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Characterization of Angiotensin II molecular determinants involved in AT_1 receptor functional selectivity

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Running title: Functional selectivity of AngII analogs

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Abbreviations

AngII, angiotensin II; AT_1 , angiotensin II receptor; BRET, bioluminescence resonance energy transfer; DAG, diacylglycerol ; DMEM, Dulbecco's modified Eagle's medium; EGFR, epidermal growth factor receptor; ERK1/2, extracellular signal–regulated kinases 1/2; FBS, fetal bovine serum; GPCR, G protein-coupled receptor; GRK, G protein–coupled receptor kinases; HEK, human embryonic kidney; IP_1, inositol 1-phosphate; PEI, polyethyleneimine; RLuc, *Renilla* luciferase; PKC, protein kinase C

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ABSTRACT

The octapeptide angiotensin II (AngII) exerts a variety of cardiovascular effects through the activation of the AngII type 1 receptor (AT_1), a G protein-coupled receptor. The AT_1 receptor engages and activates several signalling pathways, including heterotrimeric G proteins Gq and G_{12}, as well as the ERK1/2 pathway. Additionally, following stimulation, β-arrestin is recruited to the AT_1 receptor, leading to receptor desensitization. It is increasingly recognized that specific ligands selectively bind and favour the activation of some signalling pathways over others, a concept termed ligand bias or functional selectivity. A better understanding of the molecular basis of functional selectivity may lead to the development of better therapeutics with fewer adverse effects. In the present study, we developed assays allowing the measurement of 6 different signalling modalities of the AT_1 receptor. Using a series of AngII peptide analogs that were modified in positions 1, 4 and 8, we sought to better characterize the molecular determinants of AngII that underlie functional selectivity of the AT_1 receptor in HEK293 cells. The results reveal that position 1 of AngII does not confer functional selectivity, while position 4 confers a bias towards ERK signalling over Gq signalling and that position 8 confers a bias towards βarrestin recruitment over ERK activation and Gq signalling. Interestingly, the analogs modified in position 8 were also partial agonists of the PKC-dependent ERK pathway via atypical PKC isoforms PKCζ and PKCι.

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INTRODUCTION

The octapeptide hormone angiotensin II (AngII) is the active component of the renin-angiotensin system, responsible for controlling blood pressure and water retention via smooth muscle contraction and ion transport. It exerts a wide variety of physiological effects, including vascular contraction, aldosterone secretion, neuronal activation, and cardiovascular cell growth and proliferation. Virtually all the known physiological effects of AngII are produced through the activation of the AT_1 receptor, which belongs to the G protein-coupled receptor (GPCR) superfamily (de Gasparo, et al., 2000).

The AT_1 receptor interacts with the G protein $G_{q/11}$, which activates a phospholipase C, which in turn generates inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) from the cleavage of phosphatidylinositol 4,5-bisphosphate (Hunyady and Catt, 2006). The second messenger IP_3 binds to the calcium channel receptor IP_3R present at the surface of the endoplasmic reticulum, thus liberating Ca^{2+} into the cytosol. G protein–coupled receptor kinases (GRKs) phosphorylate the receptor, leading to β-arrestin recrutement and functional uncoupling of G protein signaling. The AT_1 receptor also activates the G protein G_{12} (Sagara, et al., 2007; Suzuki, et al., 2009; Ushio-Fukai, et al., 1998; Gohla, et al., 2000). It is known that $G_{12,}$ through the regulation of RhoGEF proteins, leads to the activation of RhoA/Rho kinase and cytoskeleton reorganisation (Siehler, 2009). The AT_1 receptor also activates extracellular signal–regulated kinases 1 and 2 (ERK1/2) (Tian, et al., 1998; Wei, et al., 2003). ERK1/2 activation by the AT_1 receptor is complex and can be mediated by PKC (G protein-dependent) or by EGFR transactivation (G protein-independent) (Luttrell, 2002; Miura, et al., 2004).

Recent evidence has demonstrated that different ligands for a GPCR can stabilize the receptor under distinct conformations that promote the activation of some signalling pathways over others (Kenakin, 1995; Galandrin, et al., 2007; Shonberg, et al., 2014). This phenomenon is referred to as ligand bias or functional selectivity. Biased ligands have been proposed to stabilize receptor conformations that are distinct from those induced by unbiased ligands and selectively change the propensity of GPCR coupling to various effectors, leading to different signalling outcomes. Several biased agonists have been recently described for numerous GPCRs and such

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ligands show much therapeutic promise which could translate to compounds having less off target effects (van der Westhuizen, et al., 2014; Rominger, et al., 2014).

The goal of the present study was to better comprehend the molecular basis underlying AT_1 receptor functional selectivity. Early structure-activity relashionship studies using assays measuring rabbit aorta strip contraction and rat blood pressure have shown that position 8 of AngII is essential for ligand activity, while position 4 is critical for ligand affinity, as well as some activity (Regoli, et al., 1974). Position 1 of AngII analogs is often substituted with sarcosine (*N*-methylglycine), which allows the peptides to resist aminopeptidase degradation. AngIII, also known as AngII (2-8), is an endogenous AngII peptide where the aspartic acid at position 1 is removed by animopeptidase degradation. The ligand [$Sar^1Ile^4Ile^8$]AngII, which is unable to signal via the Gq pathway, is able to selectively recruit βarrestin (Wei, et al., 2003). Based on these observations, the goal of the study was to ascertain the impact of positions 1, 4 and 8 of AngII on the signalling profiles of the AT_1 receptor and thus gain some insight into whether these molecular determinants of AngII are involved in conferring the property of functional selectivity towards the AT_1 receptor. We therefore synthesized and investigated a series of peptide analogs containing amino acid substitutions at positions 1, 4 and 8 and determined their signalling profiles towards six different signalling pathways.

MATERIALS AND METHODS

Materials

All reagents were from Sigma-Aldrich Canada Ltd. (Oakville, ON) unless otherwise indicated. Culture media, trypsin, FBS, penicillin, and streptomycin were from Wisent (St-Bruno, Qc, Canada). OPTI-MEM and RNAi max were from Invitrogen Canada Inc. (Burlington, ON). Polyethyleneimine (PEI) was from Polysciences (Warrington, PA). All siRNAs were from Sigma-Aldrich (St-Louis, MO). Antibodies against PKCζ and PKCι were from Cell Signaling Technology (Whitby, ON). Peroxidase-conjugated donkey anti-rabbit-IgG was from GE Healthcare (Little Chalfont Buckinghamshire, UK). Western Lightning™ Chemiluminescence Reagent Plus was from Perkin Elmer (Waltham, MA). ^{125}I-AngII (specific radioactivity ~1000

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Ci/mmol) was prepared with Iodo-GEN® (Perbio Science, Erembodegem, Belgium) as reported previously.

Constructs

The cDNA clone for the human AT_1 receptor was kindly provided by Dr. Sylvain Meloche (University of Montréal). The AT_1-GFP10 construct was built by inserting the GFP10 sequence at the C-terminus of the AT_1 construct, joined by the linker GSAGT, using the In-Fusion® PCR cloning system (Clontech Laboratories, Mountain View, CA) as recommended by the manufacturer. The RLuc-βarrestin1, RLuc-βarrestin2, Gα12-RLuc, Gβ1 and Gγ1-GFP10 constructs were kindly provided by Dr. Michel Bouvier (University of Montréal). All constructs were confirmed by automated DNA sequencing by alignment with multiAlin (Corpet, 1988).

Cell culture and transfection

Human embryonic kidney (HEK) 293 cells were maintained in DMEM medium supplemented with 10% FBS, 100 IU/ml penicillin, and 100 µg/ml streptomycin at 37°C in a humidified 5% CO_2 atmosphere. HEK293 cells stably expressing the AT_1 receptor were maintained in medium containing 0.5 mg/mL G418. For βarrestin recruitment assays, HEK293 cells (3×10^6 cells) were transiently transfected with 8700 ng of AT_1-GFP10 and either 300 ng of Rluc-βarrestin1 or 300 ng of Rluc-βarrestin2 using linear polyethylenimine (1 mg/ml) (PEI:DNA ratio 4:1). For G12 activation assays, HEK293 cells (3×10^6 cells) were transiently cotransfected with the following constructs: 3000 ng of AT_1 receptor, 600 ng Gα12-RLuc, 3000 ng Gγ1-GFP10 and 1800 ng Gβ1, using linear polyethylenimine (PEI:DNA ratio 4:1). For siRNA transfection, HEK293 cells stably expressing the AT_1 receptor were seeded in a 96-well plate (50,000 cells/well) and each well was transfected with 1 pmol of the indicated siRNA using RNAi max (0.3 uL).

Binding Experiments

For binding experiments, broken cells were gently scraped into washing buffer (25 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$), centrifuged at 2500 × *g* for 15 min at 4 °C, and resuspended in binding buffer (25 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 0.1% bovine serum albumin, 0.01% bacitracin, 0.01% soybean trypsin inhibitor). Dose displacement

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experiments were done by incubating broken cells (20–40 μg of protein) for 1 h at room temperature with 0.8 nM ^{125}I-AngII as tracer and increasing concentrations of AngII. Bound radioactivity was separated from free ligand by filtration through GF/C filters presoaked for at least 3 h in binding buffer. Receptor-bound radioactivity was evaluated by γ counting. Results are presented as means ± S.D. The K_d values in the displacement studies were determined from the IC_{50} values using the Cheng-Prusoff equation.

Gq signalling

Gq signalling was evaluated by the measurement of inositol 1-phosphate (IP_1) production using the IP-One assay (Cisbio Bioassays, Bedford, MA). Necessary dilutions of each analog were prepared in stimulation buffer (10 mM Hepes, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 4.2 mM KCl, 146 mM NaCl, 5.5 mM glucose, 50 mM LiCl, pH 7.4). HEK293 cells stably expressing the AT_1 receptor were washed with PBS at room temperature, then trypsinized and distributed at 15 000 cells/well (7 μl) in a white 384-well plate in stimulation buffer. Cells were stimulated at 37 °C for 30 min with increasing concentrations of AngII or analogues. Cells were then lysed with the lysis buffer containing 3 μl of IP_1 coupled to the dye d2. After addition of 3 μl of anti-IP_1 cryptate terbium conjugate, cells were incubated for 1 h at room temperature under agitation. FRET signal was measured using a TECAN M1000 fluorescence plate reader (TECAN, Austria).

BRET-based biosensor assays

After 48 h post-transfection, cells were washed with PBS and resuspended in stimulation buffer. For the βarrestin recruitment assays, the proximity of fusion protein RLuc-βarrestin to the reporter AT_1-GFP10 was evaluated. Upon stimulation, RLuc-βarrestin was recruited to the AT1-GFP10 fusion protein, whereby the BRET signal was increased. For the G12 activation assay, the biosensor measures the proximity of the fusion protein RLuc-$G\alpha_{12}$ to GFP10-Gγ. Upon activation, both RLuc-$G\alpha_{12}$ and GFP10-Gγ move away from each other, which causes a decrease in the measured BRET. For both the βarrestin recruitment assays and G12 activation assay, cells transfected with the appropriate constructs were stimulated with the indicated ligands in 96-well white plates (50 000 cells/well) for 8 min, and then coelentherazine 400A was added at a final concentration of 5 uM. All BRET signals were measured using a TECAN M1000 fluorescence plate reader. The BRET ratio was calculated as the GFP10 emission over luminescence emission.

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Net BRET ratio was calculated by subtracting the BRET ratio under basal conditions from the BRET ratio upon maximal stimulation. All data were expressed as a percentage of maximal AngII response.

ERK1/2 activation assay

ERK1/2 activation was measured using the ERK1/2 AlphaScreen Surefire kit (PerkinElmer, Waltham, MA). HEK293 cells stably expressing the AT_1 receptor were seeded into 96-well plates at a density of 125 000 cells/well. After 24 h, cells were starved for at least 16 h in phenol red-free media before stimulation. Where specified, protein kinase C inhibitor Go6983 (1 uM), EGFR tyrosine kinase inhibitor PD168393 (250 nM) or combination of both inhibitors was added 30 min before stimulation. For time-course experiments, 100 nM AngII was added for the indicated times. For concentration-response experiments, cells were stimulated with increasing concentrations of indicated ligand. Cells were incubated at 37°C either for 2 min (PKC-ERK) or 5 min (EGFR-ERK), as determined by the peak responses obtained in the time-course assays. Stimulation of cells was terminated by the addition of lysis buffer to each well. The plate was then agitated at room temperature for 10 min and 4 µl of lysate was transferred to 384-well ProxiPlates (PerkinElmer, Waltham, MA) and 5 ul of the assay reaction mix was added to each well (reaction buffer : activation buffer : donor beads : acceptor beads = 120 : 40 : 1 : 1). The plate was then incubated in the dark at room temperature for 24 h under agitation and the signal was measured with an Enspire alpha reader (PerkinElmer, Waltham, MA) using standard AlphaScreen settings. All data were expressed as a percentage of maximal AngII-induced ERK1/2 phosphorylation.

Western blotting

HEK293 cells stably expressing the AT_1 receptor that had been transfected with siRNA as described above for 48 h were washed with PBS and then solubilized with lysis buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, 5 mM EDTA, pH 7.4) for 30 min at 4°C. Insoluble material was precipitated by centrifugation at 15 000 *g* for 20 min at 4°C. Cell lysates (30 ug) were separated on a 8% SDS-PAGE gel and were transferred to a 0.2-µm PVDF membrane in a 96 mM glycine, 10 mM Tris-base and 20% methanol buffer for 60 min at 0.4 A at 4°C. The membranes were blocked for 1 h at room temperature with TBS-T buffer (20 mM

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Tris-base, 150 mM NaCl, and 0.1% Tween-20) containing 5% (w/v) skim milk. They were then incubated for 2 h at room temperature with rabbit anti-PKCζ (1:1000) or rabbit anti-PKCι (1:250) in TBS-T supplemented with 5% skim milk. After three washes with TBS-T, the membranes were incubated with a peroxidase-conjugated donkey anti-rabbit-IgG (1:2000) for 30 min at room temperature in TBS-T supplemented with 5% skim milk. They were then washed three times with TBS-T, and the immune complexes were visualized using Western Lightning™ Chemiluminescence Reagent Plus.

Data analysis

Binding data were analyzed with Prism version 6.0 for Windows (GraphPad Software, San Diego CA), using a one-site binding hyperbola nonlinear regression analysis. Transduction ratios and bias factors were calculated based on the method of Kenakin (Kenakin, et al., 2012), as described in detail by van der Westhuizen *et al* (van der Westhuizen, et al., 2014). Transduction ratios [$\log(\tau/K_A)$] were first derived using the operational model equation in GraphPad Prism. The transduction ratio is an assessment of the effect (potency and efficacy) of a compound on receptor conformation and the subsequent ligand-receptor interaction with downstream effectors. In order to assess true ligand bias, system and observational bias which may be present owing to the different sensitivities of the assays used must be eliminated by comparing ligand activity at a given signalling pathway to that of a reference agonist. AngII, which yielded similar potencies and maximally activated all the pathways, was the reference compound. By subtracting $\log(\tau/K_A)$ of AngII to the $\log(\tau/K_A)$ value of each analog for a given pathway, a *within-pathway* comparison was first established, yielding $\Delta\log(\tau/K_A)$. Finally, *between-pathway* comparisons were achieved for a given ligand in the form of $\Delta\Delta\log(\tau/K_A)$ and the bias factor BF. $\Delta\Delta\log(\tau/K_A)$ were calculated by substracting $\Delta\log(\tau/K_A)$ values of one signalling pathway from the $\Delta\log(\tau/K_A)$ of the signalling pathway to which it is compared. BF values are the base 10 values of $\Delta\Delta\log(\tau/KA)$ and are the actual bias factors.

RESULTS

Binding properties of AngII peptide analogs

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We evaluated the binding properties of 8 selected peptide analogs of AngII (Table 1). AngII (K_d = 1.1 nM), and its analogs [Sar^{1}Ile^8]AngII (K_d = 1.7 nM), [Sar^1]AngII (K_d = 1.8 nM), [Ile^8]AngII (K_d = 3.2 nM), AngIII (K_d = 5.2 nM), [Sar^{1}Ile^8]AngIII (K_d = 12 nM) showed high binding affinity in the low nanomolar range whereas analogs [Sar^{1}Ile^4]AngII (K_d = 78 nM) and [Ile^4]AngII (K_d = 894 nM) showed lower binding affinities. These analogs modified at positions 1, 4 or 8 were appropriate to compare their relative efficacies in the different signalling pathways.

Gq signalling

To assess AT_1 receptor signalling via the heterotrimeric G protein Gq, we measured IP_1 production after a 30 min stimulation with each analog, using AngII as a reference. The AngII dose-response curve shown at Fig. 1A revealed a maximal response of 730.2 nM of IP_1 produced (efficacy normalized to 100%) with a half-maximal response (EC_{50}) at a concentration of 3.1 nM. [Sar^1]AngII also showed a high affinity with a good efficacy (Fig. 1B) whereas AngIII had a good efficacy but a low affinity (Table 2). [Sar^{1}Ile^4]AngII and [Ile^4]AngII were partial agonists with low affinities (Fig. 1C and Table 2). [Sar^{1}Ile^8]AngII (Fig. 1D), [Ile^8]AngII (Fig. 1E) and [Ile^8]AngIII (Fig. 1F) did not produce any measurable IP_1. All these data are summarized in Table 2. These results indicate that position 8 of AngII is critical for activating the Gq pathway. These results also indicate that position 4 of AngII affects both the potency and the efficacy of the peptide in the activation of the Gq pathway whereas modifications at position 1 did not show any impact.

βarrestin recruitment

To evaluate the capacity of the receptor to recruit and engage with either βarrestin1 or βarrestin2 following an 8 min stimulation with each analog, a BRET-based βarrestin recruitment assay was used. The AngII dose-response curve for βarrestin1 recruitment revealed a maximal net BRET ratio of 0.085 (efficacy normalized to 100%) with an EC_{50} of 6.1 nM (Fig. 2A), while that for βarrestin2 recruitment revealed a maximal net BRET ratio of 0.106 (efficacy normalized to 100%) with an EC_{50} of 4.6 nM (Fig. 3A). [Sar^1]AngII showed a full efficacy and a high affinity for the recruitment of both βarrestins (Fig. 2B and Fig. 3B). AngIII showed a high efficacy and a low affinity for the recruitment of both βarrestins (Table 2). [Sar^{1}Ile^4]AngII

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showed a high efficacy but a low affinity for the recruitment of both βarrestins (Fig. 2C and Fig. 3C). [Ile^4]AngII also showed a good efficacy but a very low affinity for the recruitment of both βarrestins (Table 2).

Dose-response curves were performed with three analogs modified at position 8. [Sar^{1}Ile^8]AngII was a partial agonist with a high affinity for the recruitment of both βarrestins (Fig. 2D and Fig. 3D). [Ile^8]AngII was also a partial agonist with a high affinity for the recruitment of both βarrestins (Fig. 2E and Fig. 3E) whereas [Ile^8]AngIII was a partial agonist with a low affinity for the recruitment of both βarrestins (Fig. 2F and Fig. 3F). All these results are summarized in Table 2. These results indicate that the analogs modified at position 8 of AngII are partial agonists for the recruitment of βarrestins with relatively high potencies but with efficacies lower (~50%) than that of AngII. The analogs modified at position 4 are also partial agonists for the recruitment of βarrestins although with relatively high efficacies (~80% compared to AngII) and with low potencies. Modifications at position 1 of AngII did not show any impact on the recruitment of βarrestins.

G_{12} activation

The ability of the AT_1 receptor to engage with and activate the G_{12} heterotrimer was evaluated using a BRET-based biosensor assay. The AngII dose-response curve shown at Fig. 4A revealed a net BRET ratio of 0.096 (efficacy normalized to 100%) with an EC_{50} of 4.7 nM. [Sar^1]AngII (Fig. 4B) showed a full efficacy and a high affinity, whereas AngIII (Table 2) showed a full efficacy but a low affinity for the activation of G_{12}. [Sar^{1}Ile^4]AngII (Fig. 4C) and [Ile^4]AngII (Table 2) were partial agonists with low affinities for the activation of G_{12}. [Sar^{1}Ile^8]AngII (Fig. 4D), and [Ile^8]AngII (Fig. 4E) were partial agonists with high affinities whereas [Ile^8]AngIII (Fig. 4F) was a partial agonist with a low affinity for the activation of G_{12}. All these results are summarized in Table 2. These results indicate that the analogs modified at position 8 of AngII are partial agonists for G_{12} activation with relatively high potencies but with efficacies lower (~40%) than that of AngII. The analogs modified at position 4 are also partial agonists for G_{12} activation with efficacies lower (~60%) than that of AngII and with low potencies. Modifications at position 1 of AngII did not show any impact on the activation of G_{12}.

ERK response

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We evaluated the capacity of the selected AngII analogs to activate the ERK1/2 pathway by measuring ERK phosphorylation levels upon stimulation, a hallmark of ERK activity. Figure 5 shows that under control conditions (circles), the addition of AngII increased ERK phosphorylation which reached a maximal value at 5 min and then slowly declined towards the basal level. In the presence of the EGFR tyrosine kinase inhibitor PD168393 (triangles), the addition of AngII increased ERK phosphorylation which reached a maximal value at 2 min and then slowly declined towards the basal level. In the presence of the PKC inhibitor Go6983 (squares), the addition of AngII increased ERK phosphorylation which reached a maximal value at 5 min and then slowly declined towards the basal level. In the presence of both PD168393 and Go6983 (diamonds), the addition of AngII did not produce any measurable ERK phosphorylation. These results suggest that in our experimental model, within the limits of our time-course, AngII activates the ERK1/2 pathway via PKC following Gq activation and also via the transactivation of EGFR. Since the ERK1/2 response is completely abolished in the presence of both inhibitors, we reasoned that the ERK response in the presence of Go6983 was mediated by EGFR (pathway EGFR-ERK) while the ERK response in the presence of PD168393 was mediated by PKC (pathway PKC-ERK).

EGFR-mediated ERK response

To evaluate the EGFR-ERK pathway, HEK293 cells stably expressing the AT_1 receptor were treated with increasing concentrations of each analog for 5 min, in the presence of Go6983 (Fig. 6 and Table 3). The AngII dose-response curve shown at Fig. 6A shows a maximal response of 49 677 luminescence arbitrary units (efficacy normalized to 100%) with an EC_{50} of 2.8 nM. [Sar^1]AngII (Fig. 6B) showed a high efficacy and a high affinity whereas AngIII (Table 3) showed a high efficacy but a low affinity for the activation of the EGFR-ERK pathway. [Sar^1Ile^4]AngII (Fig. 6C), and [Ile^4]AngII (Table 3) showed high efficacies but low affinities for the activation of the EGFR-ERK pathway. [Sar^1Ile^8]AngII (Fig. 6D), [Ile^8]AngII (Fig. 6E), and [Ile^8]AngIII (Fig. 6F) were partial agonists with relatively low affinities for the activation of the EGFR-ERK pathway. All these results are summarized in Table 3. These results indicate that the analogs modified at position 4 are partial agonists, although with very high efficacies (~94%) for the EGFR-ERK pathway. Furthermore, [Sar^1Ile^4]AngII showed a higher potency (~10 times) in the EGFR-ERK pathway than the Gq, G12 and βarrestin pathways. The analogs modified at

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position 8 of AngII are partial agonists of the EGFR-ERK pathway, with relatively low potencies (~10 times lower than that of AngII) and with low efficacies (~30% of that of AngII). Modifications at position 1 of AngII had no major impact on the EGFR-ERK pathway.

PKC-mediated ERK response

To evaluate the PKC-ERK pathway, HEK293 cells stably expressing the AT_1 receptor were treated with increasing concentrations of each analog for 2 min, in the presence of PD168393 (Fig. 7 and Table 3). The AngII dose-response curve shown at Fig. 7A shows a maximal response of 58 391 luminescence arbitrary units (efficacy normalized to 100%) with an EC_{50} of 1.2 nM. [Sar^1]AngII (Fig. 7B) showed a high efficacy and a high affinity whereas AngIII (Table 3) also showed a high efficacy but a low affinity for the activation of the PKC-ERK pathway. [Sar^1Ile^4]AngII (Fig. 7C), and [Ile^4]AngII (Table 3) showed high efficacies but low affinities for the activation of the PKC-ERK pathway. [Sar^1Ile^8]AngII (Fig. 7D), [Ile^8]AngII (Fig. 7E), and [Ile^8]AngIII (Fig. 7F) were partial agonists with relatively low affinities for the activation of the PKC-ERK pathway. These results indicate that the analogs modified at position 4 are partial agonists, although with high efficacies (~85%) for the PKC-ERK pathway. Furthermore, the analog [Sar^1Ile^4]AngII showed a higher potency (~10 times) in the PKC-ERK pathway than the Gq, G12 and βarrestin pathways. The analogs modified at position 8 of AngII are partial agonists of the PKC-ERK pathway, with relatively low potencies (~10 times lower than AngII) and with low efficacies (~30% that of AngII). Modifications at position 1 of AngII had no major impact on the PKC-ERK pathway.

Role of atypical PKC isoforms in the PKC-mediated ERK response

It is generally accepted that Gq activation leads to the production of DAG and IP_3, leading to the elevation of intracellular Ca^{2+} concentration. DAG and Ca^{2+} in turn lead to the activation of most isoforms of PKC. How then can a ligand such as [Sar^1Ile^8]AngII, that does not engage Gq, activate the PKC-ERK pathway? Actually, PKC are comprised of at least 15 isomers which can be classified into 3 families: conventional PKCs (cPKCs) including PKCα, PKCβ and PKCγ that are activated by Ca^{2+} and DAG, novel PKCs (nPKCs) including PKCδ, PKCε, PKCη and PKCθ that are activated by DAG only and atypical PKCs (aPKCs) including

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PKCζ and PKCι that are not activated by Ca^{2+} nor by DAG, but by other, less understood mechanisms (Wu-Zhang and Newton, 2013). We therefore hypothesized that [Sar^1Ile^8]AngII could activate atypical PKC isoforms PKCι or PKCζ. To test this hypothesis, we used siRNA directed against either PKCι or PKCζ, and then measured the PKC-dependent ERK response. Figure 8A shows that upon PKCι knockdown, the PKC-dependent ERK activity promoted by [Sar^1Ile^8]AngII was reduced by 55%. When PKCζ was knocked down, PKC-dependent ERK activity promoted by [Sar^1Ile^8]AngII was reduced by 60%. Upon knockdown of both isoforms simultaneously, the PKC-dependent response elicited with [Sar^1Ile^8]AngII was further diminished by 75%. Western blotting analysis showed that both PKCζ (Figure 8B) and PKCι (Figure 8C) were both efficiently knocked down. These results support a role for both atypical PKC isoforms PKCι and PKCζ in AT_1 receptor signalling that may have previously been underestimated.

Quantification of ligand bias

Large variations in the potencies and efficacies of AngII analogs towards the different signalling pathways were observed (Tables 2 and 3), which suggests the presence of signalling bias. In order to clearly establish whether an analog was biased towards one pathway over the others, the bias factors were determined for each analog and for all the signalling pathways. Transduction ratios [$\log(\tau/K_A)$] were first derived using the operational model (Table 4). The $\log(\tau/K_A)$ of the reference compound AngII was then subtracted from the $\log(\tau/K_A)$ value of each analog for a given pathway, yielding $\Delta\log(\tau/K_A)$ as a *within-pathway* comparison for each signalling pathway. The $\Delta\log(\tau/K_A)$ value is indicative of how well a given signalling pathway can be activated by a ligand, where a value of 0 indicates that a given ligand activates a pathway to the same degree as the reference compound, a positive value indicating that the ligand more strongly activates the signalling pathway than the reference compound and an increasingly negative value indicating that the ligand poorly activates the signalling pathway, if at all. The $\Delta\log(\tau/K_A)$ values of each analog calculated for every signalling pathway was represented on a radar plot, adapted from the 'web of efficacy' (Evans, et al., 2010; Zhou, et al., 2013) (Figure 9). This allowed a graphic representation highlighting to what extent each pathway can be activated

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(or not) by a given ligand. Ultimately, a *between-pathway* comparison was achieved for a given ligand in the form of $\Delta\Delta\log(\tau/K_A)$, which is the actual bias factor (Tables 5 and 6).

The different analogs were partitioned into 3 groups, based on their functional selectivity profiles. The first group was comprised of the analogs modified solely at position 1, [Sar^1]AngII and AngIII (Table 5 and Fig. 9A). The analog [Sar^1]AngII showed $\Delta\log(\tau/K_A)$ values ranging from -0.25 to 0.29, indicating that it is balanced relative to AngII (Fig. 9A). These results suggest that position 1 of AngII is likely not an important molecular determinant for functional selectivity towards the AT_1 receptor. A second group of analogs included [Sar^{1}Ile^4]AngII (Fig. 9B) and [Ile^4]AngII that are modified at position 4 (Table 6). Figure 9B shows that [Sar^{1}Ile^4]AngII was a strong activator of both EGFR-dependent and PKC-dependent ERK signalling with $\Delta\log(\tau/K_A)$ values respectively of -1.51 and -1.72. The rank order of pathways activated were as follows: βarrestin2 recruitment (($\Delta\log(\tau/K_A)$ of -2.35) , βarrestin1 recruitment (($\Delta\log(\tau/K_A)$ of -2.39), the G_{12} pathway ($\Delta\log(\tau/K_A)$ of -2.49) and the Gq pathway ($\Delta\log(\tau/K_A)$ of -3.47). Table 6 shows that the biases of [Ile^4]AngII and [Sar^{1}Ile^4]AngII towards EGFR-ERK over Gq were 14-fold and 93-fold, respectively. The bias of [Ile^4]AngII towards PKC-ERK over Gq was 26-fold while for [Sar^{1}Ile^4]AngII it was 57-fold. These represented the strongest biases for AngII analogs modified at position 4. These results suggest that position 4 of AngII is likely an important molecular determinant involved in functional selectivity towards the AT_1 receptor. The third group of analogs included [Sar^{1}Ile^8]AngII (Figure 9C), [Ile^8]AngII (Figure 9D) and [Ile^8]AngIII (Figure 9E), that are modified at position 8. Figure 9C shows that for [Sar^{1}Ile^8]AngII, the rank order of pathways activated were as follows : βarrestin2 recruitment $\Delta\log(\tau/K_A)$ of -0.77), βarrestin1 recruitment ($\Delta\log(\tau/K_A)$ of -0.79), the G_{12} pathway (($\Delta\log(\tau/K_A)$ of -1.93) , EGFR-ERK (($\Delta\log(\tau/K_A)$ of -4.03) and PKC-ERK ($\Delta\log(\tau/K_A)$ of -4.13). Analogs [Ile^8]AngII and [Ile^8]AngIII showed the same rank order of pathway activation than [Sar^{1}Ile^8]AngII, but were less proficient at recruiting both βarrestins and activating the G_{12} pathway. Since none of the analogs in this group activated the Gq pathway, no bias factor could be calculated. However, this in itself represents an obviously strong bias of these ligands for all the other pathways over the Gq pathway. Table 7 shows that the bias of [Sar^{1}Ile^8]AngII towards βarrestin2 over PKC-ERK was 2252-fold, while the bias of βarrestin1 over PKC-ERK was 2147-fold. These represented the strongest biases found for AngII analogs modified at position 8.

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These results confirm that position 8 of AngII is likely an important molecular determinant involved in functional selectivity towards the AT_1 receptor.

DISCUSSION

Functional selectivity is a relatively new concept and its therapeutic potential is becoming more and more acknowledged (Rominger, et al., 2014). Ligand bias is the ability of certain ligands to stabilize distinct receptor-transducer pairs at the expense of others, leading to signal pathway selectivity. The goal of our study was to identify biased ligands of the AT_1 receptor and to better characterize the molecular determinants of AngII that underlie functional selectivity. A better understanding of the mechanisms leading to the recognition of distinct receptor-effector states by biased ligands should lead to the development of better therapeutics with less off-target effects. For this study, eleven AngII peptide analogs together with the reference ligand AngII were selected to assess their functional selectivity profiles towards 6 different AT_1 receptor signalling outcomes. The pathways investigated were IP_1 production (as a reporter of Gq activation), βarrestin1 and βarrestin2 recruitment, G_{12} activation as assessed by a BRET biosensor assay, and the ERK1/2 activation via either PKC activation or EGFR activation. The ligands were chosen based on the previously characterised importance of positions 1, 4 and 8 for AngII activity, as demonstrated by previous structure-activity relationship studies (Regoli, et al., 1974; Holloway, et al., 2002).

We showed that the substitution of phenylalanine at position 8 of AngII for isoleucine abolishes Gq signalling, whereas it maintains βarrestin recruitment, G_{12} and ERK activation. The radar plot shown at Fig. 9 further indicates the strongest bias towards βarrestin recruitment. [Ile^8]AngII, [Sar^1Ile^8]AngII and [Ile^8]AngIII were antagonists for the Gq pathway, partial agonists with relatively high efficacies for βarrestin recruitment and with lower efficacies for the other pathways. It was known for a long time that AngII analogs containing an aliphatic residue at position 8 are antagonists of the Gq pathway (Regoli, et al., 1974; Miura, et al., 1999). It was only recently shown that these analogs can activate some pathways downstream of the AT_1 receptor. For instance, it was shown that [Sar^1Ile^8]AngII can activate total ERK (Holloway, et al., 2002; Ahn, et al., 2004a) and recruit βarrestin (Zimmerman, et al., 2012; Ahn, et al., 2004b).

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TRV027, an analog that contains D-Ala at position 8, is another recently developed biased ligand towards β-arrestin recruitment (Violin, et al., 2010). These results suggest that the molecular determinants present at position 8 of AngII contribute to AT_1 receptor functional selectivity, whereby Gq activation is abolished, while the $G_{12,}$ and βarrestin pathways are maintained and ERK activity is strongly decreased.

Of particular interest, we found that [Sar^1Ile^8]AngII, despite being unable to generate a Gq-dependent response, was still able to activate ERK in an atypical PKC-dependent manner. It was previously shown that PKCζ plays a role in AT_1 receptor ERK activation in vascular smooth muscle cells (Kim, et al., 2009; Liao, et al., 1997; Zhao, et al., 2005). These results support a role for both atypical PKC isoforms PKCι and PKCζ in AT_1 receptor signalling that may have previously been underestimated.

We showed that the substitution of aspartate at position 1 for sarcosine ([Sar^1]AngII) or its removal (AngIII) had very little impact on the capacity of the AT_1 receptor to signal through the different pathways tested (Table 5 and Fig. 9A). It has been long known that sarcosine at position 1 of AngII peptides confers resistance to aminopeptidase degradation in vivo (Hall, et al., 1974). Substitution at position 1 of AngII was seen to have little or no impact on smooth muscle contraction (an assay dependent on Gq activation) (Hall, et al., 1974), on inositol phosphates production (Holloway, et al., 2002) or on total ERK activation (Miura, et al., 2004). Here, we further showed that substitution at position 1 of AngII also had no impact on G_{12} activation, EGFR transactivation and βarrestin recruitment. Altogether, these results suggest that position 1 of AngII peptides has very little impact on AT_1 receptor functional selectivity.

We showed that the substitution of tyrosine at position 4 of AngII for isoleucine caused a bias towards ERK signalling. Both [Ile^4]AngII and [Sar^1Ile^4]AngII are full agonists for PKC-dependent and EGFR-dependent ERK activity, but partial agonists for the G_{12}, βarrestin and Gq pathways. Although the substitution of Tyr^4 for Ile^4 decreased the potency for activation of all the pathways, this decrease was markedly less for the ERK pathway. This modification at position 4 appears to stabilize a receptor conformation that preferentially interacts with the effectors of the ERK pathway. To our knowledge, we are the first to evaluate and compare the impact of position 4 of AngII on different signalling pathways using a single model. We are also the first to evaluate the impact of position 4 on G_{12} activation. It was previously shown that

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[Sar^{1}Ile^4]AngII is a partial agonist of the Gq pathway in CHO-K1 cells (Miura, et al., 2004). Substitution at position 4 was also shown to decrease AngII potency and efficacy in smooth muscle contraction assays (Regoli, et al., 1974; Samanen, et al., 1989). A previous report indicated that substitutions at position 4 of AngII had very little impact on ERK activation in CHO-K1cells (Holloway, et al., 2002). These results suggest that the molecular determinants present at position 4 of AngII contribute to AT_1 receptor functional selectivity, whereby ERK activity is maintained while the $G_{12,}$ Gq and βarrestin pathways are negatively impacted.

In conclusion, the purpose of our study was to establish the impact of positions 1, 4 and 8 of AngII on different signalling pathways downstream of the AT_1 receptor and thus gain some insight into whether these molecular determinants of AngII were involved in conferring functional selectivity. Our study reveals that position 1 of AngII does not confer functional selectivity, while position 4 confers a bias towards ERK signalling over Gq signalling and that position 8 confers a bias towards βarrestin recruitment over ERK activation and Gq signalling.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design : Domazet, Holleran, Lavigne, Escher, Leduc and Guillemette

Conducted experiments : Domazet, Holleran, Richard, Vandenberghe

Performed data analysis : Domazet, Holleran, Richard, Vandenberghe, Lavigne, Escher, Leduc and Guillemette

Contributed to the writing of the manuscript : Domazet, Holleran, Leduc and Guillemette

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This article has not been copyedited and formatted. The final version may differ from this version.

REFERENCES

Ahn S, Shenoy SK, Wei H and Lefkowitz RJ (2004a) Differential kinetic and spatial patterns of beta-arrestin and G protein-mediated ERK activation by the angiotensin II receptor. *J Biol Chem* **279:**35518-35525.

Ahn S, Wei H, Garrison TR and Lefkowitz RJ (2004b) Reciprocal regulation of angiotensin receptor-activated extracellular signal-regulated kinases by beta-arrestins 1 and 2. *J Biol Chem* **279:**7807-7811.

Corpet F (1988) Multiple sequence alignment with hierarchical clustering. *Nucleic Acids Res* **16:**10881-10890.

de Gasparo M, Catt KJ, Inagami T, Wright JW and Unger T (2000) International union of pharmacology. XXIII. The angiotensin II receptors. *Pharmacol Rev* **52:**415-472.

Evans BA, Sato M, Sarwar M, Hutchinson DS and Summers RJ (2010) Ligand-directed signalling at beta-adrenoceptors. *Br J Pharmacol* **159:**1022-1038.

Galandrin S, Oligny-Longpre G and Bouvier M (2007) The evasive nature of drug efficacy: implications for drug discovery. *Trends Pharmacol Sci* **28:**423-430.

Gohla A, Schultz G and Offermanns S (2000) Role for G(12)/G(13) in agonist-induced vascular smooth muscle cell contraction. *Circ Res* **87:**221-227.

Hall MM, Khosla MC, Khairallah PA and Bumpus FM (1974) Angiotensin analogs: the influence of sarcosine substituted in position 1. *J Pharmacol Exp Ther* **188:**222-228.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 10, 2024

Molecular Pharmacology Fast Forward. Published on March 25, 2015 as DOI: 10.1124/mol.114.097337
This article has not been copyedited and formatted. The final version may differ from this version.

Holloway AC, Qian H, Pipolo L, Ziogas J, Miura S, Karnik S, Southwell BR, Lew MJ and Thomas WG (2002) Side-Chain Substitutions within Angiotensin II Reveal Different Requirements for Signaling, Internalization, and Phosphorylation of Type 1A Angiotensin Receptors. *Mol Pharmacol* **61:**768-777.

Hunyady L and Catt KJ (2006) Pleiotropic AT1 Receptor Signaling Pathways Mediating Physiological and Pathogenic Actions of Angiotensin II. *Molecular Endocrinology* **20:**953-970.

Kenakin T (1995) Agonist-receptor efficacy. II. Agonist trafficking of receptor signals. *Trends Pharmacol Sci* **16:**232-238.

Kenakin T, Watson C, Muniz-Medina V, Christopoulos A and Novick S (2012) A simple method for quantifying functional selectivity and agonist bias. *ACS Chem Neurosci* **3:**193-203.

Kim J, Ahn S, Rajagopal K and Lefkowitz RJ (2009) Independent beta-arrestin2 and Gq/protein kinase Czeta pathways for ERK stimulated by angiotensin type 1A receptors in vascular smooth muscle cells converge on transactivation of the epidermal growth factor receptor. *J Biol Chem* **284:**11953-11962.

Liao DF, Monia B, Dean N and Berk BC (1997) Protein kinase C-zeta mediates angiotensin II activation of ERK1/2 in vascular smooth muscle cells. *J Biol Chem* **272:**6146-6150.

Luttrell LM (2002) Activation and targeting of mitogen-activated protein kinases by G-protein-coupled receptors. *Can J Physiol Pharmacol* **80:**375-382.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 10, 2024

Molecular Pharmacology Fast Forward. Published on March 25, 2015 as DOI: 10.1124/mol.114.097337
This article has not been copyedited and formatted. The final version may differ from this version.

Miura S, Zhang J, Matsuo Y, Saku K and Karnik SS (2004) Activation of extracellular signal-activated kinase by angiotensin II-induced Gq-independent epidermal growth factor receptor transactivation. *Hypertens Res* **27:**765-770.

Miura S, Feng Y, Husain A and Karnik SS (1999) Role of Aromaticity of Agonist Switches of Angiotensin II in the Activation of the AT1 Receptor. *Journal of Biological Chemistry* **274:**7103-7110.

Regoli D, Park WK and Rioux F (1974) Pharmacology of angiotensin. *Pharmacol Rev* **26:**69-123.

Rominger DH, Cowan CL, Gowen-MacDonald W and Violin JD (2014) Biased ligands: pathway validation for novel GPCR therapeutics. *Curr Opin Pharmacol* **16C:**108-115.

Sagara Y, Hirooka Y, Nozoe M, Ito K, Kimura Y and Sunagawa K (2007) Pressor response induced by central angiotensin II is mediated by activation of Rho/Rho-kinase pathway via AT1 receptors. *J Hypertens* **25:**399-406.

Samanen J, Cash T, Narindray D, Brandeis E, Yellin T and Regoli D (1989) The role of position 4 in angiotensin II antagonism: a structure-activity study. *J Med Chem* **32:**1366-1370.

Shonberg J, Lopez L, Scammells PJ, Christopoulos A, Capuano B and Lane JR (2014) Biased agonism at G protein-coupled receptors: the promise and the challenges--a medicinal chemistry perspective. *Med Res Rev* **34:**1286-1330.

Siehler S (2009) Regulation of RhoGEF proteins by G12/13-coupled receptors. *Br J Pharmacol* **158:**41-49.

Downloaded from molpharm.aspetjournals.org at ASPET Journals on April 10, 2024

Molecular Pharmacology Fast Forward. Published on March 25, 2015 as DOI: 10.1124/mol.114.097337
This article has not been copyedited and formatted. The final version may differ from this version.

Suzuki H, Kimura K, Shirai H, Eguchi K, Higuchi S, Hinoki A, Ishimaru K, Brailoiu E, Dhanasekaran DN, Stemmle LN, Fields TA, Frank GD, Autieri MV and Eguchi S (2009) Endothelial nitric oxide synthase inhibits G12/13 and rho-kinase activated by the angiotensin II type-1 receptor: implication in vascular migration. *Arterioscler Thromb Vasc Biol* **29:**217-224.

Tian Y, Smith RD, Balla T and Catt KJ (1998) Angiotensin II activates mitogen-activated protein kinase via protein kinase C and Ras/Raf-1 kinase in bovine adrenal glomerulosa cells. *Endocrinology* **139:**1801-1809.

Ushio-Fukai M, Griendling KK, Akers M, Lyons PR and Alexander RW (1998) Temporal dispersion of activation of phospholipase C-beta1 and -gamma isoforms by angiotensin II in vascular smooth muscle cells. Role of alphaq/11, alpha12, and beta gamma G protein subunits. *J Biol Chem* **273:**19772-19777.

van der Westhuizen ET, Breton B, Christopoulos A and Bouvier M (2014) Quantification of ligand bias for clinically relevant beta2-adrenergic receptor ligands: implications for drug taxonomy. *Mol Pharmacol* **85:**492-509.

Violin JD, DeWire SM, Yamashita D, Rominger DH, Nguyen L, Schiller K, Whalen EJ, Gowen M and Lark MW (2010) Selectively engaging beta-arrestins at the angiotensin II type 1 receptor reduces blood pressure and increases cardiac performance. *J Pharmacol Exp Ther* **335:**572-579.

Wei H, Ahn S, Shenoy SK, Karnik SS, Hunyady L, Luttrell LM and Lefkowitz RJ (2003) Independent beta-arrestin 2 and G protein-mediated pathways for angiotensin II activation of extracellular signal-regulated kinases 1 and 2. *Proc Natl Acad Sci U S A* **100:**10782-10787.

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Molecular Pharmacology Fast Forward. Published on March 25, 2015 as DOI: 10.1124/mol.114.097337
This article has not been copyedited and formatted. The final version may differ from this version.

Wu-Zhang AX and Newton AC (2013) Protein kinase C pharmacology: refining the toolbox. *Biochem J* **452:**195-209.

Zhao Y, Liu J, Li L, Liu L and Wu L (2005) Role of Ras/PKCzeta/MEK/ERK1/2 signaling pathway in angiotensin II-induced vascular smooth muscle cell proliferation. *Regul Pept* **128:**43-50.

Zhou L, Lovell KM, Frankowski KJ, Slauson SR, Phillips AM, Streicher JM, Stahl E, Schmid CL, Hodder P, Madoux F, Cameron MD, Prisinzano TE, Aube J and Bohn LM (2013) Development of functionally selective, small molecule agonists at kappa opioid receptors. *J Biol Chem* **288:**36703-36716.

Zimmerman B, Beautrait A, Aguila B, Charles R, Escher E, Claing A, Bouvier M and Laporte SA (2012) Differential beta-arrestin-dependent conformational signaling and cellular responses revealed by angiotensin analogs. *Sci Signal* **5:**ra33.

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Molecular Pharmacology Fast Forward. Published on March 25, 2015 as DOI: 10.1124/mol.114.097337
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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Inositol 1-phosphate production induced by AngII analogs. HEK293 cells expressing the AT_1 receptor were stimulated with increasing concentrations of AngII (A), Sar^1-AngII (B), Sar^1Ile^4-AngII (C), Sar^1Ile^8-AngII (D), Ile^8-AngII (E) and Ile^8-AngIII (F) for 30 min at 37°C. IP_1 accumulation was measured with the IP-One assay, as described in the methods. Data are expressed as a percentage of AngII maximal response. The dotted line represents the AngII dose-response curve. Data are the mean ± SD of 3-6 independent experiments performed in triplicate.

Figure 2. βarrestin1 recruitment to the AT_1 receptor by AngII analogs. HEK293 cells co-transfected with fusion protein RLuc-βarrestin and the reporter AT_1-GFP10 were stimulated with increasing concentrations of AngII (A), Sar^1-AngII (B), Sar^1Ile^4-AngII (C), Sar^1Ile^8-AngII (D), Ile^8-AngII (E) and Ile^8-AngIII (F) for 8 min at 37°C. βarrestin1 recruitment was measured as described in the methods. Data are expressed as a percentage of AngII maximal response. The dotted line represents the AngII dose-response curve. Data are the mean ± SD of 3-6 independent experiments performed in triplicate.

Figure 3. βarrestin2 recrutment to the AT_1 receptor by AngII analogs. HEK293 cells co-transfected with fusion protein RLuc-βarrestin and the reporter AT_1-GFP10 were stimulated with increasing concentrations of AngII (A), Sar^1-AngII (B), Sar^1Ile^4-AngII (C), Sar^1Ile^8-AngII (D), Ile^8-AngII (E) and Ile^8-AngIII (F) for 8 min at 37°C. βarrestin2 recruitment was measured as described in the methods. Data are expressed as a percentage of AngII maximal response. The dotted line represents the AngII dose-response curve. Data are the mean ± SD of 3-6 independent experiments performed in triplicate.

Figure 4. G_{12} activation by AngII analogs. HEK293 cells expressing the AT_1 receptor and co-transfected with Gα12-RLuc, Gγ1-GFP10 and Gβ1 were stimulated with increasing concentrations of AngII (A), Sar^1-AngII (B), Sar^1Ile^4-AngII (C), Sar^1Ile^8-AngII (D), Ile^8-AngII (E) and Ile^8-AngIII (F) for 8 min at 37°C. G_{12} activity was measured as described in the methods.

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Data are expressed as a percentage of AngII maximal response. The dotted line represents the AngII dose-response curve. Data are the mean ± SD of 3-6 independent experiments performed in triplicate.

Figure 5. AngII-induced ERK activation. HEK293 cells expressing the AT_1 receptor were pretreated as indicated for 30 min and then were stimulated with 100 nM AngII for different periods of time. ERK activity was measured as described in the methods. Data are expressed as a percentage of AngII maximal response. Data are the mean ± SD of 3-6 independent experiments performed in triplicate.

Figure 6. EGFR-dependent ERK activation by AngII analogs. HEK293 cells expressing the AT_1 receptor were pretreated with 1 uM Go6983 for 30 min and then were stimulated with increasing concentrations of AngII (A), Sar^1-AngII (B), Sar^1Ile^4-AngII (C), Sar^1Ile^8-AngII (D), Ile^8-AngII (E) and Ile^8-AngIII (F) for 5 min at 37°C. ERK activity was measured as described in the methods. Data are expressed as a percentage of AngII maximal response. The dotted line represents the AngII dose-response curve. Data are the mean ± SD of 3-6 independent experiments performed in triplicate.

Figure 7. PKC-dependent ERK activation by AngII analogs. HEK293 cells expressing the AT_1 receptor were pretreated with 250 nM PD168393 for 30 min and then were stimulated with increasing concentrations of AngII (A), Sar^1-AngII (B), Sar^1Ile^4-AngII (C), Sar^1Ile^8-AngII (D), Ile^8-AngII (E) and Ile^8-AngIII (F) for 2 min at 37°C. ERK activity was measured as described in the methods. Data are expressed as a percentage of AngII maximal response. The dotted line represents the AngII dose-response curve. Data are the mean ± SD of 3-6 independent experiments performed in triplicate.

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Figure 8. Atypical PKC-dependent ERK activation. HEK293 cells expressing the AT_1 receptor were transfected with siRNA against the indicated PKC isoforms as described in the methods. (panel A) Cells were stimulated with 100 nM Sar^1Ile^8-AngII for 2 min at 37°C. ERK activity was measured as described in the methods. Data are expressed as a percentage of AngII maximal response. Data are the mean ± SD of 3-6 independent experiments performed in triplicate. Western-bot analysis of PKCζ (panel B) and PKCι (panel C) following treatment with the indicated siRNA.

Figure 9. Effects of AngII analogs on AT_1 signalling pathways. Radar graph representations summarizing the calculated $\Delta\log(\tau/K_A)$ values of the different ligand-activated pathways for Sar^1-AngII (A), Sar^1Ile^4-AngII (B), Sar^1Ile^8-AngII (C), Ile^8-AngII (D) and Ile^8-AngIII (E). The balanced reference analog AngII is represented in blue.

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TABLES

Table 1

Binding properties of AT_1 receptor ligands

	K_d (nM)	n
AngII	1.1 ± 0.3	12
[Sar^1]-AngII	1.8 ± 0.1	3
[Ile^4]-AngII	894 ± 132	3
[Ile^8]-AngII	3.2 ± 1.1	3
[Sar^{1}Ile^4]-AngII	78 ± 11	3
[Sar^{1}Ile^8]-AngII	1.7 ± 0.8	3
[Ile^{4}Ile^8]-AngII	1254 ± 198	4
[Sar^{1}Ile^{4}Ile^8]-AngII	223 ± 38	3
AngIII	5.2 ± 2.1	4
[Ile^4]-AngIII	3689 ± 2027	3
[Ile^8]-AngIII	12.3 ± 5.1	3
[Ile^{4}Ile^8]-AngIII	5783 ± 1408	3

HEK293 cells stably expressing the AT_1 receptor were assayed as described in "Experimental Procedure." Binding affinities (K_d) are expressed as the means ± SD of values obtained in *n* independent experiments performed in duplicate.

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Table 2

Activation of Gq, βarrestin1, βarrestin2 and G_{12} by AT_1 receptor ligands

	Gq		βarrestin1		βarrestin2		G_{12}	
	EC_{50} (nM)	E_{max} (% AngII)	EC_{50} (nM)	E_{max} (% AngII)	EC_{50} (nM)	E_{max} (% AngII)	EC_{50} (nM)	E_{max} (% AngII)
AngII	2.4 ± 1.6	100	6.1 ± 1.7	100	4.6 ± 1.9	100	4.7 ± 1.9	100
[Sar^1]-AngII	3.3 ± 0.8	98 ± 8	6.4 ± 3.0	100 ± 1	5.6 ± 2.3	94 ± 5	5.1 ± 2.0	104 ± 8
[Ile^4]-AngII	3104 ± 106	67 ± 2	2802 ± 670	72 ± 6	3829 ± 649	84 ± 8	1056 ± 321	60 ± 9
[Sar^{1}Ile^4]-AngII	686 ± 52	43 ± 7	429 ± 126	81 ± 4	458 ± 158	86 ± 3	228 ± 62	65 ± 5
[Ile^8]-AngII	NR	NR	7.0 ± 1.6	54 ± 6	5.7 ± 2.7	59 ± 6	3.5 ± 1.4	42 ± 5
[Sar^{1}Ile^8]-AngII	NR	NR	4.5 ± 1.9	56 ± 6	5.6 ± 2.0	61 ± 8	1.0 ± 0.6	44 ± 6
AngIII	51 ± 19	87 ± 8	54 ± 8	94 ± 3	48 ± 16	93 ± 9	90 ± 33	98 ± 7
[Ile^8]-AngIII	NR	NR	11.3 ± 1.0	50 ± 10	49 ± 2	48 ± 11	12.3 ± 7.1	38 ± 6

HEK293 cells expressing the AT_1 receptor were assayed as described in the methods. EC_{50} and E_{max} are expressed as the means ± SD of values obtained in at least 3 independent experiments performed in triplicate. NR, no response.

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Table 3

Activation of Gq, PKC-ERK and EGFR-ERK by AT_1 receptor ligands

	Gq		PKC-ERK		EGFR-ERK	
	EC_{50} (nM)	E_{max} (% AngII)	EC_{50} (nM)	E_{max} (% AngII)	EC_{50} (nM)	E_{max} (% AngII)
AngII	2.4 ± 1.6	100	1.2 ± 0.1	100	2.8 ± 0.5	100
[Sar^1]-AngII	3.3 ± 0.8	98 ± 8	3.0 ± 0.9	92 ± 4	4.2 ± 1.9	94 ± 2
[Ile^4]-AngII	3104 ± 106	67 ± 2	523 ± 93	80 ± 10	1327 ± 264	93 ± 8
[Sar^{1}Ile^4]-AngII	686 ± 52	43 ± 7	32 ± 5	94 ± 7	50 ± 9	95 ± 5
[Ile^8]-AngII	NR	NR	55 ± 13	38 ± 4	40 ± 14	27 ± 3
[Sar^{1}Ile^8]-AngII	NR	NR	45 ± 14	28 ± 4	11 ± 3	27 ± 1
AngIII	51 ± 19	87 ± 8	4.0 ± 0.6	88 ± 3	9.2 ± 1.9	88 ± 3
[Ile^8]-AngIII	NR	NR	40 ± 5	25 ± 3	21 ± 4	30 ± 4

HEK293 cells stably expressing the AT_1 receptor were assayed as described in the methods. EC_{50} and E_{max} are expressed as the means ± SD of values obtained in at least 3 independent experiments performed in duplicate. NR, no response.

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Table 4

Transduction ratios of AT_1 receptor ligands

	Gq		βarrestin1		βarrestin2		G_{12}		PKC-ERK		EGFR-ERK	
	$\log(\tau/K_A)$	$\Delta\log(\tau/K_A)$	$\log(\tau/K_A)$	$\Delta\log(\tau/K_A)$	$\log(\tau/K_A)$	$\Delta\log(\tau/K_A)$	$\log(\tau/K_A)$	$\Delta\log(\tau/K_A)$	$\log(\tau/K_A)$	$\Delta\log(\tau/K_A)$	$\log(\tau/K_A)$	$\Delta\log(\tau/K_A)$
AngII	8.46 ± 0.07	0.00 ± 0.10	8.29 ± 0.12	0.00 ± 0.16	8.42 ± 0.06	0.00 ± 0.08	8.25 ± 0.07	0.00 ± 0.10	8.96 ± 0.04	0.00 ± 0.06	8.76 ± 0.04	0.00 ± 0.06
[Sar^1]-AngII	8.75 ± 0.07	0.29 ± 0.09	8.40 ± 0.28	0.11 ± 0.29	8.24 ± 0.06	-0.18 ± 0.08	8.48 ± 0.22	0.23 ± 0.23	8.72 ± 0.12	-0.25 ± 0.12	8.55 ± 0.15	-0.22 ± 0.15
[Ile^4]-AngII	4.50 ± 0.41	-3.95 ± 0.41	5.25 ± 0.07	-3.04 ± 0.13	5.27 ± 0.08	-3.15 ± 0.10	5.02 ± 0.22	-3.23 ± 0.22	6.43 ± 0.24	-2.53 ± 0.24	5.94 ± 0.13	-2.82 ± 0.13
[Sar^1Ile^4]-AngII	4.98 ± 0.23	-3.47 ± 0.23	5.90 ± 0.17	-2.39 ± 0.20	6.07 ± 0.18	-2.35 ± 0.18	5.76 ± 0.16	-2.49 ± 0.18	7.24 ± 0.36	-1.72 ± 0.36	7.26 ± 0.22	-1.51 ± 0.10
[Ile^8]-AngII	ND	ND	6.81 ± 0.36	-1.48 ± 0.37	7.63 ± 0.20	-0.79 ± 0.20	5.15 ± 0.45	-3.10 ± 0.45	4.98 ± 0.20	-3.99 ± 0.20	4.96 ± 0.22	-3.81 ± 0.22
[Sar^1Ile^8]-AngII	ND	ND	7.50 ± 0.38	-0.79 ± 0.39	7.65 ± 0.22	-0.77 ± 0.23	6.32 ± 0.56	-1.93 ± 0.56	4.84 ± 0.16	-4.13 ± 0.16	4.74 ± 0.04	-4.03 ± 0.05
AngIII	6.65 ± 0.12	-1.80 ± 0.14	7.29 ± 0.19	-0.99 ± 0.22	7.32 ± 0.16	-1.10 ± 0.16	7.25 ± 0.27	-1.00 ± 0.27	8.26 ± 0.05	-0.70 ± 0.06	8.39 ± 0.15	-0.37 ± 0.15
[Ile^8]-AngIII	ND	ND	5.66 ± 0.16	-2.63 ± 0.19	5.55 ± 0.20	-2.87 ± 0.21	4.64 ± 0.15	-3.61 ± 0.16	4.55 ± 0.08	-4.42 ± 0.09	4.95 ± 0.18	-3.81 ± 0.18

HEK293 cells expressing the AT_1 receptor were stimulated with the different analogs and responses were measured for 6 distinct signalling pathways. Data were analysed by nonlinear regression using the Operational Model equation as described in the methods to determine $\log(\tau/K_A)$. $\Delta\log(\tau/K_A)$ were calculated from $\log(\tau/K_A)$ using AngII as the reference ligand. Data are the mean ± SEM of 3-6 independent experiments performed in triplicate. ND, Cannot be determined.

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Table 5

Bias factors of AT_1 receptor ligands modified at position 1

	AngII		[Sar^1]-AngII		AngIII	
	$\Delta\Delta\log(\tau/K_A)$	BF	$\Delta\Delta\log(\tau/K_A)$	BF	$\Delta\Delta\log(\tau/K_A)$	BF
EGFR/Gq	0.00 ± 0.12	1.00	-0.51 ± 0.18	0.31	1.43 ± 0.21	26.99
PKC/Gq	0.00 ± 0.12	1.00	-0.54 ± 0.16	0.29	1.10 ± 0.16	12.63
G_{12}/Gq	0.00 ± 0.14	1.00	-0.06 ± 0.25	0.86	0.80 ± 0.31	6.35
βarrestin1/Gq	0.00 ± 0.19	1.00	-0.19 ± 0.32	0.65	0.80 ± 0.27	6.34
βarrestin2/Gq	0.00 ± 0.14	1.00	-0.48 ± 0.13	0.33	0.70 ± 0.22	5.04
EGFR/βarrestin2	0.00 ± 0.11	1.00	-0.03 ± 0.18	0.93	0.73 ± 0.23	5.36
EGFR/βarrestin1	0.00 ± 0.17	1.00	-0.33 ± 0.34	0.47	0.63 ± 0.27	4.24
EGFR/G_{12}	0.00 ± 0.12	1.00	-0.45 ± 0.28	0.93	0.63 ± 0.32	4.26
PKC/βarrestin2	0.00 ± 0.11	1.00	-0.06 ± 0.15	0.87	0.40 ± 0.18	2.51
PKC/βarrestin1	0.00 ± 0.17	1.00	-0.36 ± 0.32	0.44	0.30 ± 0.18	1.98
PKC/G_{12}	0.00 ± 0.12	1.00	-0.48 ± 0.26	0.33	0.30 ± 0.29	1.99
EGFR/PKC	0.00 ± 0.09	1.00	-0.03 ± 0.20	0.94	0.33 ± 0.23	2.14
βarrestin1/βarrestin2	0.00 ± 0.19	1.00	0.30 ± 0.31	1.98	0.10 ± 0.28	1.74
βarrestin1/G_{12}	0.00 ± 0.19	1.00	-0.12 ± 0.38	0.75	0.10 ± 0.36	1.50
βarrestin2/G_{12}	0.00 ± 0.14	1.00	-0.42 ± 0.25	0.38	0.01 ± 0.33	1.01

$\Delta\Delta\log(\tau/K_A)$ and BF values were calculated as described in the methods. Data are the mean ± SEM of 3-6 independent experiments performed in triplicate.

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Table 6

Bias factors of AT_1 receptor ligands modified at position 4

	AngII		[Ile^4]-AngII		[Sar^{1}Ile^4]-AngII	
	$\Delta\Delta\log(\tau/K_A)$	BF	$\Delta\Delta\log(\tau/K_A)$	BF	$\Delta\Delta\log(\tau/K_A)$	BF
EGFR/Gq	0.00 ± 0.12	1.00	1.13 ± 0.44	13.53	1.97 ± 0.26	92.69
PKC/Gq	0.00 ± 0.12	1.00	1.42 ± 0.48	26.24	1.76 ± 0.43	57.10
βarrestin2/Gq	0.00 ± 0.14	1.00	0.80 ± 0.43	6.36	1.12 ± 0.30	13.27
βarrestin1/Gq	0.00 ± 0.19	1.00	0.92 ± 0.44	8.23	1.08 ± 0.31	12.11
G_{12}/Gq	0.00 ± 0.14	1.00	0.72 ± 0.47	5.29	0.98 ± 0.30	9.66
EGFR/G_{12}	0.00 ± 0.12	1.00	0.41 ± 0.27	2.56	0.98 ± 0.20	9.60
EGFR/βarrestin1	0.00 ± 0.17	1.00	0.22 ± 0.19	1.64	0.88 ± 0.23	7.66
EGFR/βarrestin2	0.00 ± 0.11	1.00	0.33 ± 0.17	2.13	0.84 ± 0.21	6.99
PKC/G_{12}	0.00 ± 0.12	1.00	0.70 ± 0.33	4.96	0.77 ± 0.40	5.91
PKC/βarrestin1	0.00 ± 0.17	1.00	0.50 ± 0.28	3.19	0.67 ± 0.42	4.72
PKC/βarrestin2	0.00 ± 0.11	1.00	0.62 ± 0.26	4.13	0.63 ± 0.41	4.30
βarrestin2/G_{12}	0.00 ± 0.14	1.00	0.08 ± 0.25	1.20	0.14 ± 0.26	1.37
βarrestin1/G_{12}	0.00 ± 0.19	1.00	0.19 ± 0.27	1.56	0.10 ± 0.27	1.25
βarrestin1/βarrestin2	0.00 ± 0.19	1.00	0.11 ± 0.17	1.29	-0.04 ± 0.28	0.91
PKC/EGFR	0.00 ± 0.09	1.00	0.29 ± 0.28	1.94	-0.21 ± 0.38	0.62

$\Delta\Delta\log(\tau/K_A)$ and BF values were calculated as described in the methods. Data are the mean ± SEM of 3-6 independent experiments performed in triplicate.

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Table 7

Bias factors of AT_1 receptor ligands modified at position 8

	AngII		[Ile^8]-AngII		[Sar^{1}Ile^8]-AngII		[Ile^8]-AngIII	
	$\Delta\Delta\log(\tau/K_A)$	BF	$\Delta\Delta\log(\tau/K_A)$	BF	$\Delta\Delta\log(\tau/K_A)$	BF	$\Delta\Delta\log(\tau/K_A)$	BF
βarrestin2/PKC	0.00 ± 0.11	1.00	3.19 ± 0.29	1547.8	3.35 ± 0.29	2252.1	1.54 ± 0.23	34.88
βarrestin1/PKC	0.00 ± 0.17	1.00	2.50 ± 0.43	316.3	3.33 ± 0.43	2147.7	1.78 ± 0.22	60.76
βarrestin2/EGFR	0.00 ± 0.11	1.00	3.02 ± 0.31	1035.3	3.26 ± 0.24	1803.9	0.94 ± 0.28	8.74
βarrestin1/EGFR	0.00 ± 0.17	1.00	2.33 ± 0.44	211.61	3.24 ± 0.40	1720.3	1.18 ± 0.27	15.22
G_{12}/PKC	0.00 ± 0.12	1.00	0.88 ± 0.50	7.62	2.19 ± 0.59	155.00	0.81 ± 0.19	6.42
G_{12}/EGFR	0.00 ± 0.12	1.00	0.71 ± 0.51	5.10	2.09 ± 0.57	124.16	0.21 ± 0.25	1.61
βarrestin2/ G_{12}	0.00 ± 0.14	1.00	2.31 ± 0.50	203.13	1.16 ± 0.61	14.53	0.74 ± 0.27	5.43
βarrestin1/ G_{12}	0.00 ± 0.19	1.00	1.62 ± 0.59	41.52	1.14 ± 0.69	13.86	0.98 ± 0.25	9.47
PKC/EGFR	0.00 ± 0.09	1.00	0.17 ± 0.31	1.49	0.10 ± 0.18	1.25	0.60 ± 0.21	3.99
βarrestin2/ βarrestin1	0.00 ± 0.19	1.00	-0.69 ± 0.43	0.20	-0.02 ± 0.5	0.95	0.24 ± 0.29	1.74
βarrestin1 /Gq	0.00 ± 0.19	1.00	ND	ND	ND	ND	ND	ND
βarrestin2/Gq	0.00 ± 0.14	1.00	ND	ND	ND	ND	ND	ND
G_{12}/Gq	0.00 ± 0.14	1.00	ND	ND	ND	ND	ND	ND
PKC/Gq	0.00 ± 0.12	1.00	ND	ND	ND	ND	ND	ND
EGFR/Gq	0.00 ± 0.12	1.00	ND	ND	ND	ND	ND	ND

$\Delta\Delta\log(\tau/K_A)$ and BF values were calculated as described in the methods. Data are the mean ± SEM of 3-6 independent experiments performed in triplicate. ND, Cannot be determined.

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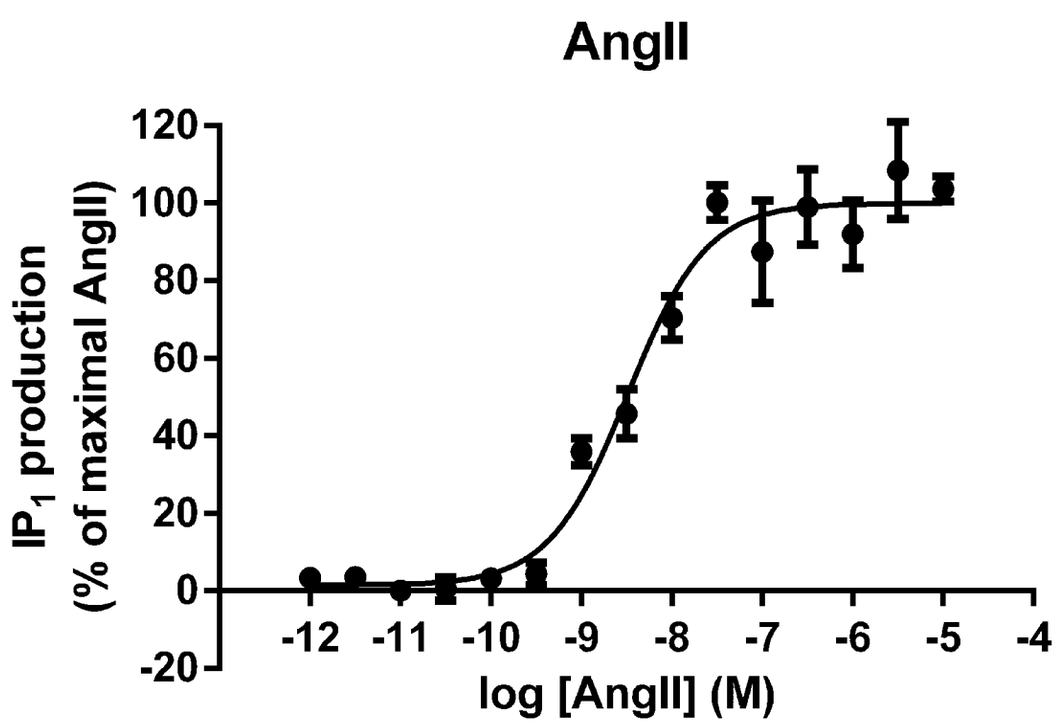
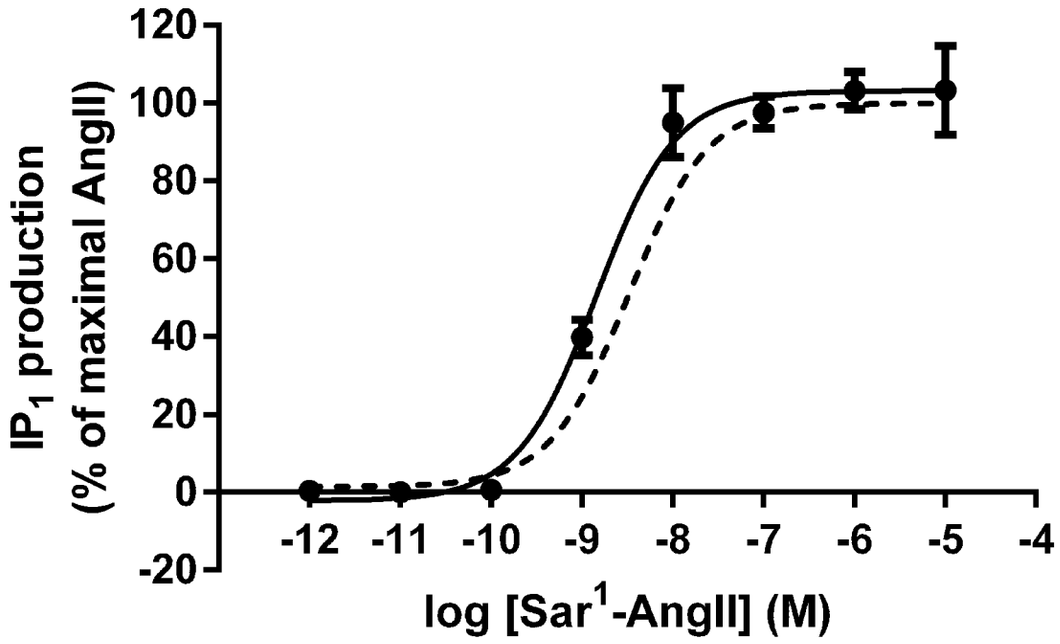
Figure 1





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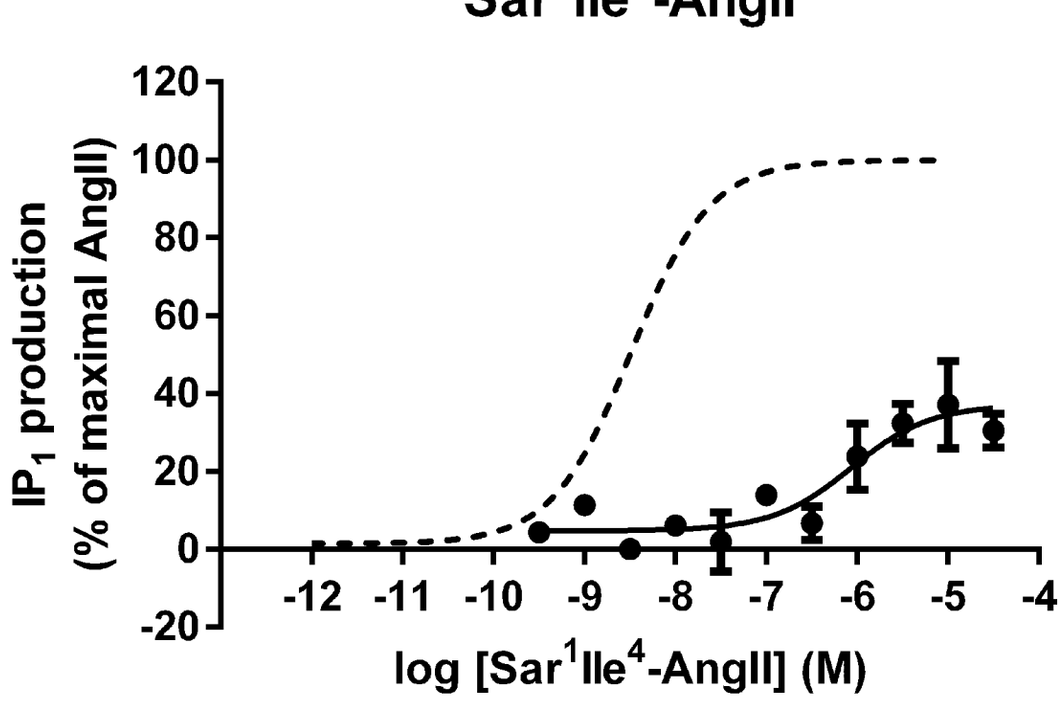

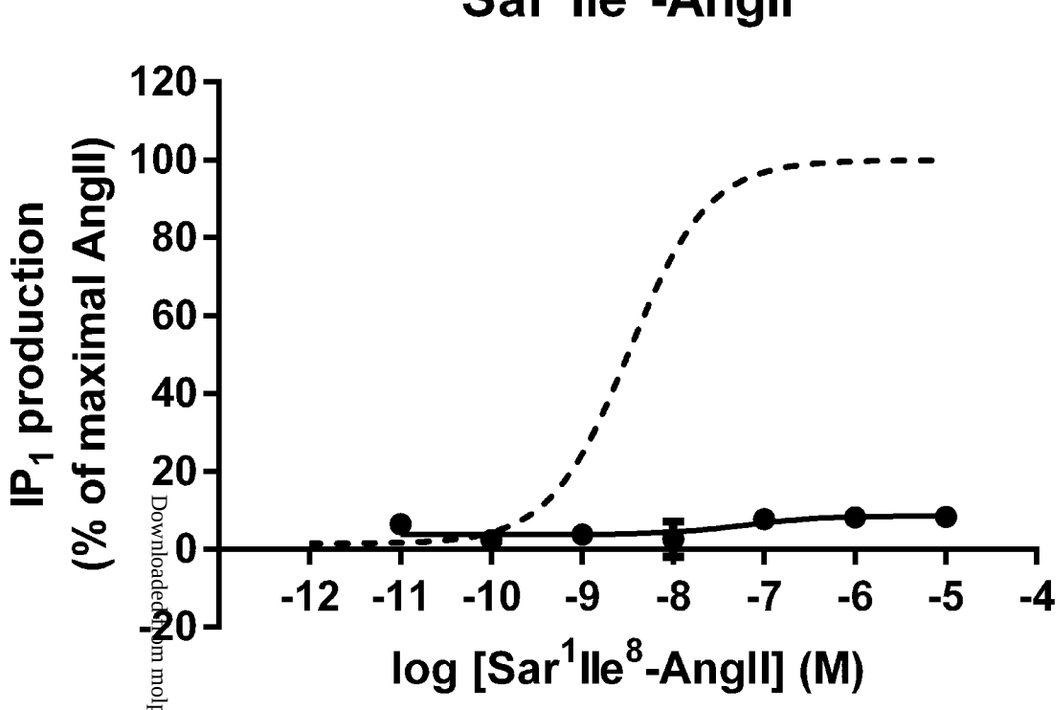

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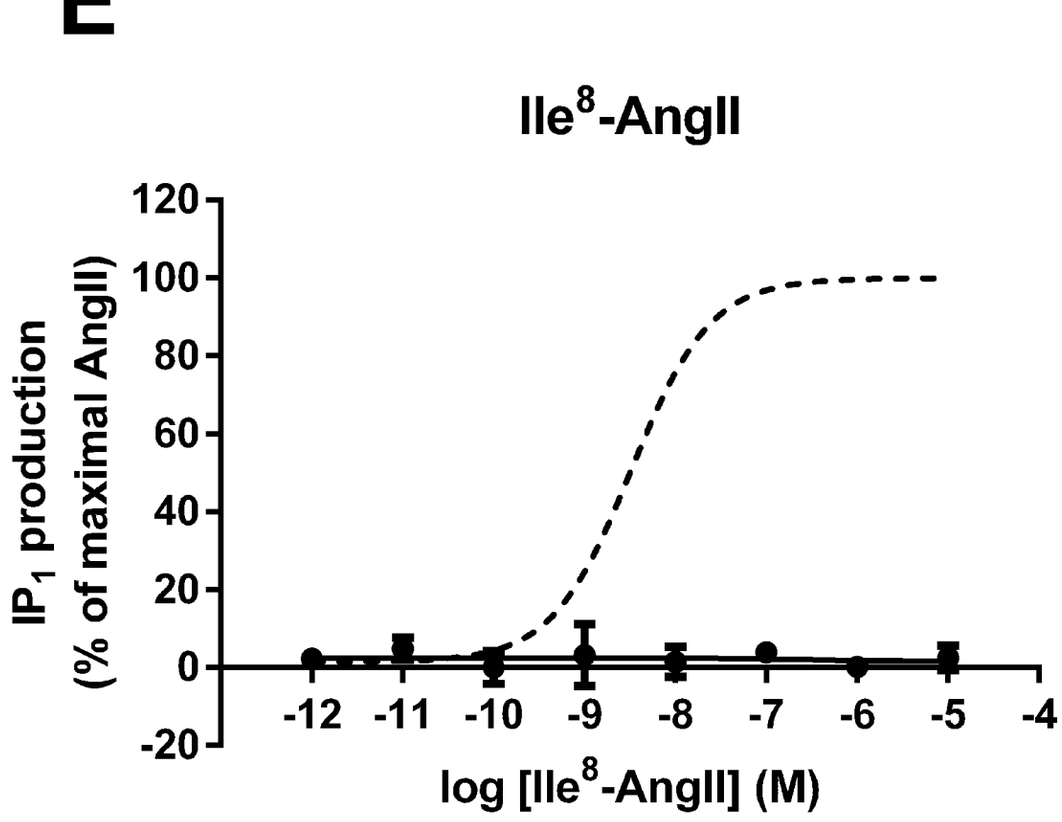

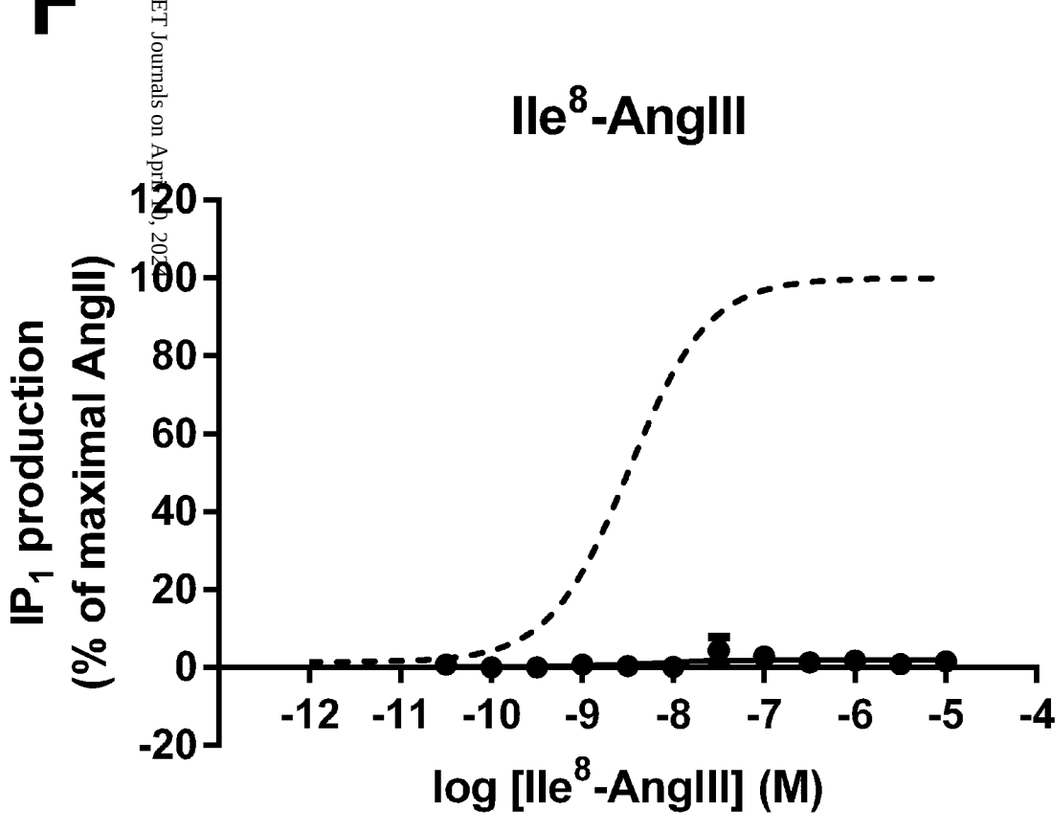

Figure 2

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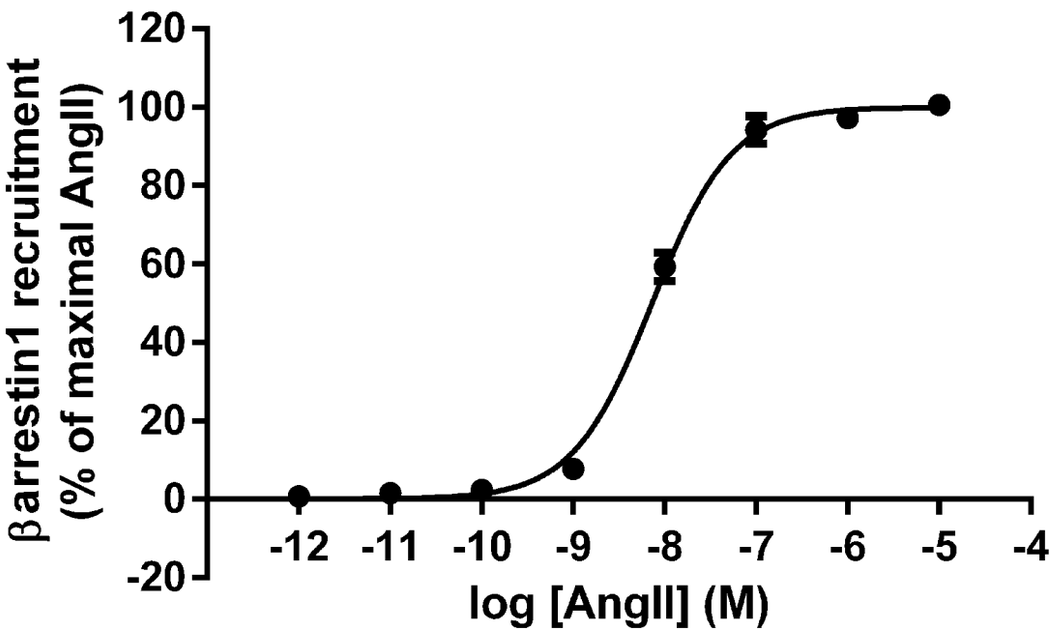

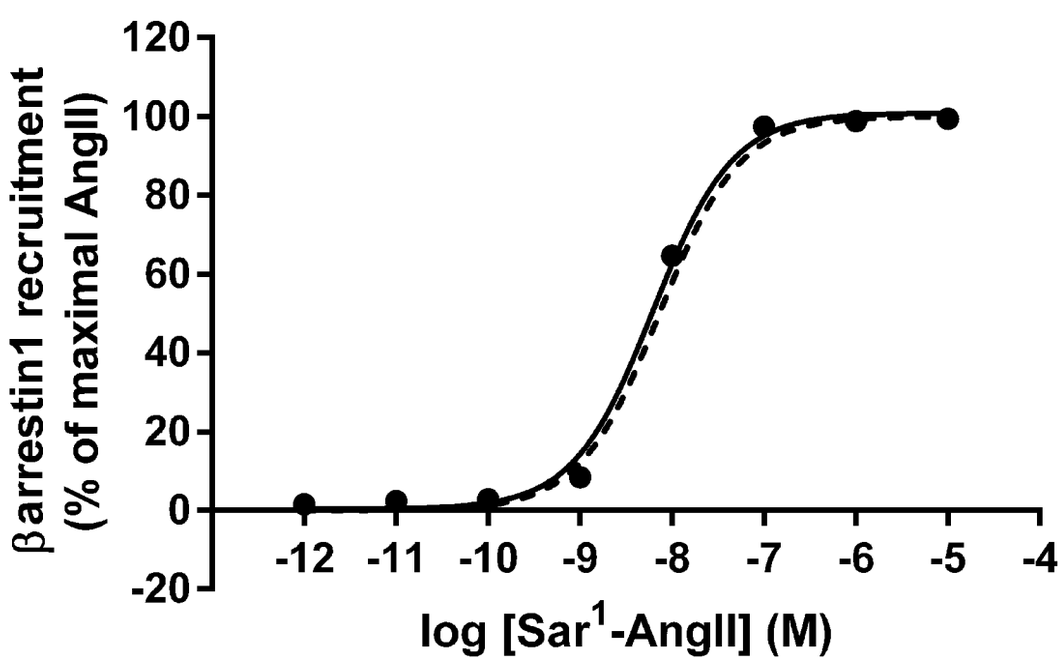

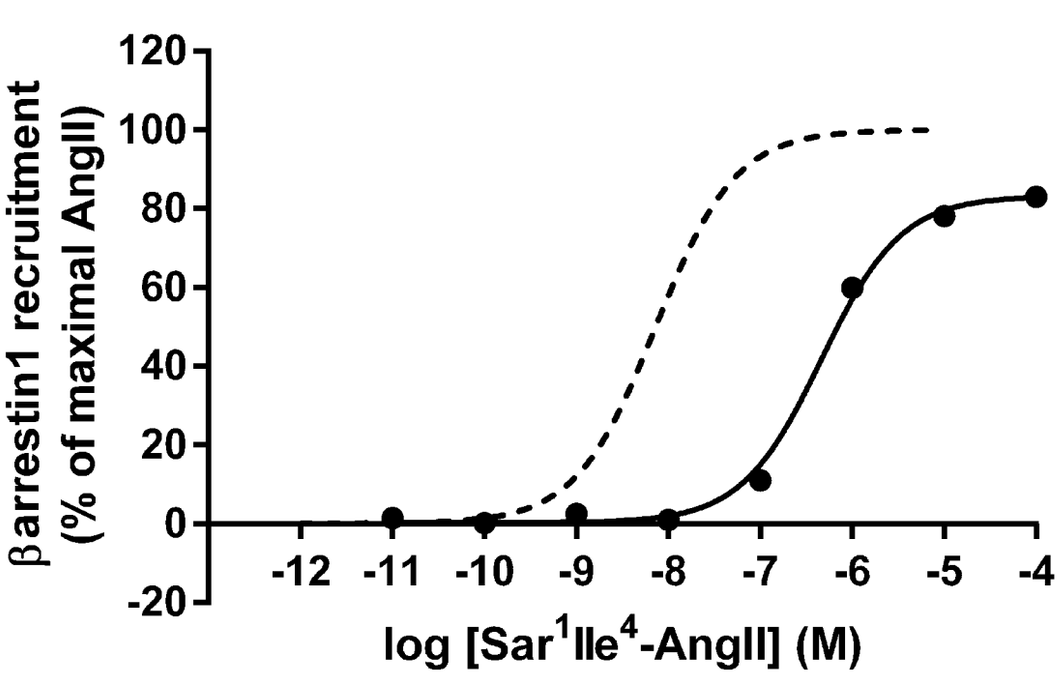

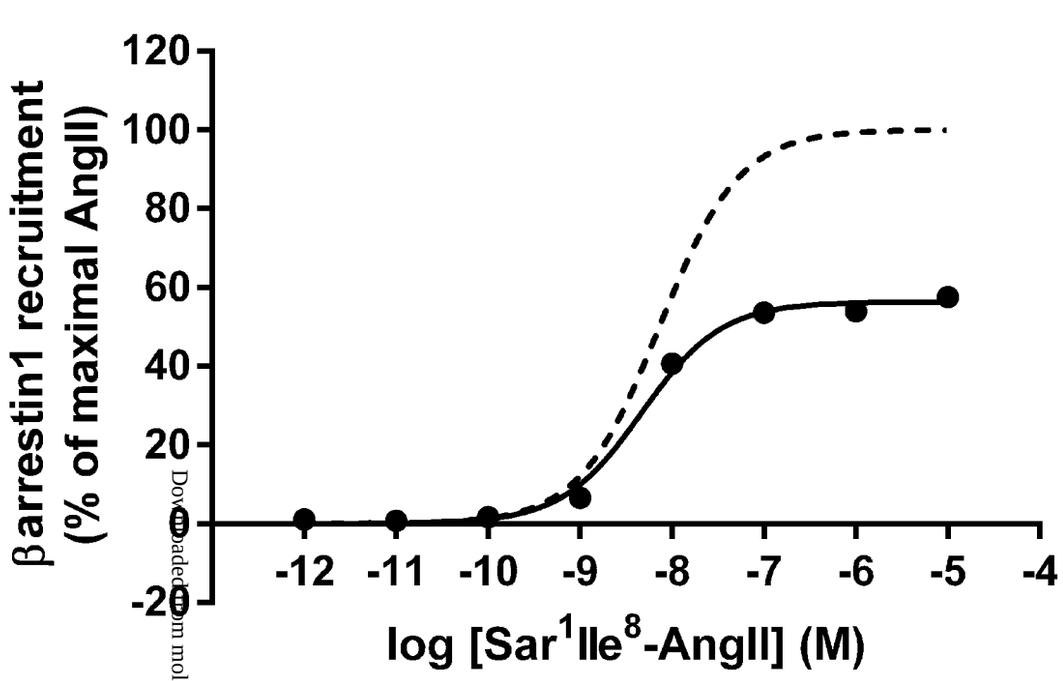

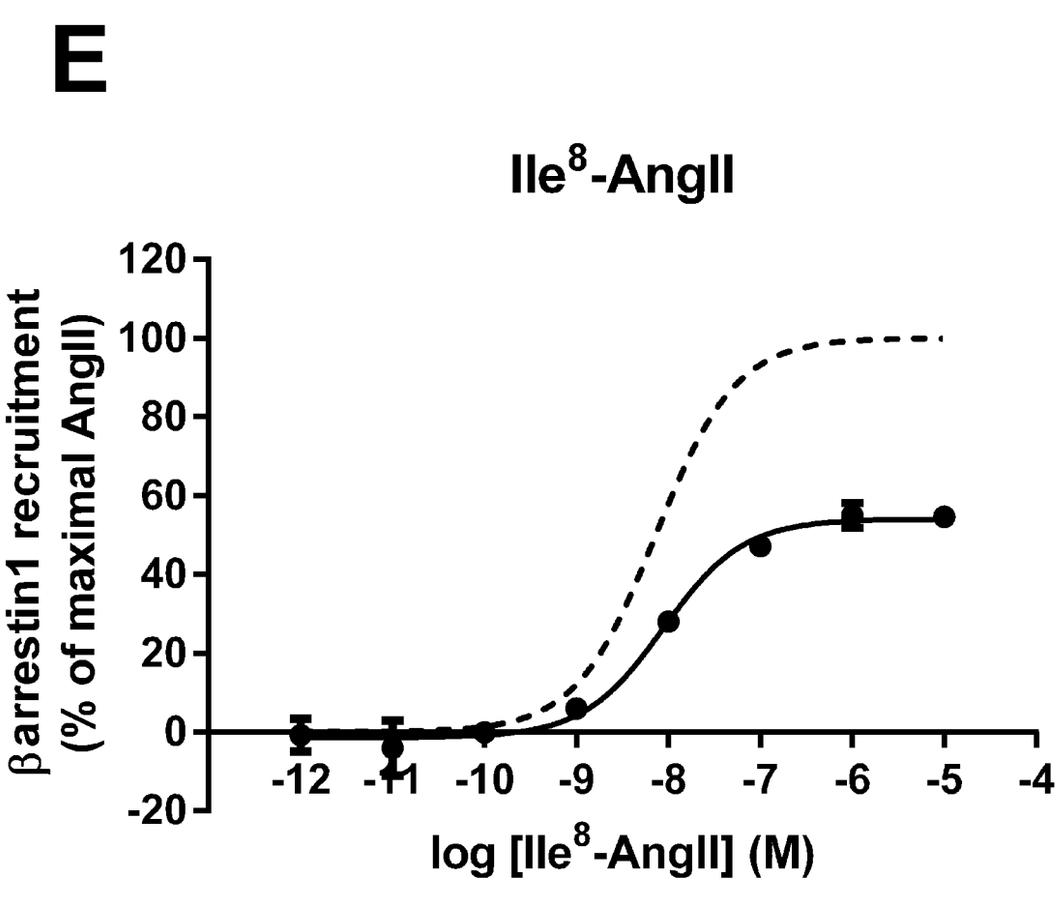

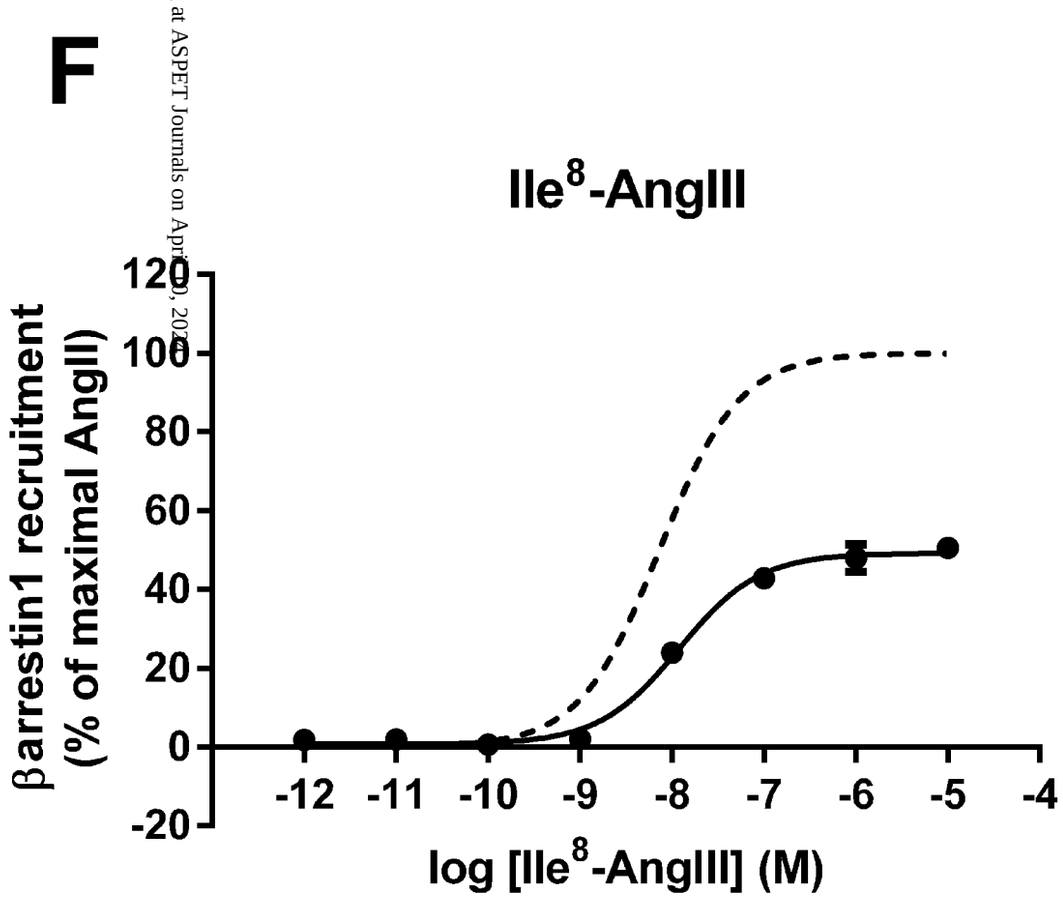

Figure 3

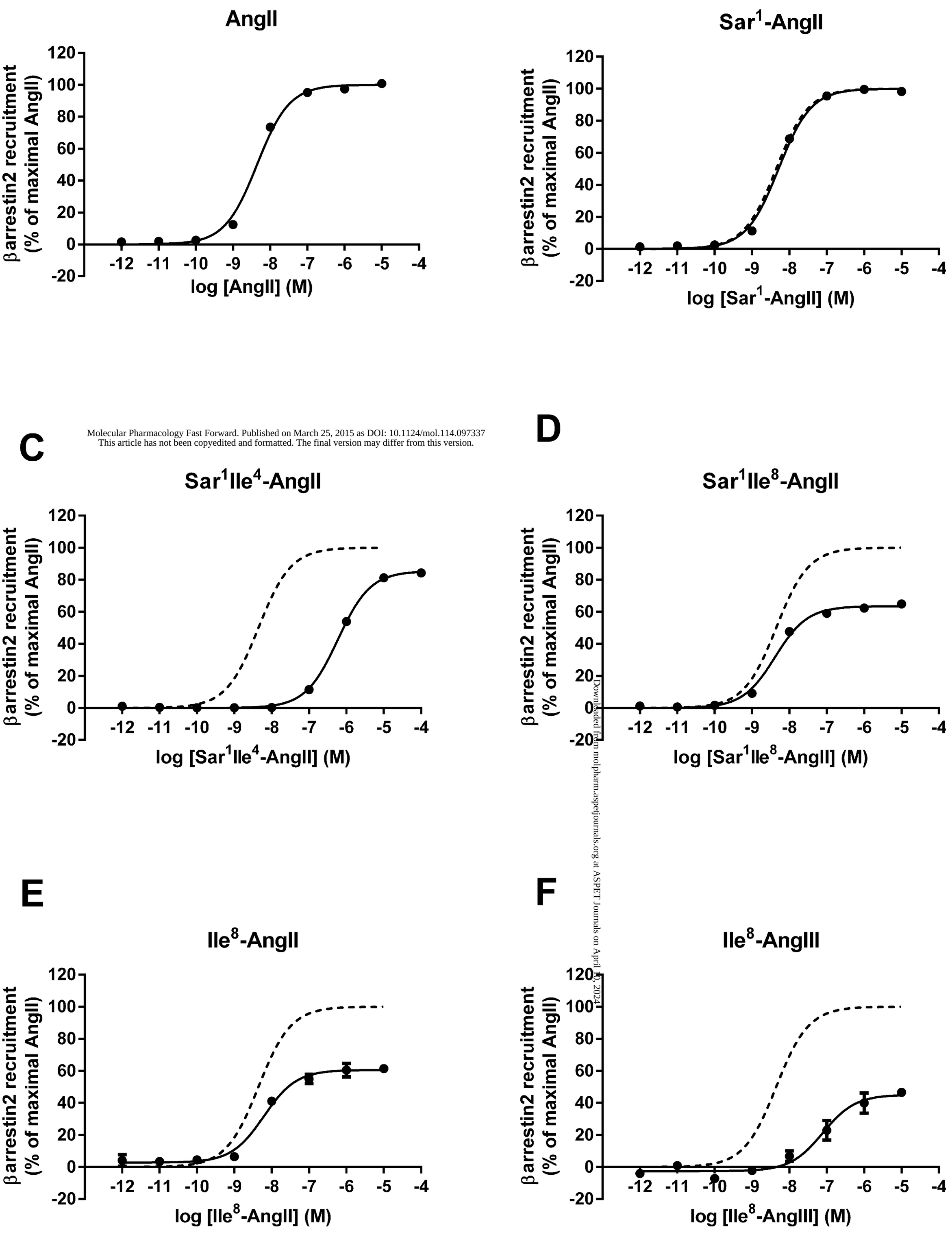

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Figure 4

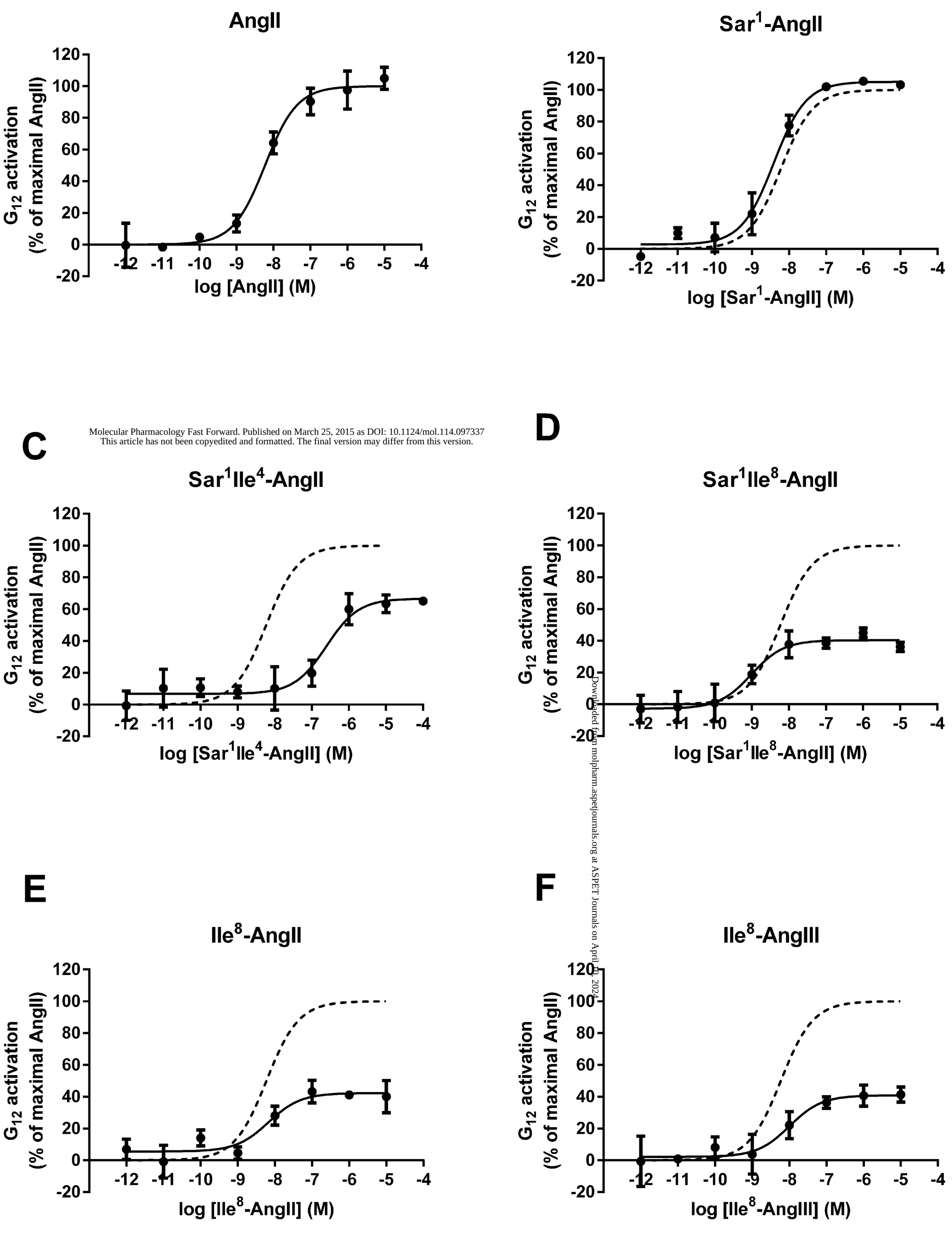

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Figure 5

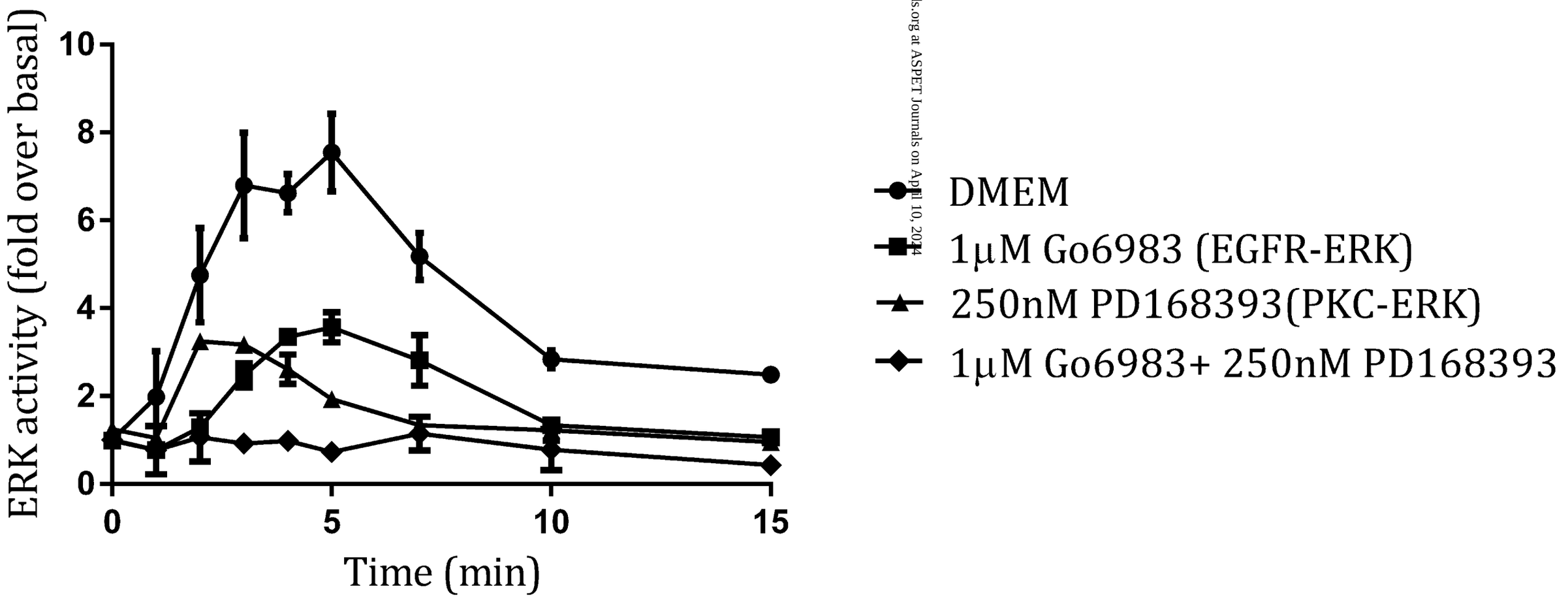

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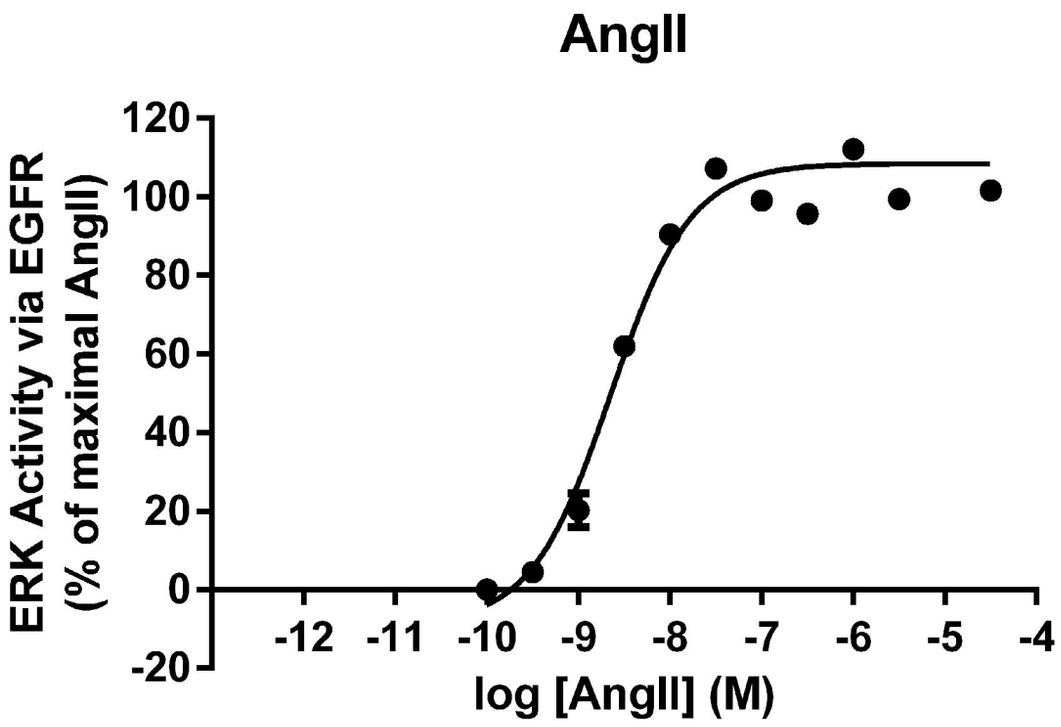
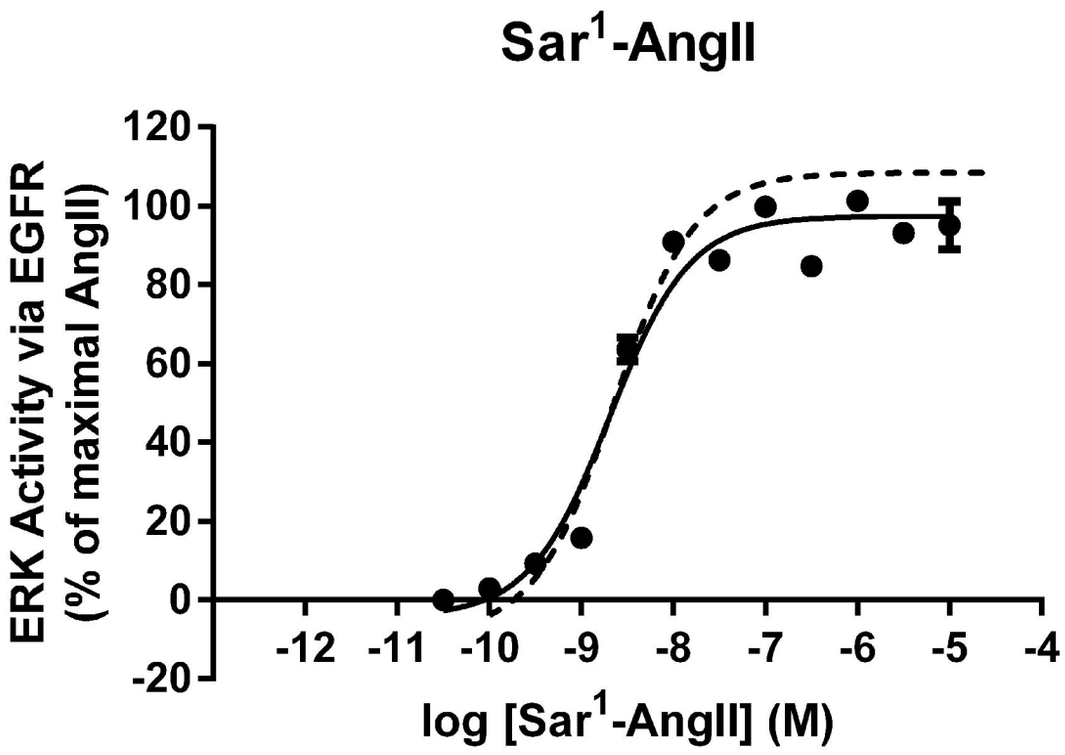
Figure 6





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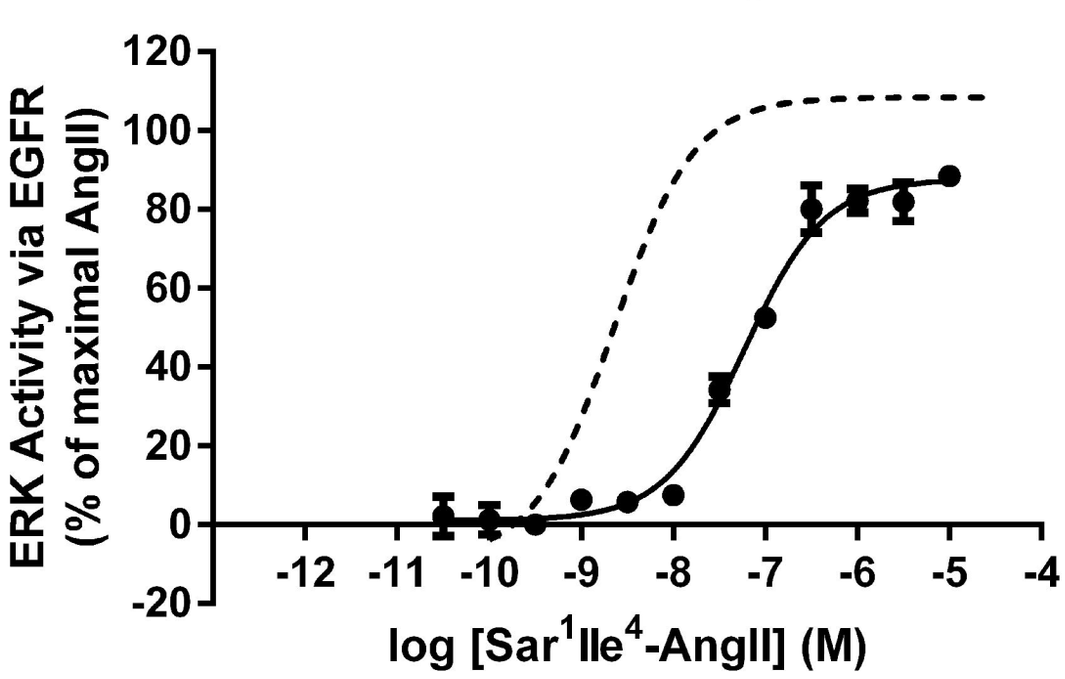

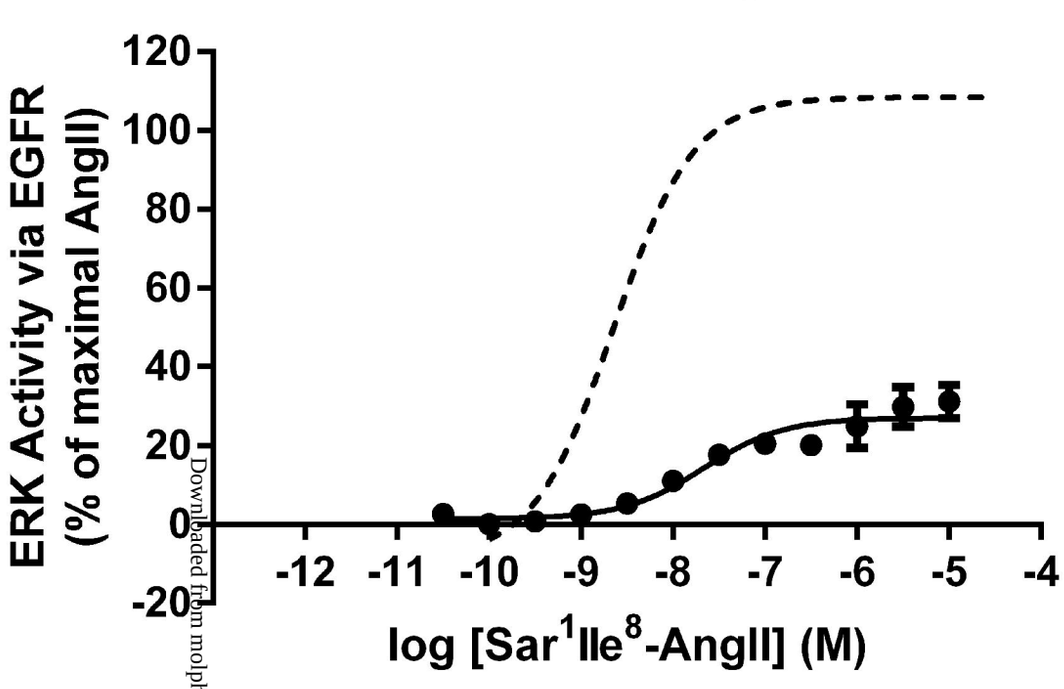

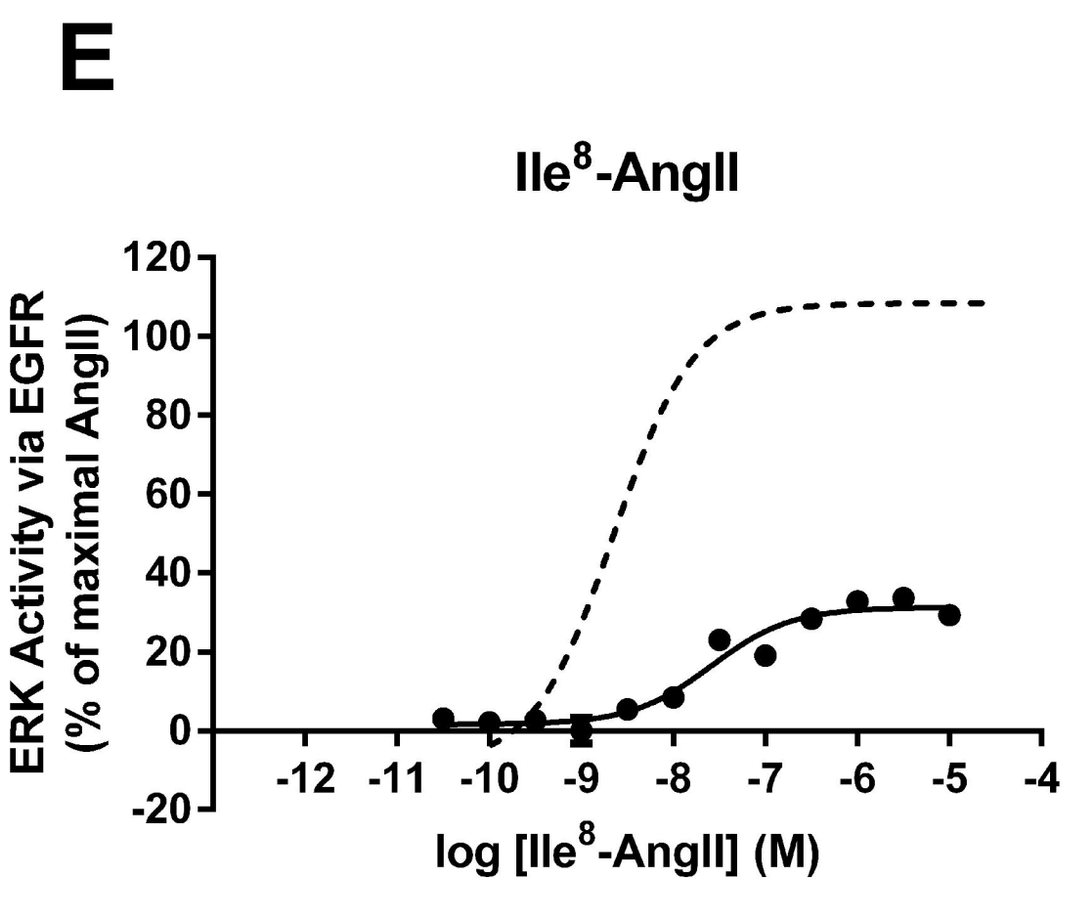

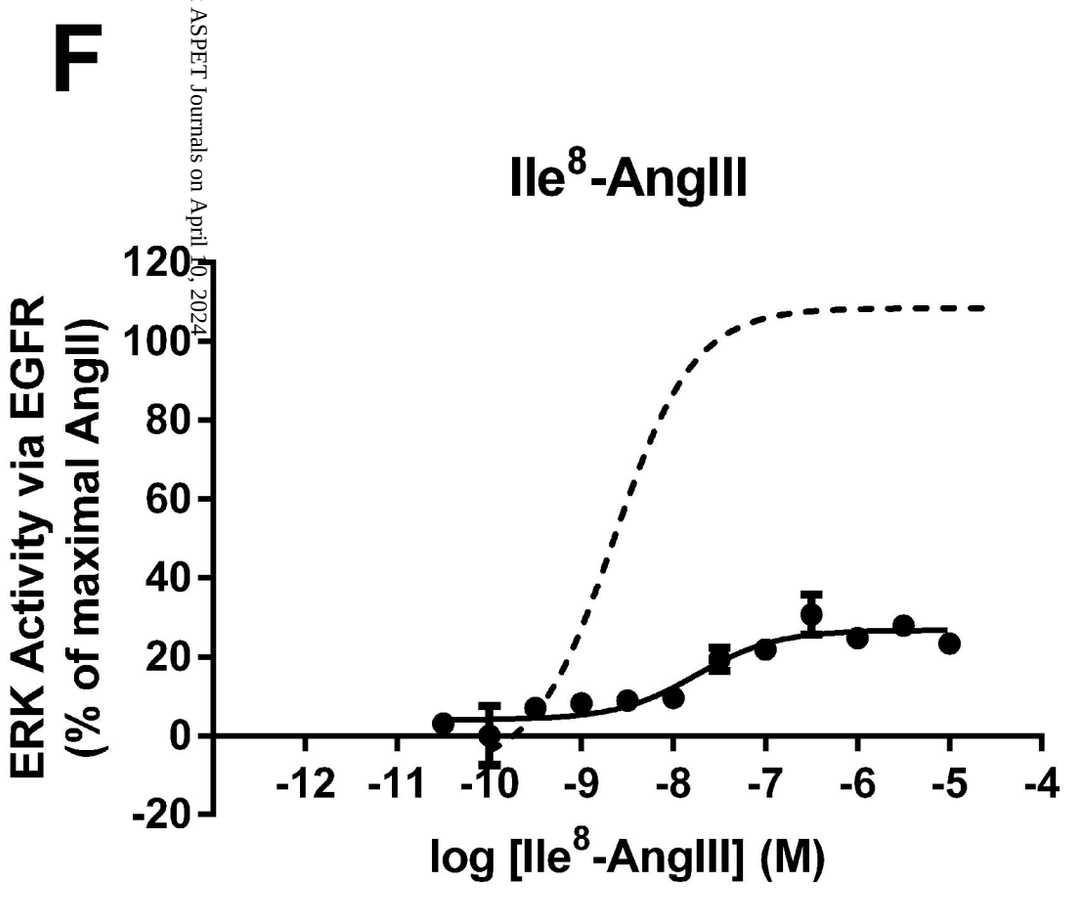

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Figure 7

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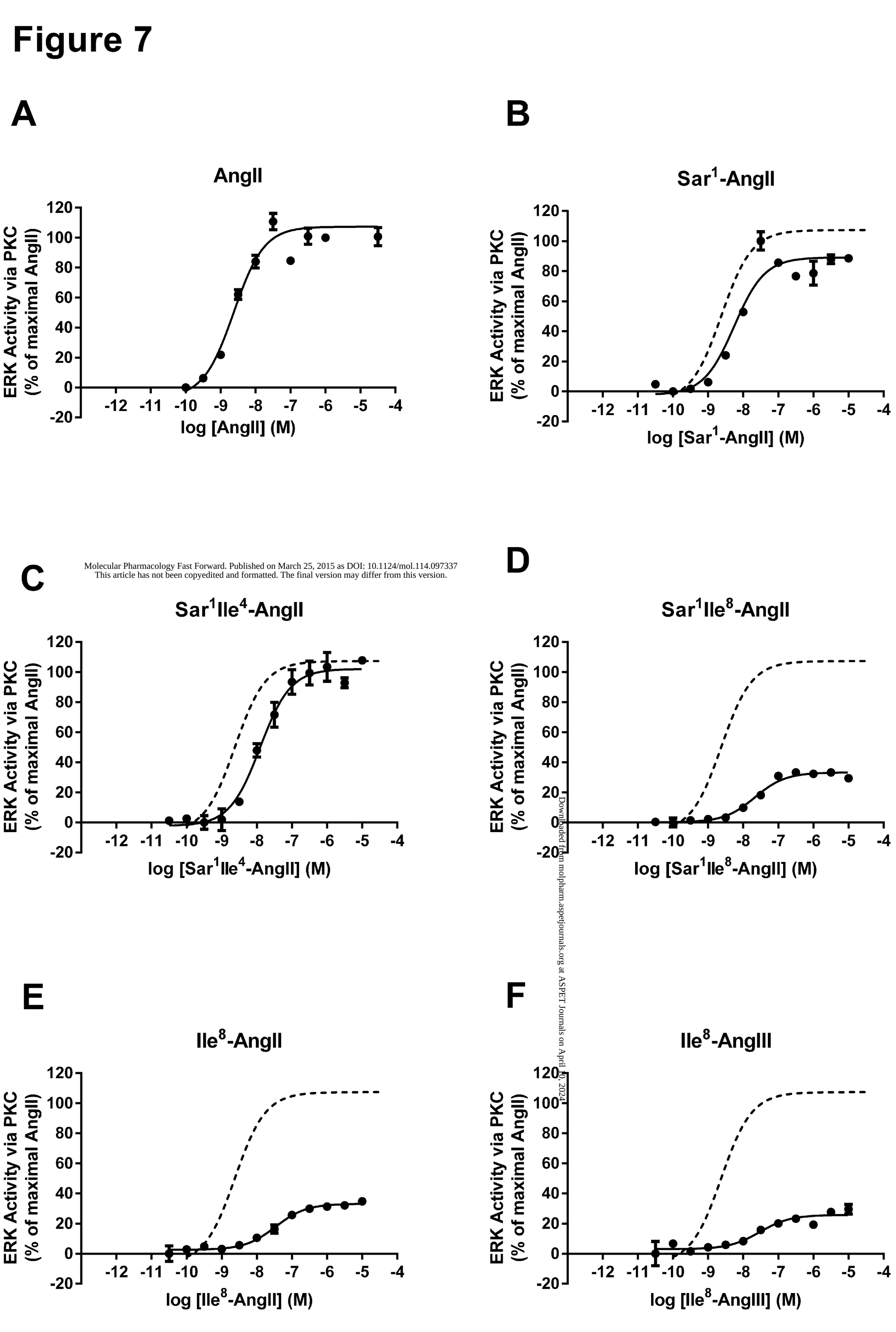


Figure 8

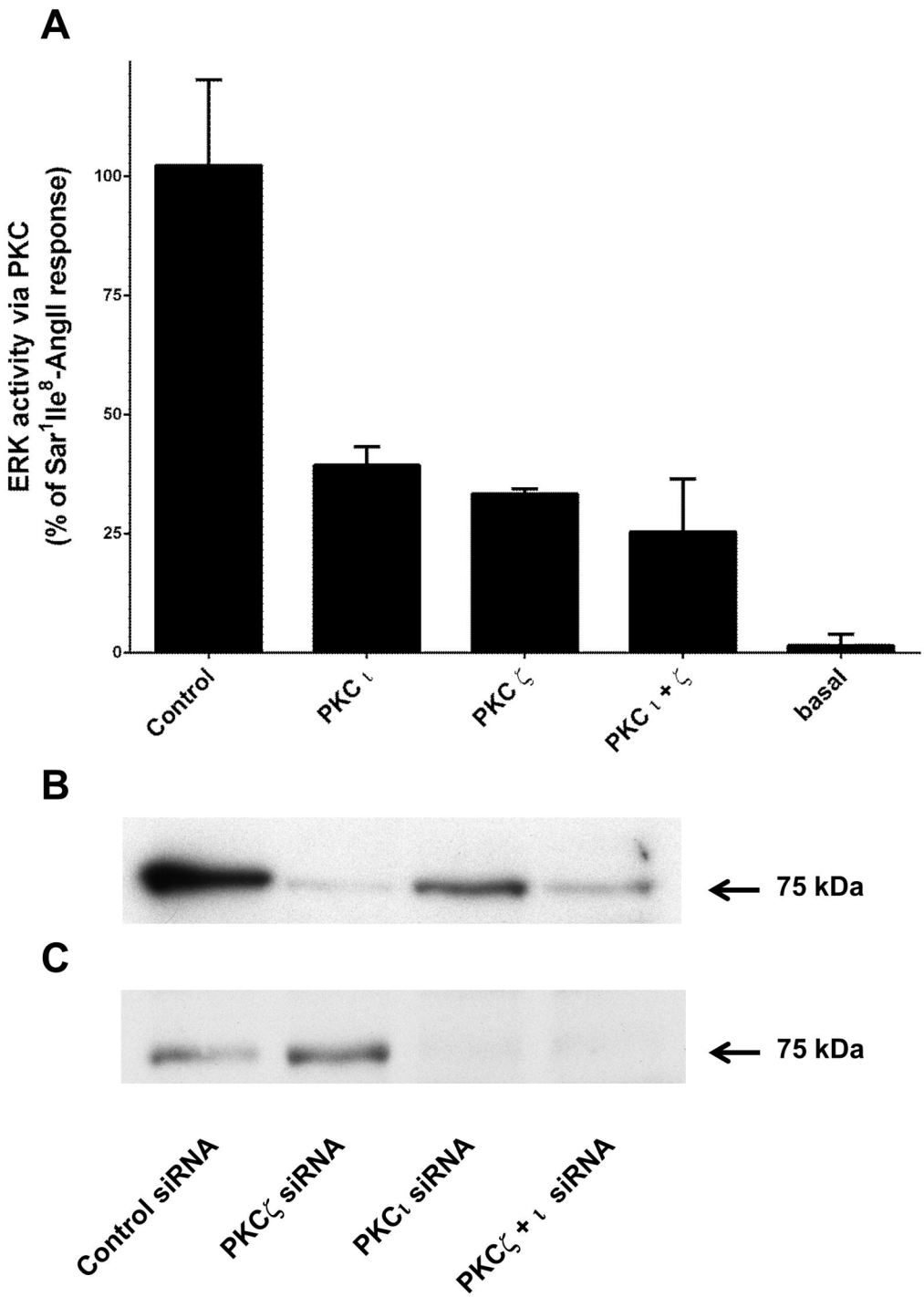
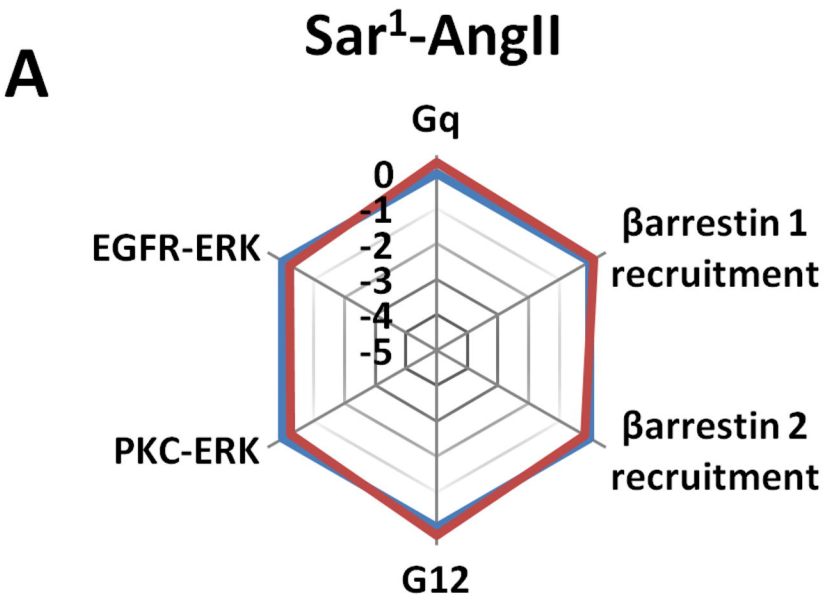
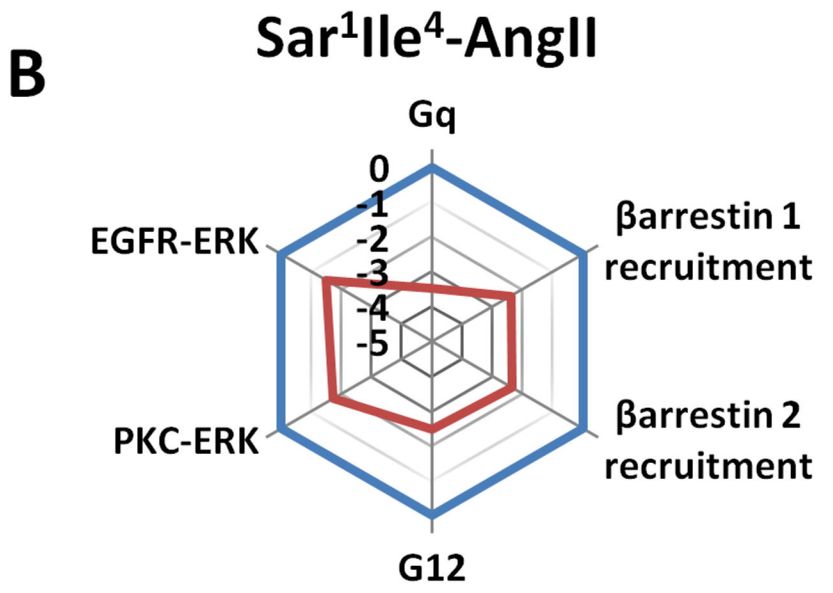
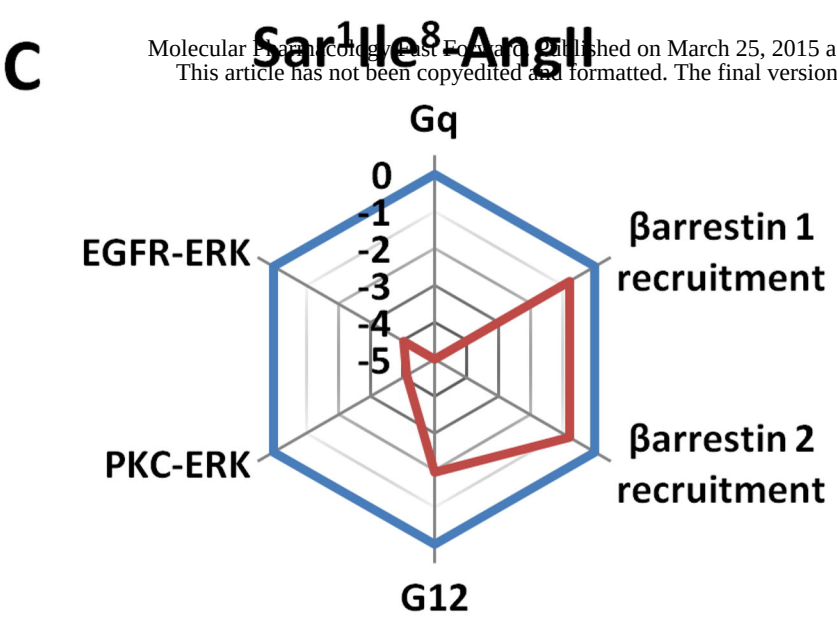
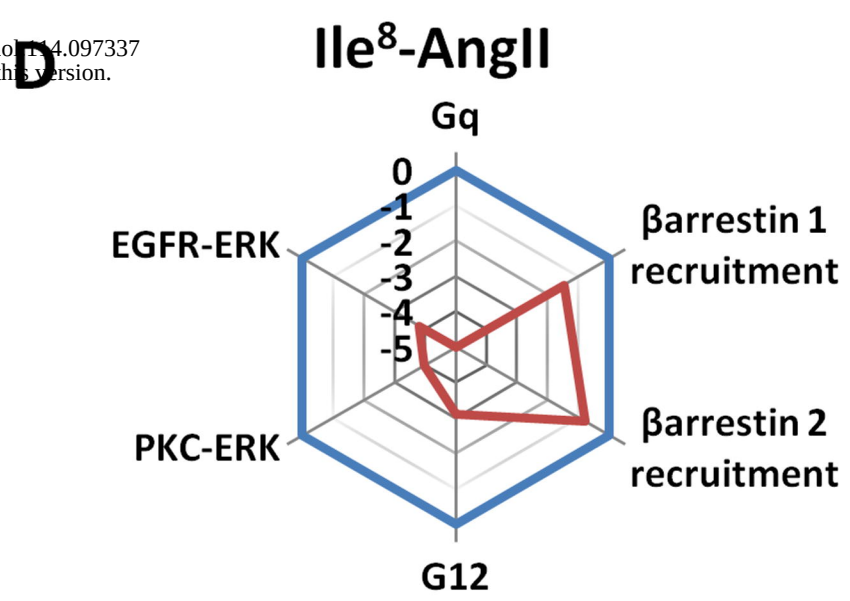

A
ERK activity via PKC
(% of Sar[1]Ile[8]-AngII response)
100
75
50
25
0
Control
PKC ι
PKC ζ
PKC ι + ζ
basal
B
75 kDa
C
75 kDa
Control siRNA
PKCζ siRNA
PKCι siRNA
PKCζ + ι siRNA

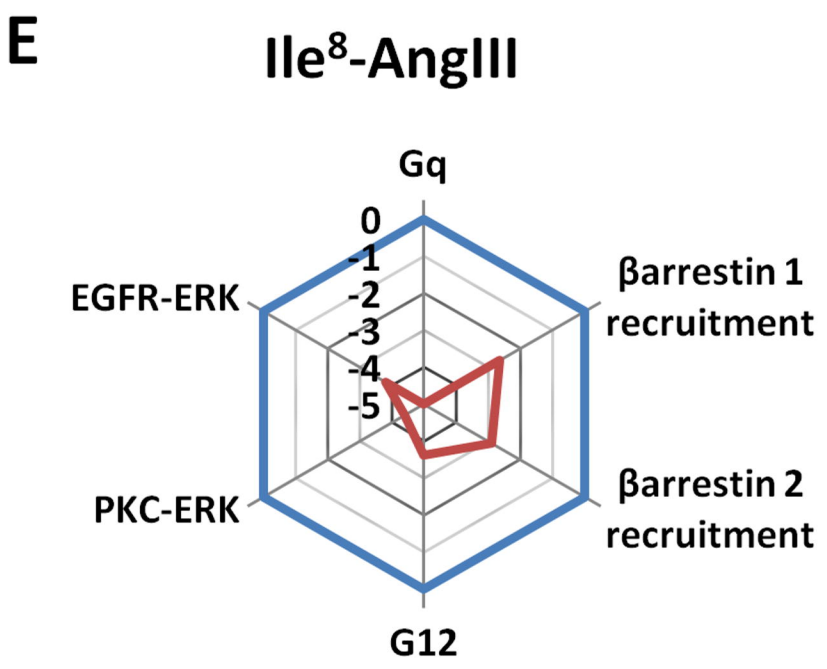
Figure 9











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