\alpha_i$  remains unknown. Consistent with the notion that the action occurs at the level of the receptor and not G $\alpha_i$  directly is supported by our observation that the pepducin promoted the direct engagement of G $\alpha_i$  to CXCR4 and that ATI-2341-induced activation of G $\alpha_i$  was seen in cells expressing CXCR4 but not the G $\alpha_i$ -coupled  $\alpha_2AAR$  or CCR2. Given that CXCR4 has been shown to form homodimers (54, 55), it can be hypothesized that pepducins might act by modulating dimerization. However, no effect of ATI-2341 could be detected on the BRET between CXCR4-Rluc and CXCR4-YFP (Fig. S5), indicating that the pepducin did not affect dimerization. The lack of effect of the pepducin is in contrast with the SDF-1-promoted increase in BRET associated with a change in conformation of the dimer (54). This finding further indicates that ATI-2341 induces conformational changes that are different from those promoted by SDF-1. When considering the BRET between CXCR4 and G $\alpha_i$ , both ATI-2341 and SDF-1 induced an increase in the maximal BRET without affecting the BRET<sub>50</sub>, suggesting that both ligands promoted a conformational rearrangement of a preexisting receptor-G protein complex rather than the recruitment of G $\alpha_i$  to the receptor. These data are consistent with the notion that GPCRs may be precoupled to G protein, even before activation (41, 56, 57). Although no detailed structure activity relationship (SAR) was performed, the composition in amino acid of the pepducins plays an important role for their activity. Indeed, a pepducin lacking the last three amino acids of ATI-2341 was

without activity and used as a negative control throughout the study. Another analog lacking the first four amino acids of ATI-2341 also showed a dramatically reduced ability to promote G $\alpha_{i1}$  engagement (Fig. S6), indicating that residues both in N- and C-terminal position of the peptide play a role for activity. Also, a pepducin differing from ATI-2341 by a substitution of the position 1 methionine for a glycine and the substitution of the serine and methionine at position 9 and 10 by D-proline and a histidine, respectively, was more efficacious than ATI-2341 for both G $\alpha_{i1}$  and  $\beta$ -arrestin engagements, suggesting a possible SAR.

A potentially important consequence of functional selectivity at GPCR is the different physiological responses that could be triggered by biased vs. balanced ligands. For instance, reduced ability of ATI-2341 to promote  $\beta$ -arrestin recruitment could result in attenuated desensitization and/or altered  $\beta$ -arrestin-dependent signaling pathways. In recent studies, whereas SDF-1 was found to promote mobilization of B and T lymphocytes, ATI-2341 did not lead to the mobilization of either lymphocyte type (18, 28, 58). However, the two drugs promote neutrophil and hematopoietic progenitor cell mobilization (18). Whether this functional difference is due to the biased signaling activity of ATI-2341 remains to be investigated. Interestingly, G $\alpha_{13}$ /Rho axis has recently been implicated in CXCR4-mediated chemotaxis and migration of metastatic breast cancer cells (38). Therefore, the observation that SDF-1 but not ATI-2341 leads to the engagement of G $\alpha_{13}$  by CXCR4 indicates that the pepducin could provide a useful tool to assess the role of the different signaling pathways in CXCR4-mediated cell migration and mobilization.

Overall, our study demonstrates that the pepducin ATI-2341 is a biased allosteric agonist, favoring G $\alpha_i$  over G $\alpha_{13}$  and  $\beta$ -arrestin pathways. In addition to identifying a biased ligand for CXCR4, thus providing a useful tool for studying the functional consequences of such bias, our study clearly illustrates how BRET-based biosensors can be used to directly assess ligand-biased signaling and their molecular basis. The study also opens up the intriguing possibility that pepducins represent a generic approach to generating biased ligands that can selectively activate specific pathways.

## Materials and Methods

**Plasmids, Cell Culture and Transfections.** The plasmids used in this study are described in *SI Materials and Methods*. Transiently and stably transfected Human Embryonic Kidney 293 (HEK293T) cells as well as SUP-T1 cells were used in this study. Cell culture and transfection conditions are described in *SI Materials and Methods*.

**Bioluminescence Resonance Energy Transfer Measurement.** BRET<sub>480-YFP</sub> and BRET<sub>400-GFP2/10</sub> were used in this study (59) and experiments were performed as described in *SI Materials and Methods*.

**Western Blotting.** The CXCR4 specific phosphorylation state of Ser-324/5 or Ser-330 was measured by Western blotting as previously described (45) (*SI Materials and Methods*).

**Flow Cytometry.** To measure cell surface receptor expression, flow-cytometry experiments were performed on HEK293T and SUP-T1 cells using a LSR II flow-cytometer (BD Biosciences) and data were analyzed using the BD FACSDiva and FlowJo software (*SI Materials and Methods*).

**Calcium Measurements.** Obelin biosensor was used as a calcium reporter (60) in HEK293T cells while calcium mobilization was measured using a calcium 4 dye in SUP-T1 cells (*SI Materials and Methods*).

**MAPK Experiments.** Dose-response experiments were performed in HEK293 cells stably expressing human CXCR4 using the Cellul'erk kit (Cisbio). Kinetics experiments were performed in HEK293T cells endogenously expressing CXCR4 using the AlphaScreenSureFire kit (Perkin Elmer) (*SI Materials and Methods*).

**Data and Statistical Analysis.** Data analysis, statistical significance and bias factors were calculated using GraphPad Prism (GraphPad Software, Inc.) (*SI Materials and Methods*).

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