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Supplementary Materials for

Differential β-Arrestin–Dependent Conformational Signaling and Cellular Responses Revealed by Angiotensin Analogs

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Published 24 April 2012, *Sci. Signal.* **5**, ra33 (2012)
DOI: 10.1126/scisignal.2002522

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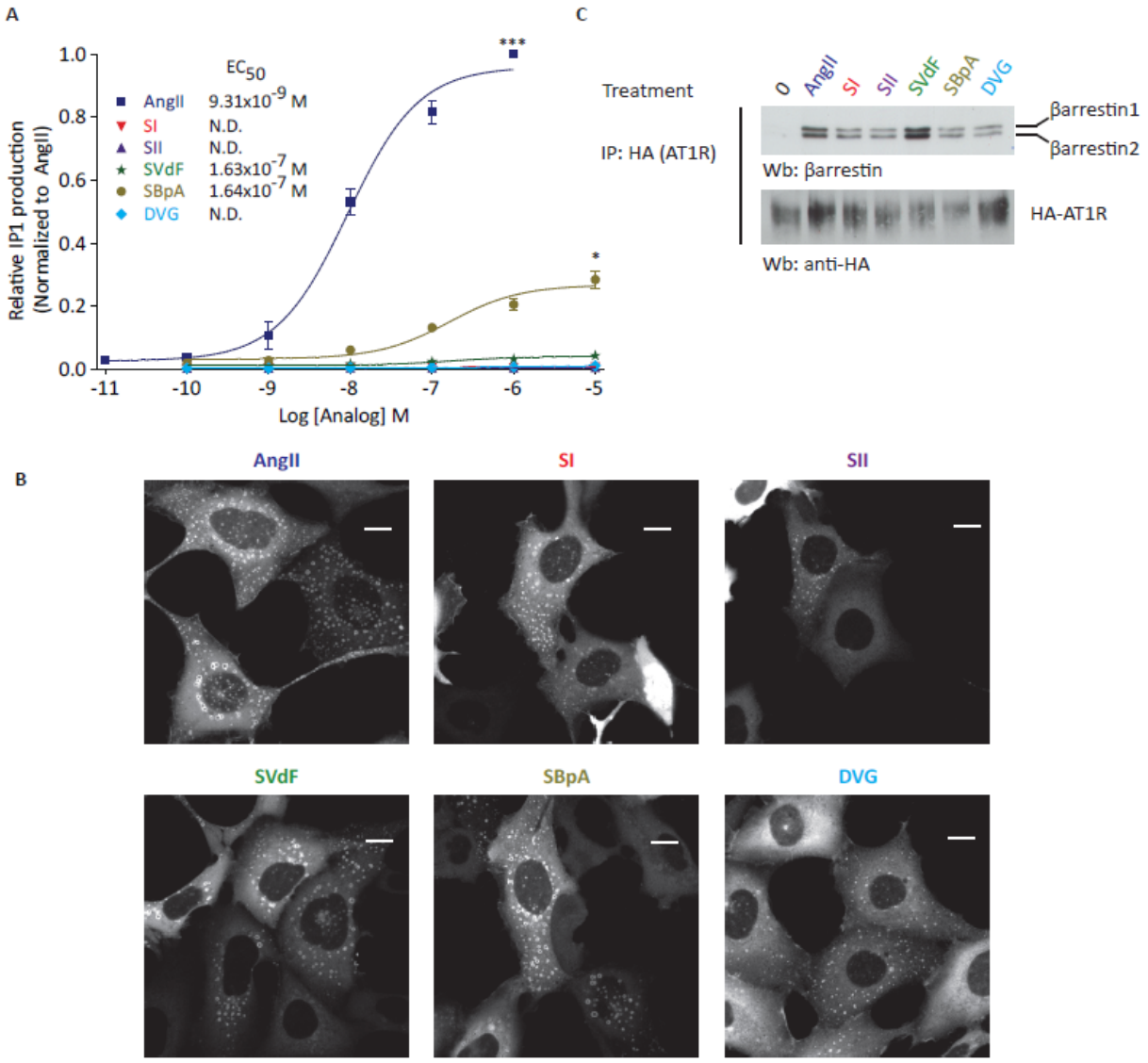

Figure S1. Analog-induced production of IP_1 and recruitment of β-arrestin as analyzed by confocal microscopy and immunoprecipitation.

A, HEK293 cells stably expressing HA-AT1R were used to measure IP_1 production downstream of the AT1R using CisBio's IP-one HTRF assay. Cells were treated with increasing concentrations of AngII or the analogs. AngII-induced production of IP_1 was set as the maximum. Data is representative of 3 independent experiments performed in duplicate. Statistical analysis was performed on maximal ligand concentration using a one-way ANOVA followed by a Dunnett's post-hoc test with untreated as control. *, $p<0.05$, ***, $p<0.001$. N.D. = Not determined. B, HA-AT1R cells transfected βarr2-YFP were stimulated with AngII (1 µM) or analogs (10 µM) for 15 min. Images are representative of 3 independent experiments. Scale bars are indicative of 10 µm. C, HEK293 cells stably expressing HA-AT1R were stimulated for 15 min with either 1 µM AngII or 10 µM analog. Receptor immunoprecipitates were immunoblotted for β-arrestin and AT1R. Representative blot is shown. n=3.

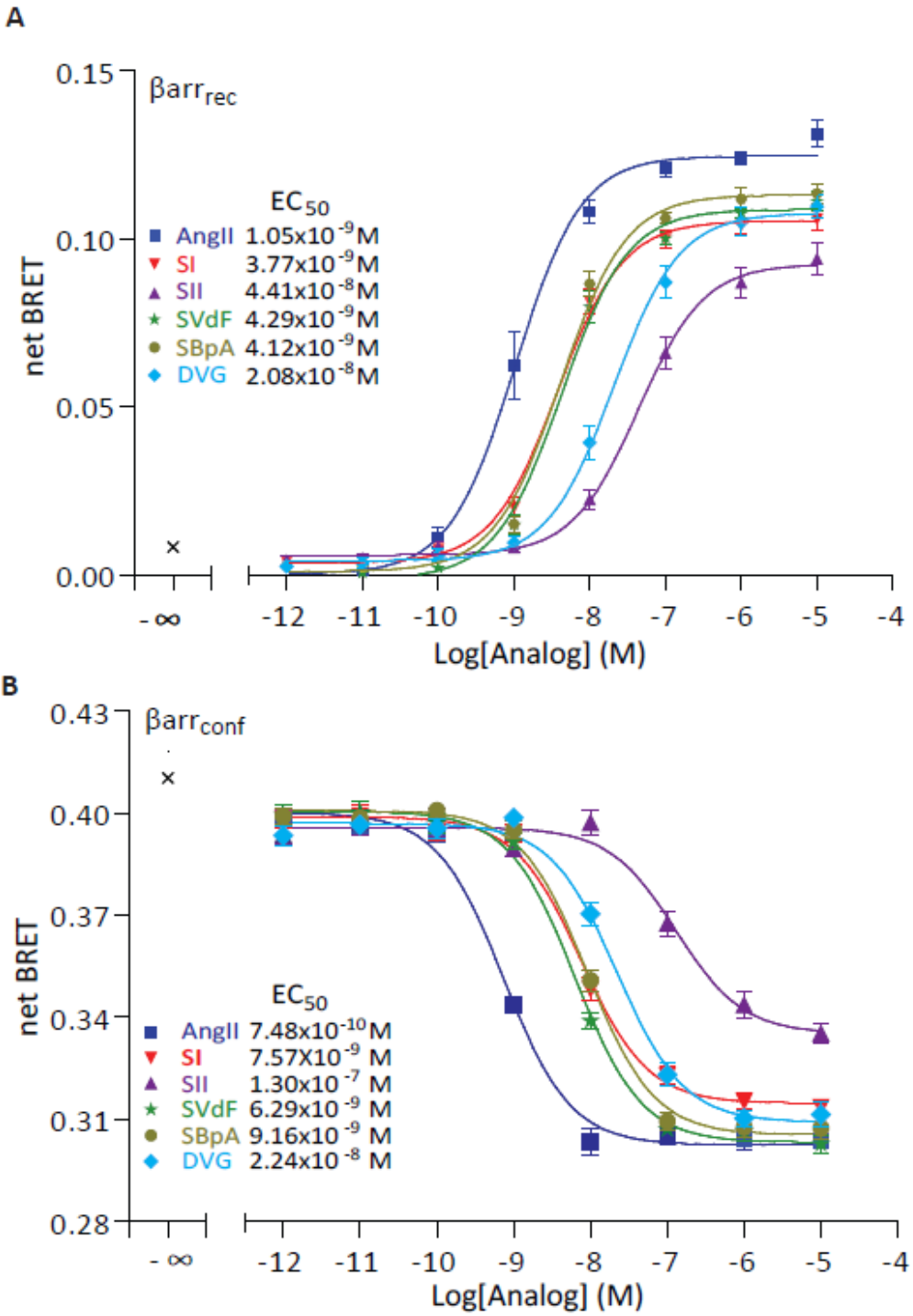

Fig. S2. Recruitment of β-arrestin to AT1R and conformational changes induced by AngII analogs.

BRET analysis showing dose-dependent agonist-promoted increased recruitment and conformational change of βarrestin to AT1R. A, Cells transfected with AT1R-YFP and βarr1-RlucII were stimulated with increasing concentrations of AngII and analogs for 25 min. The "x" represents the baseline signal from vehicle-treated cells. Data are the mean ± S.E.M. of 3 independent experiments. Curves were fitted using nonlinear regression. B, Dose-dependent agonist-promoted decrease of β-arrestin intramolecular BRET. As in A, but cells were transfected with HA-AT1R and GFP10-βarr1-RlucII.

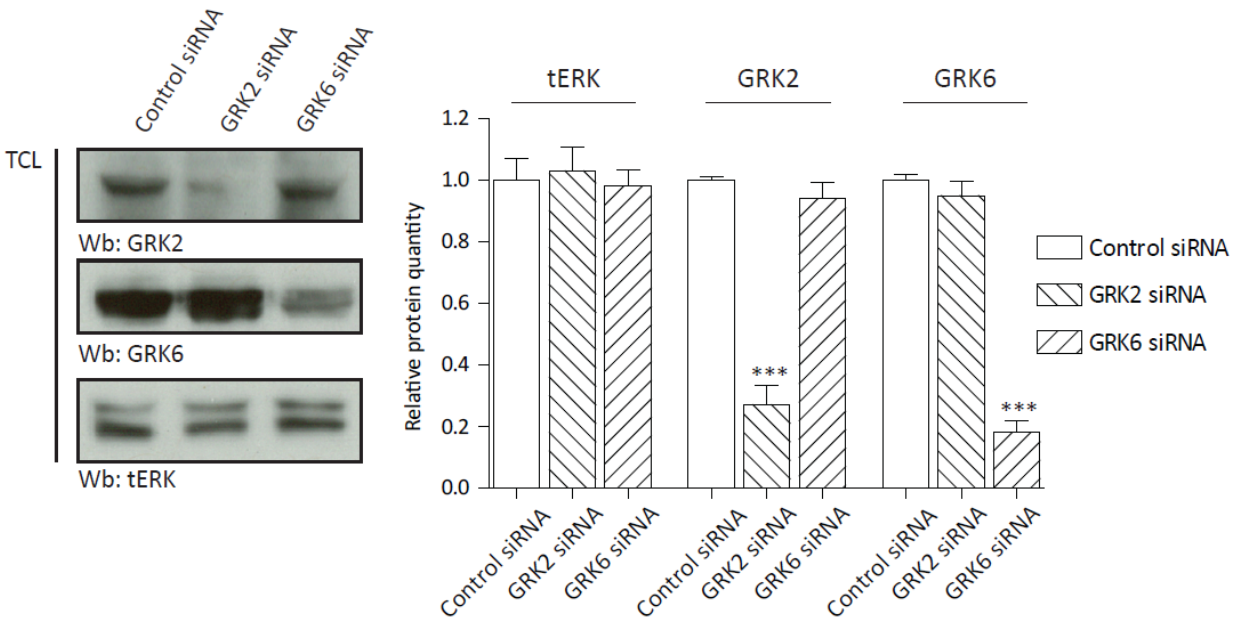

Figure S3. siRNA-mediated knockdown of GRK2 and GRK6.

Representative blot of GRK2 and GRK6 knockdown. Quantification of 3 independent experiments from cells used in BRET data presented in Fig. 3. Total ERK (tERK) was used as the loading control. One-way ANOVA followed by a Dunnett's post-hoc test was performed. ***, $p<0.001$

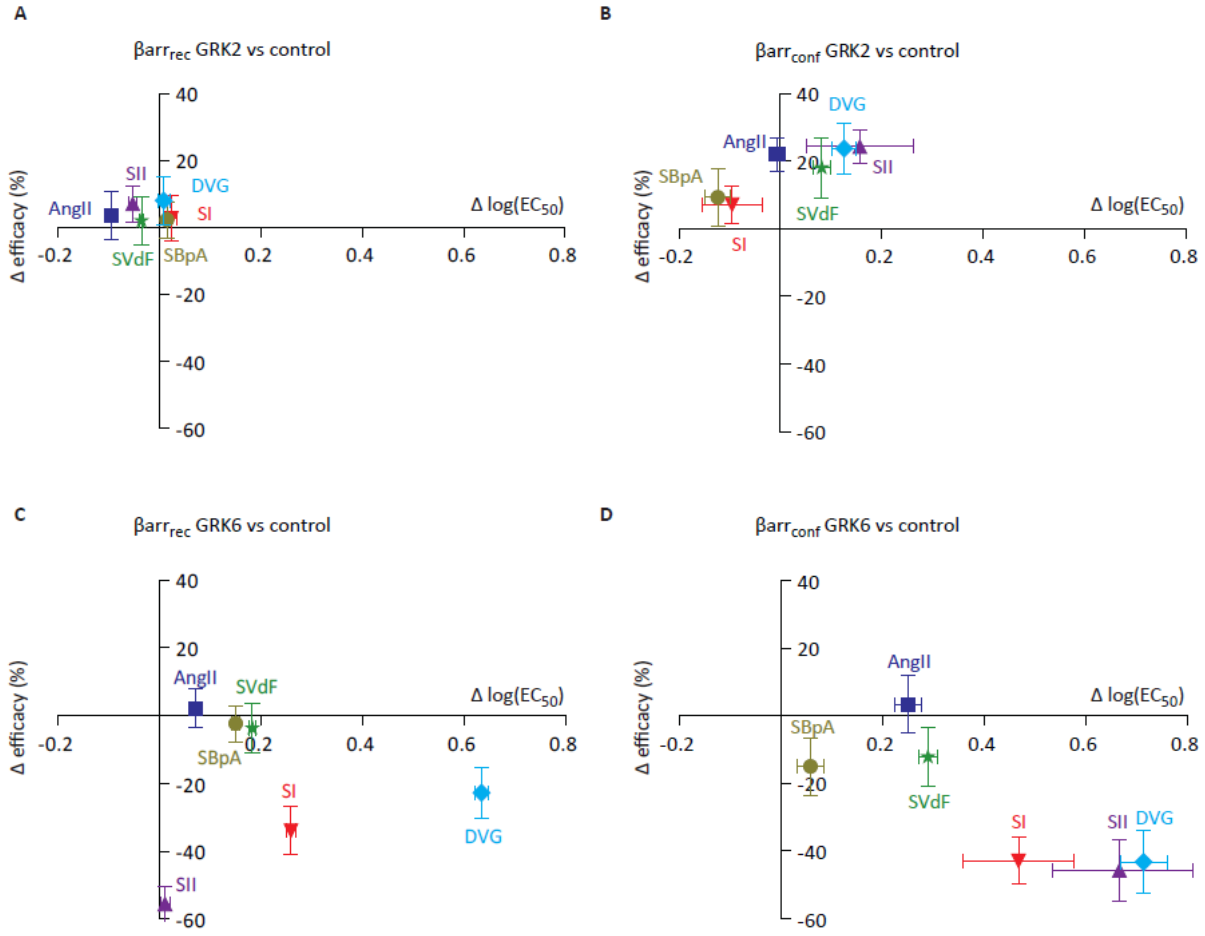

Figure S4. Role of GRK2 and GRK6 in the efficacy and potency on ligand-induced β-arrestin recruitment and conformational change.

The log(EC50) and the efficacy of the analogs to induce β-arrestin recruitment (A) or conformational change (B) were subtracted between the GRK2 knockdown and control conditions. The data are presented as the change in potency and efficacy. The same analysis was performed in (C) and (D) respectively, in reference to GRK6 depleted cells compared to control cells.

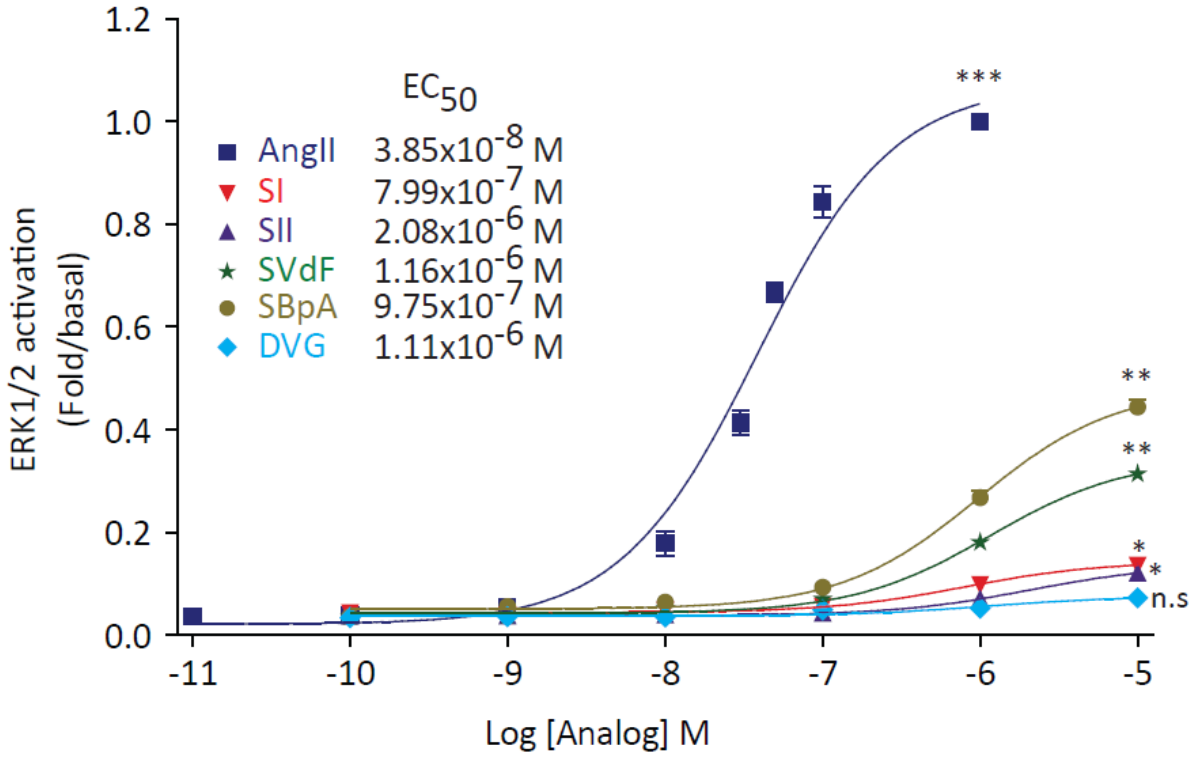

Figure S5. Dose-response relationship of ERK1/2 activation by AngII and analogs.

Perkin Elmer's AlphaScreen technology was used to measure ERK1/2 activation triggered by increasing concentrations of AngII and the analogs. AngII-mediated activation of ERK1/2 was set as the maximum. Data is representative of 3 independent experiments done in duplicate. Statistical analysis was performed on maximal ligand concentration using a one-way ANOVA followed by a Dunnett's post-hoc test with untreated as control. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$, n.s., non-significant.

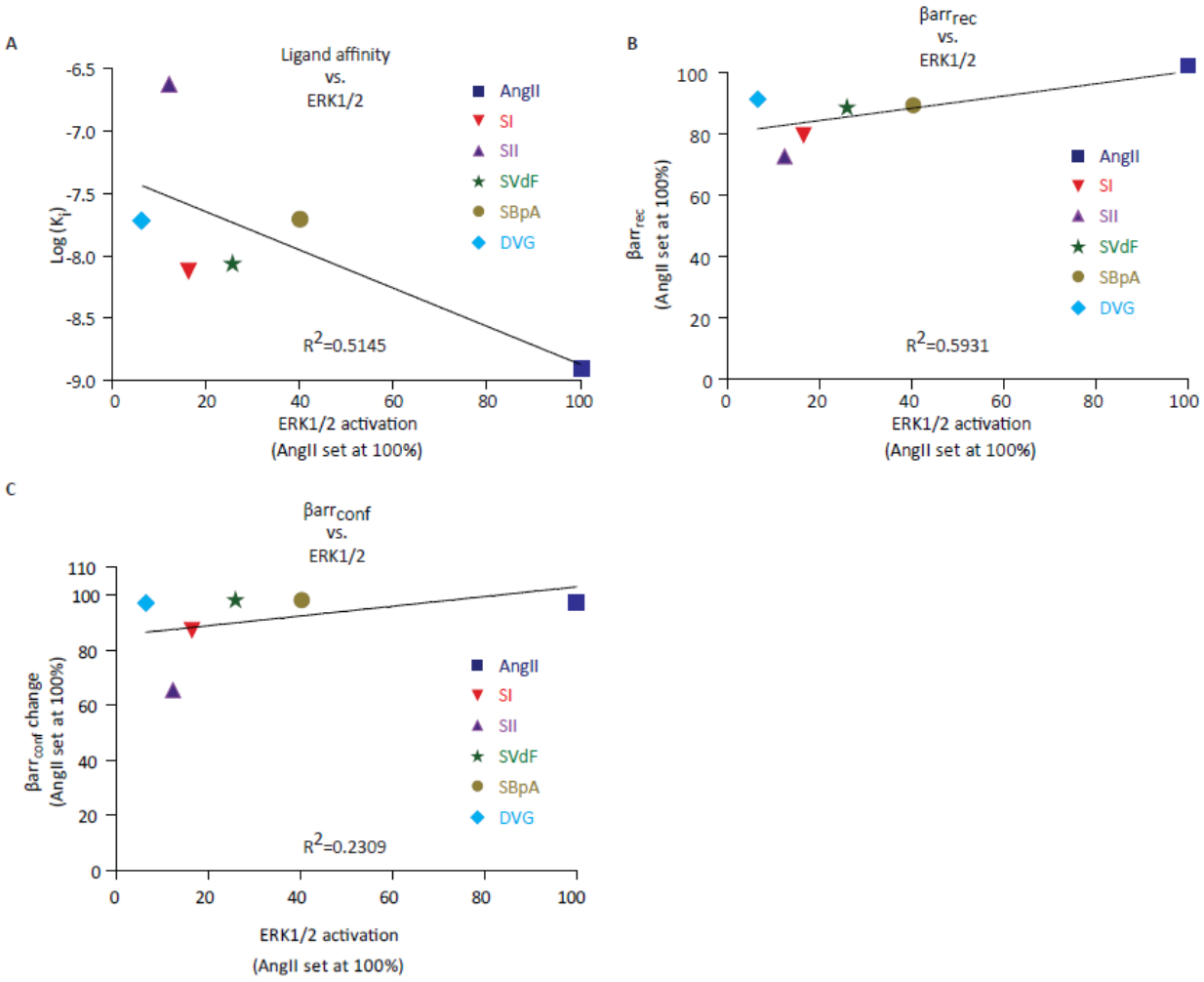

Figure S6. Correlation plots between ligand affinity, BRET values, and ERK1/2 activation.

A, Values of ERK1/2 activation at 15 min stimulation were correlated with the log of the Ki of binding. B, Values of ERK1/2 activation at 15 min stimulation were correlated with the efficacy of βarrestin recruitment to AT1R by BRET. C, Values of ERK1/2 activation at 15 min stimulation were correlated with the efficacy of βarrestin conformational change by BRET.

Table S1. IP_1 dose-response curve data used to calculate σ values and β factors.
n.d. = not determined

Ligand	Emax	LogKi	log(τ)	SEM log τ	R^2
AngII	95.970	-8.938	0.919	0.068	0.981
SI	0.605	-8.155	n.d.	n.d.	0.451
SII	0.499	-6.672	n.d.	n.d.	0.353
SVdF	3.944	-8.101	0.513	0.109	0.852
SBpA	26.710	-7.749	0.863	0.079	0.918
DVG	1.078	-7.763	n.d.	n.d.	0.310

Table S2. BRET dose-response data used to calculate σ values and β factors.

	βarr_{rec}				βarr_{conf}			
	Ligand	log(τ)	SEM log τ	R^2	Ligand	log(τ)	SEM log τ	R^2
Control siRNA	AngII	0.057	0.026	0.993	Ang II	-0.078	0.111	0.804
	SI	0.029	0.040	0.989	SI	-0.555	0.096	0.696
	SII	0.195	0.028	0.983	SII	-0.439	0.075	0.626
	SVdF	0.084	0.013	0.992	SVdF	-0.209	0.104	0.848
	SBpA	0.318	0.024	0.994	SBpA	-0.151	0.094	0.833
	DVG	-0.193	0.060	0.987	DVG	-0.391	0.144	0.840
GRK2 siRNA	AngII	0.148	0.027	0.994	Ang II	-0.055	0.045	0.900
	SI	-0.076	0.025	0.984	SI	-0.492	0.063	0.778
	SII	0.238	0.037	0.953	SII	-0.486	0.107	0.601
	SVdF	0.119	0.024	0.988	SVdF	-0.287	0.085	0.860
	SBpA	0.301	0.020	0.990	SBpA	-0.058	0.080	0.875
	DVG	-0.122	0.042	0.991	DVG	-0.504	0.037	0.905
GRK6 siRNA	AngII	-0.014	0.010	0.976	Ang II	-0.238	0.156	0.882
	SI	-0.267	0.012	0.952	SI	-1.046	0.052	0.546
	SII	0.175	0.055	0.826	SII	-0.876	0.121	0.160
	SVdF	-0.099	0.050	0.976	SVdF	-0.504	0.096	0.832
	SBpA	0.162	0.051	0.968	SBpA	-0.226	0.092	0.789
	DVG	-0.719	0.063	0.944	DVG	-1.046	0.049	0.629