Aptamer-assisted two-point immobilized agonist-bound angiotensin II type 1 receptor for a second-site modulator discovery

Highlights

- An online SELEX approach was proposed for conformation selective aptamer screening
- Aptamer and peptide were introduced for functional protein immobilization
- Simultaneous screening of antagonists and allosteric ligands by affinity chromatography
- A potential allosteric modulator was first reported from the herbal extract
Aptamer-assisted two-point immobilized agonist-bound angiotensin II type 1 receptor for a second-site modulator discovery

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SUMMARY

Methods of immobilized proteins are challenged by the way how to capture the proteins in their intact functional states. Here we present a two-point, high-specific method for the immobilization of conformationally specific angiotensin II type 1 receptor (AT1R) on amino-functionalized polystyrene microspheres. We identified a selective DNA aptamer of AT1R by a column-based SELEX approach with micromolar affinity. Two single-stranded DNA strands were utilized to introduce the AT1R aptamer and angiotensin II 3-8 peptide to the microsphere surface, resulting in the two surface-positioned sites. The two-point immobilized AT1R exhibited enhanced ligand-binding activity and stability in comparison with that prepared by a one-positioned site. Ginsenoside Rg1 and rosmarinic acid were screened from the herbal extract and proved to bind with AT1R through the allosteric and orthosteric sites of the receptor, respectively. These provide a generally applicable approach for functional protein immobilization with enhanced conformation stability, ligand binding activity, and screening efficiency.

INTRODUCTION

Immobilized proteins have outstanding practical advantages in developing biotechnological methodologies for fundamental research and industry applications, owing to their easier reusability, enhanced activity and recovery, optimized specificity, and improved stability under diverse conditions (Smith et al., 2013; Redeker et al., 2013). Despite these merits, the immobilization of proteins on the surface is possible to cause a loss of protein activity and stability due to the interaction between the proteins and the surface (Fruh et al., 2011). This necessitates increasing explorations for the way to capture proteins onto solid support with simple operation and minimized loss of protein properties.

With the understanding that protein orientation, conformation, and surface density determine protein activity and stability, the way how to capture a protein has evolved to the oriented, site-specific, and covalent strategy. This is partly realized by the introduction of click chemistry or bioorthogonal chemistry methods for the immobilization of the protein of interest owing to the advances in protein engineering (Thirumurugan et al., 2013). Such attempts rely on the specific covalent reaction between the substrate-modified surface and the site-specifically introduced tags of the fusion proteins (Thirumurugan et al., 2013). Taking inspiration from these attempts, we previously immobilized a few GPCRs like beta2-adrenoceptor (β2-AR), endothelin A receptor (ETaR), angiotensin II type 1 receptor (AT1R), and type 2 receptor (AT2R) on silica gel surface (Zeng et al., 2018; Wang et al., 2019; Zhao et al., 2020). We observed improved ligand-binding activity in comparison with the receptors immobilized by physical adsorption and typical covalent methods. As previously reported, the orientation of the protein is hard to be fully controlled by such a single tethering site (Bilal et al., 2019; Rodrigues et al., 2021). The orientation flexibility might make the immobilized protein an unfavorable orientation whereby the active site interacts with the solid surface or neighboring proteins on the surface, thus leading to the loss of the protein properties (Kortt et al., 1997). New strategies are urgently needed to better control the orientation of the proteins during their immobilization.

The most straightforward method is the introduction of two tethering points on the surface that simultaneously contribute to protein immobilization. Excellent works have suggested: the properties of the immobilized protein are improved on heterofunctional support; the protein stabilization depends strongly on the position and number of the tethering sites; and there is a balance between the activity and stability of the
immobilized protein (Zhang et al., 2006). Despite numerous attempts, very few of them have resulted in good protein stabilities with defined control of the orientation (Zou et al., 2018; Gao et al., 2021). The reason behind the size of the protein and the chemically reactive groups on the surface. The groups are often designed with molecular weight far smaller than the protein, making it hard to fully anchor the protein on the surface even though two tethering sites are applied. Besides, the flexible properties of the groups may also contribute to the conformational change of the immobilized protein.

In recent decades, aptamers have evolved into promising large molecules that recognize and bind to their corresponding targets with affinity and specificity comparable to antibodies (Ni et al., 2021). Recent work by Lefkowitz et al. has demonstrated that aptamers bind to β2-adrenoceptor at defined surfaces with nanomolar affinity, thereby allosterically stabilizing active, inactive, and ligand-specific receptor conformations (Kahsai et al., 2016). This inspired us to hypothesize that the introduction of a protein conformational stabilizer is possible to enhance the conformation selectivity and stability of the immobilized protein. To this end, we screened an aptamer of AT1R with antagonist binding conformation by a purified receptor-based Systematic Evolution of Ligand Exponential Enrichment (SELEX) approach. We designed a two-point strategy for the immobilization of the receptor with the specific conformation by using the aptamer and angiotensin II (3-8) to tether AT1R on amino-functionalized polystyrene microspheres (APS). We observed enhanced ligand-binding activity and stability of the immobilized receptor by a systematic characterization using X-ray photoelectron spectroscopy (XPS), cryo-field emission scanning electron microscopy (cryo- FESEM), Fourier transform infrared spectroscopy (FTIR), competitive study, and chromatographic analysis. Using the two-point immobilized AT1R, we screened potent allosteric modulator (ginsenoside Rg1) and antagonist (rosmarinic acid) from Salviae Miltiorrhizae Radix et Rhizoma extract. We envisioned that the current method has the potential to become a generally applied, orientation- and conformation-specific strategy for the immobilization of GPCRs with enhanced conformation stability, ligand binding activity, and screening efficiency for both allosteric modulators and orthosteric ligands.

RESULTS AND DISCUSSION
Preparation of the angiotensin II type 1 receptor target
AT1R is a member of the rhodopsin-like GPCRs that serve as a primary regulator for blood pressure maintenance (Zhang et al., 2016). The structures of AT1R have been solved at high resolution in the antagonist, inverse agonist, and agonist binding states (Zhang et al., 2015; Wingler et al., 2019, 2020). In this work, we purified his-tagged AT1R from the E.coli cell lysate stably expressing the receptor. We analyzed the expression of the receptor by SDS-PAGE and western blot (Figures S1A and S1B). We found the receptor mainly expressed in the supernatant of the cell lysate, indicating that most of the AT1R are in soluble form. After purification by Ni Sepharose 6 Fast Flow medium, a sharp band around 45 kDa appeared in the gel, which is consistent with the predicted molecular weight of his-tagged AT1R (42 kDa). The purity of the receptor was analyzed as 98% by size exclusion chromatography (Figure S1C). We maintained the purified AT1R in an amphiphilic detergent, maltose-neopentyl glycol (MNG), which could enhance receptor stability.

Enrichment of specific DNA aptamers of angiotensin II type 1 receptor
We improved the traditional SELEX approach to rapidly identify high-affinity, specific DNA aptamers that bind to the receptor. As illustrated in Figure 1A, our approach introduced affinity chromatographic assay in the enrichment of the aptamer candidates, rather than the iterative in vitro selection by bead-based methods. The affinity chromatographic stationary phase was synthesized through the reaction between his-tag at the C-terminal of AT1R and nitrilotriacetic acid (NTA) modified on the APS surface (Figure S2) (Gao et al., 2015). This allowed us to monitor the selection efficiency online. To isolate DNA aptamers that bind to the AT1R at structurally relevant sites, we applied a highly diverse DNA library containing approximately 1015 unique sequences to the column packed with site-specific immobilized his-tagged AT1R. The length of the single-stranded DNA (ssDNA) in the initial library was 80 nt. To obtain enough ssDNA in each round of selection, we used asymmetric PCR (Figure S3). We performed seven rounds of positive selection against AT1R and AT1R bound to telmisartan. To deplete non-specific binding DNA strands, we carried out the negative selection on control columns containing bare silica gel in prior to each round of positive selection. In rounds 4, 6, and 8 (R4, R6, and R8), we performed counter-selections against immobilized unrelated GPCR (AT2R) to further enrich the population of aptamers binding to AT1R or AT1R-telmisartan. We monitored the enrichment of AT1R or AT1R-telmisartan-specific sequences by the retention times and peak profiles of each aptamer pool. This is theoretically reasonable since less
purity of the specific aptamers gives rise to wider peaks with shorter retention times, while higher purity leads to narrower profiles with longer retention.

As illustrated in Figures 1B and 1C, the initial library presented two peaks on both the AT1R column and the AT1R-telmisartan column. On each column, the first peak displayed intensive adsorption with a retention time of 0.6 ± 0.1 min while the second displayed weak signals with a retention time of 3.1 ± 0.2 min.

We attributed the first peak to the non-specific DNA molecules since the retention time was close to the void time of the chromatographic system that was determined by the DNA strand containing the same sequence as the two primers designed for the library DNA molecules. Unlike the receptor columns, we observed only one peak with approximately an equal retention time to the void time when the control columns were used.

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column was applied. These results indicated that the initial library has a minimal binding affinity to immobilized AT1R and AT1R-telmisartan. At R6, we observed a noticeable decrease in peak width and an increase in retention times and peak area of the second peak. Such growth appeared to be continuous until R11. Further rounds of the pools exhibited little changes in these parameters of the second peak. We collected the second peak of these pools to determine their binding affinities to the two selection targets. By an enzyme-linked oligonucleotide assay (ELONA) (Drolet et al., 1996; Torrini et al., 2019), we found increasing binding affinities of the selected DNA pools (R6, R9, and R11) of which the most prominent enrichments occurred at round 11 (Figures 1D and 1E). The aptamer pool of this round exhibited association constants of \((1.85 \pm 0.13) \times 10^5 \text{M}^{-1}\) and \((2.01 \pm 0.20) \times 10^5 \text{M}^{-1}\) to the immobilized AT1R and AT1R-telmisartan.

To validate the feasibility of the chromatographic method in the enrichment of target-specific DNA aptamers, we analyzed the same DNA library by a typical SELEX protocol. We synthesized AT1R and AT1R-telmisartan conjugated APS by the same immobilization strategy. The enrichment efficiency of each round was monitored by the equilibrium association constants of the aptamer pools binding to the immobilized AT1R, AT1R-telmisartan, which was determined by ELONA (Table S1). We observed minimal binding of the initial library and the pools from R6 to R9 to the immobilized receptor. Instead, all selected DNA pools from R6 to R11 demonstrated a progressive binding affinity to AT1R and AT1R-telmisartan. The strongest binding of the DNA pools to the two selection targets appeared to occur at round 13 without significant differences between the binding affinities. The association constants of R13 aptamer pools to immobilized AT1R and AT1R-telmisartan were \((1.90 \pm 0.28) \times 10^5 \text{M}^{-1}\) and \((2.01 \pm 0.13) \times 10^5 \text{M}^{-1}\), respectively.

We sequenced the aptamer pools of chromatographic selection (R6-R11) and bead-based enrichment (R6-R11) by the next-generation sequencing method (NGS) (Goodwin et al., 2016), which is provided by Shanghai Sangon Biotech. The sequencing was achieved by preparing multiple barcoded, Illumina-compatible double-stranded DNA (dsDNA) libraries derived from each pool and subjecting them to multiplexed paired-end sequencing analysis on an Illumina Miseq platform (Figure S4). The barcodes allowed us to analyze the aptamer pools in a single flow cell lane. We acquired 67,065 raw sequences from the initial pool. Over the course of the selections, we observed a substantial lessening of the sequence diversity of the pools and an increase in copy numbers of unique sequences. This indicated that both the chromatographic method and the typical SELEX approach enrich specific binders of the immobilized receptors over the course of the selections. To identify the specific aptamers, we analyzed the enrichment of individual sequences across successive selection rounds. This was performed by calculating the fold enrichment for every sequence, which we defined as the ratio of the percent frequency of a given sequence in the later round (R11) to that of the earlier round (R6). According to the fold enrichment across multiple selection rounds, we ranked the top 4 sequences in Figure 2. We found that the enriched aptamer sequences are selective toward the two immobilized receptors, indicating a conformational specificity of the aptamers to the receptor.

Man-made aptamers prove to act as ligands of diverse targets including proteins, cells, and small molecules (Gotrik et al., 2016). In comparison with naturally occurring antibodies, they display several advantages, like low toxicity, high stability, and maximizing function to regulate biological pathways (Wu et al., 2021). These make man-made aptamers with a high potential for biological detection, diagnostics, and targeted therapy (Meng et al., 2016). Applying the immobilized receptor as the stationary phase, we selected AT1R aptamers with high affinity and conformational specificity. Although similar results were also achieved by receptor-conjugated beads, chromatographic selection needed fewer rounds than the bead-based method. These results demonstrated that the chromatographic approach has a high potential to improve the efficiency of typical SELEX protocol since the specific aptamers can be rapidly enriched with the simultaneous removal of non-specific binders. Apart from this, previous studies have reported that more selection rounds attenuate the efficiency of the selection once maximal molecular enrichment is achieved (Meng et al., 2016). In this study, the enrichment of target-specific aptamers was monitored during the selection process. As such, it is possible to efficiently enrich high-affinity aptamers of GPCRs with conformational specificity.

**Angiotensin II type 1 receptor aptamer stabilizes specific conformation of the receptor**

We characterized the top four aptamers by examining their binding and specificity to AT1R and AT1R-telmisartan by ELONA and isothermal titration calorimetry (ITC) (Bottari et al., 2020). We observed that the four aptamer candidates exhibit diverse AT1R binding affinity and specificity in the absence and presence of telmisartan. Of which, one aptamer (A2) demonstrated conformational selectivity for AT1R-telmisartan while the other three aptamers displayed binding to both the two immobilized receptors without preference. Unlike them, the
Figure 2. Top 4 candidate DNA aptamers from next-generation sequencing (NGS) analysis

(A and B) Isothermal titration calorimetry (ITC) data for the titration of AT1R into aptamer solution in the absence (A1-A4) and presence (B1-B4) of losartan. 

(C) Calculated free energies and binding constants of the aptamers with AT1R. In c, the free energies of the aptamers were predicted by the DNA folding algorithm Mfold. The binding constants were calculated by ELONA.
control aptamer (collected from the initial library), showed unobservable binding to either AT1R or AT1R-telmisarten. According to their sequences, we predicted the secondary structure of the four aptamers (Figure S5). With only two base pairings in their secondary structures, A1 and A4 demonstrated lower affinity and selectivity to the two receptors due to their instability. Unlike them, A2 and A3 displayed eight base pairings, thus exhibiting higher affinity and selectivity to the two selection targets.

**Two-point strategy for the immobilization of angiotensin II type 1 receptor**

As illustrated in Figure 3A, we developed a two-point strategy for AT1R immobilization by the utilization of Ang II (3-8) and the aptamer. i) We designed a plain-extended short DNA strand (CS 1) that can never form any secondary structures. Ang II (3-8) and an amino group were then modified at the 5'-end and 3'-end of CS 1 strand, respectively. The amino group was utilized to link CS 1 onto APS activated by ethyl chlorooxoxoacetate. Ang II (3-8) can recognize AT1R by ligand-receptor binding interaction. ii) We also rationally designed Apt, which contains the sequence of A2 and a 20-nt linker with a total length of 60 nts. As the 20-nt linker is complementary to CS 1, they hybridize with each other. The aptamer at the 3'-end of Apt was able to fold the secondary structure when a quenching method is applied. iii) The quenched Apt was mixed with purified AT1R to pursue the receptor in a stabilized conformation. iv) Once the mixture was added to the Ang II (3-8)-modified APS, a double strand formed, and the two-point immobilization was achieved through aptamer-receptor and ligand-receptor interactions. To this end, we immobilized AT1R on the double-tethered APS by the two-point strategy.

The morphologies of the surface coatings on APS were directly visualized by cryo-FESEM (Figures 3B2–3G2). The naked APS showed a relatively uniform, porous, and spherical morphology (Figure 3B2). With the sequential surface modification by ethyl chlorooxooacetate, Apt, CS 1, and Ang II (3-8), we observed more compact structures within the pores of the APS. After modification with the receptor, the surfaces of the microspheres became rough and covered with demonstratable particles. We considered these particles as immobilized AT1R.

To understand the morphology, we used FTIR and XPS to determine the functional groups (Figures 3B3–3G3) and elemental composition (Figures 3B4–3G4) of the modified APS, respectively. In the FTIR image of APS, we observed the characteristic fingerprints of polystyrenes and aminos: i) stretching vibration of aromatic groups (ν(C-C): 1,430–1,610 cm⁻¹); ii) stretching vibration of methylene groups (ν(−CH2): 2,925 cm⁻¹); iii) asymmetric stretching vibration of amino groups (ν(-NH2): 3,432 cm⁻¹); iv) stretching vibration of aromatic amines (ν(C-N): 1,200 cm⁻¹). After being reacted with ethyl chlorooxooacetate, the signal of amino groups reduced dramatically, which implies that ethyl chlorooxooacetate has been successfully linked to APS. Compared with the ethyl chlorooxooacetate-modified APS, we found an obvious broad peak centered at 3,440 cm⁻¹ in the receptor-covered APS. It is rational since the introduction of the receptor results in the detection of stretching vibration of amino groups.

In the survey scan of naked APS, we observed intensive and specific signals of O 1s (531.9 eV), N 1s (399.5 eV), and C 1s (284.8 eV), implying that the microsphere contains C-NH₂, C-O, and C-C groups. The ethyl chlorooxooacetate modified APS showed a reduced signal of O 1s (from 18.30 ± 0.50)% to (5.85 ± 0.35)% and N 1s (from 3.05 ± 0.12)% to (1.03 ± 0.22)%). We also observed a new signal at 138.6 eV when APS was modified by CS 1. This peak attributes to the phosphate groups in the DNA backbone, indicating a successful coating of DNA strands. After modification with Ang II (3-8), the N 1s spectra increased from (1.64 ± 0.25)% to (3.16 ± 0.22)%). The XPS spectra of the AT1R-covered microsphere showed a signal at 163.79 eV, which represents a featured peak of the thiol group in the receptor. In addition, the contents of P 2s and N 1s ((0.54 ± 0.12)% and (5.16 ± 0.33)%) of the immobilized AT1R are much higher than the Ang II (3-8) modified support, providing additional proof of the successful immobilization of AT1R.

To prove the success of the two-point immobilization, we carried out a pulldown assay by introducing the free Ang II (3-8), the conformational specific aptamer A2, or both of them to the solution containing the microspheres with immobilized AT1R via a two-point strategy (Figure S6). In the presence of free Ang II (3-8) or A2, only a trace amount of the receptor was detected in the solution. This is predictable since only one type of competition happens and the residual affinity via the other site is still attached to most of the receptors on the APS surface. When both the peptide and aptamer A2 were added to the system, the receptor become detectable in a peptide and aptamer concentration-dependent manner since complete displacement occurred.
Immobilized angiotensin II type 1 receptor via two points exhibits enhanced activity and stability

We packed the affinity microspheres with the one-point and two-point immobilized AT1R into two stainless steel columns with a dimension of 4.6 × 30 mm. The densities of the immobilized receptor were calculated through the difference in the receptor concentrations before and after immobilization by bi-cinchoninic acid protein assay. We obtained nearly the same amount of AT1R per APS (450 ± 50 μg/g APS), which is a foundation of the comparison in the next set of experiments. The ligand recognition activities of the one-point and two-point immobilized receptors were assessed by on-column and off-column tests. In the on-column study, five antagonists of AT1R (olmesartan, irbesartan, candesartan, azilsartan, and valsartan) and a non-retained compound...
(NaNO₂) were separately loaded onto the two columns (Figure 4). As we can see, the retention times of the five antagonists on the two columns are varied and much longer than NaNO₂ (1.5 min). For olmesartan, the retention factors ($k'_0$) on the two columns are very close (one-point column: 4.4; two-point column: 4.2). It is rational since the retention factor is governed largely by the fundamental thermodynamics of the injected ligand partitioning between the mobile and stationary phases. The mobile phase and the apparent amount of the receptors on the stationary phases for the two columns are similar, resulting in the consistent retention factors of the same ligand on the two columns. For the peak shape of olmesartan on the one-point column, it gave the full width at half maxima ($W_{1/2}$) of 7.1 min, which is 3-min' wider than that on the two-point column. We also found a similar phenomenon for the other four ligands on the two columns. In chromatography, the broadening of a peak arises from many non-idealities, including axial diffusion, dispersion, resistances to mass transfer in both mobile and stationary phases, kinetic resistances to adsorption and desorption, and extra-column instrument response effects from the detector, tubing, and electronics. In this work, the chromatographic conditions and the amounts of the receptors in the columns remained identical with the only exception

| Antagonists | Data from the column with one-point immobilized AT₁R | Data from the column with two-point immobilized AT₁R | Data from literatures |
|-------------|------------------------------------------------------|-----------------------------------------------------|-----------------------|
| Olmesartan  | $K_A$ ($M^{-1}$) | $k'_0$ ($min^{-1}$) | $k_R$ ($M^{-1}min^{-1}$) | $K_A$ ($M^{-1}$) | $k'_0$ ($min^{-1}$) | $k_R$ ($M^{-1}min^{-1}$) | $IC_{50}$ (nM) | $K_A$ ($M^{-1}$) | $k'_0$ ($min^{-1}$) | Half-lives (h) |
| Olmesartan  | $3.27 \times 10^7$ | 0.45 | $1.46 \times 10^7$ | $3.13 \times 10^7$ | 3.97 | $1.24 \times 10^6$ | $\approx 7.7$ | / | $<4.2 \times 10^{-5}$ | 2.76 |
| Irbesartan  | $2.36 \times 10^7$ | 0.21 | $4.88 \times 10^6$ | $2.17 \times 10^7$ | 8.77 | $1.91 \times 10^6$ | $\approx 1.94$ | $5.26 \times 10^5$ | 0.09 | / |
| Candesartan | $1.82 \times 10^7$ | 0.30 | $5.40 \times 10^6$ | $1.78 \times 10^7$ | 9.52 | $1.69 \times 10^6$ | $\approx 0.47$ | $3.85 \times 10^5$ | $<5.2 \times 10^{-3}/0.1$ | 2.21/5-9 |
| Azilsartan  | $9.51 \times 10^6$ | 0.11 | $1.08 \times 10^6$ | $8.47 \times 10^6$ | 21.51 | $1.82 \times 10^6$ | $\approx 2.6$ | $3.23 \times 10^5$ | / | 11 |
| Valsartan   | $3.26 \times 10^6$ | 0.075 | $2.45 \times 10^6$ | $3.82 \times 10^6$ | 26.67 | $1.02 \times 10^6$ | / | $9.09 \times 10^5$ | $9.95 \times 10^{-3}$ | $1.17/6-9$ |

*Data were from the website of [https://www.invivochem.com](https://www.invivochem.com); Hypertension Research (2010) 35, 1044-1052; Hypertension Research (2013) 36, 134-139; World Journal of Nephrology (2017) 6, 1291-1296.*

**Figure 4.** Comparison of the one-point and two-point columns by chromatographic study

(A and B) Chromatograms (A) and binding parameters (B) of ligands on the one-point and two-point columns. Red: on one-point column; Blue: on two-point column; $k'_0$: retention factor; $W_{1/2}$: full width at half maxima (min). In the structures of the five antagonists, the biphenyl-linker groups, acidic tetrazole groups, and two-four carbons tails were highlighted by blue, red, and green, respectively.
of the immobilization strategy. This allowed the comparison of ligand binding kinetics on the two columns without the interferences by the thermodynamics and extra-column effects. Despite oriented immobilization, the AT₁R attached via one point remains the flexibility and tends to interact with the APS surface in all directions. Unlike this, the two-point immobilization makes the receptor only can move forward and backward. In this case, the receptor is possible to interact with the non-specific sites on APS, however, such interactions are much less compared to those of AT₁R immobilized through one site. Therefore, we suspect, the different degrees of the interactions with the APS surface are the main source for the different band broadening of the ligands on the two columns. The AT₁R immobilization via one site could lead to more unwanted orientations of the receptor and result in non-specific interactions. Thus, the specific ligands would “drizzle” on the one-point column with long extended tails.

To obtain further insight into the stability of the two-point immobilization, we compared the retention times and peak profiles of the five antagonists on the column in a one-month period. As illustrated in Figures S7E and S7F, the peak profiles and retention times of candesartan demonstrated a one-observable change in one month with relative standard deviations (RSD) of 1.3% on the two-point column. While on the one-point column, the peak shapes and retention times of candesartan remained stable only for twenty days. The RSD of the retention time of candesartan reached 13.7% for 30 days. Similar phenomena can be also found in the other four ligands and all of them showed the RSD values of retention times greater than 10% in one month. The instability of the one-point column may attribute to the medium affinities between his-tag and the Ni²⁺ (10⁶ to 10⁸ M⁻¹/C₀). With the continuous flushing of the affinity column with mobile phase in a relatively long-time course, AT₁R could be washed off from the microspheres. Ang II (3-8) is reported to be a partial agonist of AT₁R (de Gasparo et al., 2000). The aptamer A₂ binds to AT₁R with an association constant of ~10⁵ M⁻¹. We assume that there is an orthogonal affinity amplification in the two-point immobilization, the synergistic interactions of the aptamer A₂-AT₁R and Ang II (3-8)-AT₁R would make the affinity to tether AT₁R far greater than that of the his-tag system. Thus, the immobilized receptor via two points is more resistant to the elution of the mobile phase compared with the one-point tethering one.

Immobilized angiotensin II type 1 receptor is specific to recognizing the receptor antagonists

To gain further insight into the specificity of the immobilized AT₁R to recognize the receptor antagonists but not the agonists, we also studied the retention behaviors of two AT₁R agonists, Ang II and Ang II (3-8), on the one-point and two-point columns (Figure S7C and S7D). In solution, the two agonists share the same peptide backbones and bind to AT₁R via a large orthosteric ligand binding site with the key amino acids of Asp₁₇, Trp₈₄, Lys₁₉₉, Asp₂₆₃, and Asp₂₈₁ (18). The five antagonists contain the same scaffold of biphenyl-linker and acidic tetrazole groups (Figure 4). Serial femtosecond crystallography with X-ray free-electron laser and docking studies showed that antagonists associate with AT₁R in the same domain (15). The acidic tetrazole moiety forms an extensive interaction network (e.g. salt bridges) with the positively charged guanidine group of Arg₁₆₇, demonstrating that Arg₁₆₇ plays an essential role in AT₁R antagonist-binding affinity and selectivity (15). Arg₁₆₇ is located near the aforementioned five key amino acids but does not overlap with them. The five antagonists (average MW: 441 g/mol) only occupy part of the orthosteric site on AT₁R since they are much smaller than the peptide agonists (MW_Ang II: 1,046 g/mol; MW_Ang II (3-8): 775 g/mol). We found that the two peptide agonists exhibited good retentions on the one-point column. Their retention times were reduced to almost the same as NaNO₂ on the two-point column due to the occupation of the orthosteric site by Ang II (3-8). The tailed profiles of Ang II and Ang II (3-8) demonstrated their weak interactions with the immobilized AT₁R. Such interactions can be neglected compared with the binding of antagonists to the receptor. Another common feature among the five AT₁R antagonists is the short alkyl tail with two-four carbons (highlighted green in Figure 4) extending into a narrow hydrophobic pocket formed by Tyr₃₅, Phe₇₇, Val₁₀₈, Ile₂₈₈, and Tyr₂₉₂ (Kahsai et al., 2016). These interactions along with the contribution of the salt bridges formed through Arg₁₆₇, therefore explain the various retention behaviors of antagonists on the two-point column. We conclude that the two-point column can distinguish the receptor agonist and antagonist.

Immobilized angiotensin II type 1 receptor in chromatographic analysis and ligand screening

We first applied the two-point column in receptor-ligand interaction analysis by NLC (Figure S8). This function is advantageous in modeling extremely tailed, even right triangular, peaks, and is beneficial
for dealing with the chromatograms in affinity chromatography (Wade et al., 1987). Under optimized conditions, we can obtain the equilibrium and kinetic binding constants of the injected ligand to the immobilized receptor from one injection (Li et al., 2015). The binding parameters of five antagonists to AT1R were summarized in Figure 4B. The binding affinities of the five antagonists to the receptor followed the order of olmesartan > irbesartan > candesartan > azilsartan > valsartan, while the dissociation rates showed the exact opposite order on the two-point column. Although the values of the affinities and dissociation rate constants deviated from the data reported in the literature (Vanderheyden et al., 2000; Fujino et al., 2010; Miura et al., 2013; Kakuta et al., 2005; Hübner et al., 1997; Hjermitslev et al., 2017), their orders are consistent. Although the same affinity pattern, the one-point column exhibited different order of dissociation rates for the five antagonists compared with the literature. This is rational since the kinetic rates are determined by the degree of band broadening in NLC analysis (Wade et al., 1987). As we analyzed in the previous section, non-specific interactions attribute largely to the band broadening of the chromatographic peak on the one-point column, thus, resulting in inaccurate kinetic results. In this case, the two-point column is more reliable to achieve a quantitative analysis of AT1R-antagonist interaction, in particular the determination of ligand-receptor binding kinetics.

Another application of the two-point AT1R column is to screen the potential antagonists or allosteric ligands from Salviae Miltiorrhizae Radix et Rhizoma. When the herbal aqueous extract was loaded onto the AT1R column, two peaks with retention times of 1.5 min (Peak 1) and 3.0 min (Peak 2) showed up (Figure 5). We collected and analyzed Peak 2 by reverse-phase liquid chromatography coupled with tandem mass spectrometry (MS/MS). Two compounds were separated and identified ginsenoside Rg1 (G-Rg1) and rosmarinic acid (RA) (Figures 5B, 5S, and 510). We filled in the missing residues in the crystal structure of AT1R using 4YAY as the template (Waterhouse et al., 2018) and performed docking analysis for AT1R with the two compounds (Figure S11). We found that RA could stably bind to the conventional orthosteric pocket of AT1R, which was surrounded by the key amino acids of Arg167, Lys199, Asp263, and Gln267 (Figure 5D). However, G-Rg1 preferentially anchored to a topologically distinct pocket, which was located at the extracellular loop 2 (ECL2) and was reported as an allosteric site of the receptor (Singh et al., 2021) (Figure 5C).

We tested the expression of AT1R on normal and Ang II-induced mouse-derived macrophage cell lines (HBZY-1). We found that RA could dramatically decrease the expression of AT1R in both cells, demonstrating that RA may have antagonistic effects on AT1R (Figures 5E and 5F). G-Rg1 up-regulated the expression of AT1R in normal cells while displaying an indistinguishable difference from Ang II on the receptor expression when Ang II-induced HBZY-1 is applied. As Ang II binds to the orthosteric pocket of AT1R, we reasoned that G-Rg1 is an allosteric ligand of the receptor. To prove this, we measured the effects of the two compounds on the intracellular Ca2+ release of the HBZY-1 cells treated by Ang II (3-8). The Ca2+ response curves for the partial agonist Ang II (3-8) right shifted when 30 μM RA was applied to the cells (EC50 shift: 4.2). Unlike this, G-Rg1 left shifted the Ca2+ response curves in a concentration-dependent manner, with the alpha value greater than 1 (45.7). These results confirmed that RA is a potential AT1R antagonist since competition occurred between the compound and Ang II (3-8); while G-Rg1 serves as an allosteric ligand of the receptor due to the non-competitive stimulation of the cell-intrinsic activity induced by Ang II (3-8). RA was reported to have a protective effect on myocardial infarction-induced cardiac fibrosis and could decrease the expression of AT1R (Liu et al., 2016), which is highly consistent with its antagonistic effect on AT1R elucidated by our data.

Limitations of the study
The present study illustrates that aptamer can act as an agonist-bound AT1R stabilizer for controlling the conformation of the receptor with sustainable activity. Our results, therefore, establish a two-point immobilization method for AT1R specifically recognizing its antagonist and allosteric modulators. More importantly, by virtue of the recognition specificity of the immobilized AT1R, the method could be used in the discovery of receptor allosteric modulators. This represents a major step for moving the chromatographic-based methods forward to the research field of allosteric ligands since it is easily adapted to other functional proteins, which have a conformationally specific aptamer, peptide ligand, and different allosteric and orthosteric binding sites. It is even conceivable to apply the method in the discovery of a second-site ligand. Finally, the general approach in this work establishes a framework for soluble/membrane protein immobilization that undergo conformational-specific activity.
**STAR+ METHODS**

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **RESOURCE AVAILABILITY**
  - Lead contact
  - Materials availability
  - Data and code availability
EXPERIMENTAL MODEL AND SUBJECT DETAILS
- Cell lines and cell culture

METHOD DETAILS
- Expression and purification of his-tagged AT1R
- Preparation of the single-stranded DNA library
- In vitro selection of aptamers
- Binding affinity measurements by enzyme-linked oligonucleotide assay (ELONA)
- Binding affinity measurements for aptamers and AT1R by isothermal titration calorimetry (ITC)
- X-Ray photoelectron spectroscopy (XPS)
- Cryo-field emission scanning electron microscopy (cryo-FESEM)
- Fourier-transform infrared spectroscopy (FTIR) characterization
- Two-point immobilization of AT1R on APS
- Chromatographic analysis
- Molecule simulation
- Protein isolation, electrophoresis, and western blotting
- Intracellular Ca\(^{2+}\) release measurement

QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105361.

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AUTHOR CONTRIBUTIONS
Jiajun Liu: Writing-Original Draft, Visualization. Ting Li: Investigation. Ge Wang, Editing. Jiahuan Chen: PAGE analysis. Qingqing Yao: Chromatographic analysis. Qian Li and Xinfeng Zhao: Supervised the project.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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# STAR METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE                                                                 | SOURCE                        | IDENTIFIER                  |
|------------------------------------------------------------------------------------|-------------------------------|------------------------------|
| Antibodies                                                                          |                               |                              |
| Anti-Angiotensin II Type 1 Receptor                                                 | Abcam                         | Cat# ab124505; RRID:AB_10976053 |
| Biological samples                                                                  |                               |                              |
| Taq DNA Polymerase with Standard Taq Buffer                                         | New England Biolabs           | M0273L                       |
| Ang II (3–8)                                                                       | MedChemExpress                | HY-P1515                     |
| Streptavidin-horseradish peroxidase (5-HRP) conjugate                                | Cell Signaling Technology     | 39995                        |
| Chemicals, peptides, and recombinant proteins                                       |                               |                              |
| Olmesartan                                                                         | Aladdin Industrial Corporation | O124944                      |
| Irbesartan                                                                         | Aladdin Industrial Corporation | I129263                      |
| Candesartan                                                                        | Aladdin Industrial Corporation | C129307                      |
| Azilsartan                                                                         | Aladdin Industrial Corporation | A122333                      |
| Valsartan                                                                          | Aladdin Industrial Corporation | V129241                      |
| Telmisartan                                                                        | Aladdin Industrial Corporation | T129239                      |
| TMB Single-Component Substrate solution                                              | Solarbio Life Sciences        | PR1200                       |
| ELISA Stop Solution                                                                 | Solarbio Life Sciences        | C1058                        |
| Fura-2 (AM, Cell Permeant)                                                          | Yeasen                        | 40702E550                    |
| Experimental models: Cell lines                                                    |                               |                              |
| Mouse-derived macrophage cell line (HBZY-1)                                         | Procell                       | CL-0092                      |
| Oligonucleotides                                                                   |                               |                              |
| DNA library                                                                         | Sangon Biotech                | N/A                          |
| Strand A1                                                                          | Sangon Biotech                | N/A                          |
| Strand A2                                                                          | Sangon Biotech                | N/A                          |
| Strand A3                                                                          | Sangon Biotech                | N/A                          |
| Strand A4                                                                          | Sangon Biotech                | N/A                          |
| Software and algorithms                                                             |                               |                              |
| GraphPad Prism 5                                                                    | GraphPad Software, Inc.       | https://www.graphpad.com/     |
| Origin 8.0                                                                          | OriginLab Corporation         | https://www.originlab.com/Origin |
| Homology-modelling server                                                           | SWISS-MODEL                   | https://swissmodel.expasy.org/|
| AutoDockTools-1.5.6rc3                                                              | The Scripps Research Institute | https://autodocksuite.scripps.edu/adt/ |
| Other                                                                              |                               |                              |
| Amino-functionalized polystyrene microspheres (APS, 5 μm, 300 Å)                    | Suzhou Knowledge & Benefit Sphere Tech. Co., Ltd. | PS20210331                  |
| Polystyrene 96-well test plates                                                     | Wuxi NEST Biotechnology Co.   | 701001                       |

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the Lead Contact, Xinfeng Zhao (zhaoxf@nwu.edu.cn).

### Materials availability

This study did not generate new materials.
Data and code availability

- The attached supplemental information includes all datasets generated or analyzed to perform this study.
- This study does not report the original code.
- Any additional information is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell lines and cell culture

Mouse-derived macrophage cell lines (HBZY-1, Procell, Wuhan, China) were seeded in a 25-cm² flask and cultured in Dulbecco's modified Eagle's medium (DMEM, Servicebro, Wuhan, China) supplemented with 10% fetal bovine serum (FBS, Tianhang, Zhejiang, China), 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were grown in a humidified 5% CO₂ incubator at 37°C. All compounds used were dissolved in water.

METHOD DETAILS

Expression and purification of his-tagged AT₁R

The protein sequence of AT₁R (GenBank: AAA58370.1) was obtained from GenBank. We added 6×His at the C-terminal of the protein and carried out codon optimization for expression in the E.coli system. The gene was synthesized by Sangon Biotech (Shanghai, China) and inserted into the pET15b vector through the restriction sites of Nco I and Xho I. The resulting vector was transformed into BL21(DE3) and the positive colony was selected with ampicillin (100 µg/mL) resistance. We picked a single positive colony into a 50.0 mL of Luria-Bertani (LB) medium with ampicillin and cultured the strain at 37°C for 12 h at 220 rpm/ min. Large-scale production of the bacteria proceeded in a 500-mL LB medium under the same condition until the OD600 reached 0.8. The expression of the AT₁R was induced by isopropylthio-β-D-galactoside (IPTG, 2.0 mM) for 4 hours. The cell pellets were harvested by centrifugation and re-suspended in lysis buffer (20 mM Tris-HCl containing 1.0% n-dodecyl-β-D-maltoside (DDM), 500 mM NaCl, 10 mg/mL benzamidine, 10 mg/mL leupeptin, 0.1 mM PMSF, pH 7.4). To obtain functional purified AT₁R, we added telmisartan (10 µM), a long-lasting antagonist of the receptor, before loading the cell lysate to purify it from those nonfunctional ones via Ni sepharose 6 Fast Flow affinity column (Figure S1). After purification, DDM was exchanged to maltose-neopentyl glycol (MNG) by a gradual gradient of DDM:MNG buffer (20 mM Tris-HCl containing 1.0% n-dodecyl-β-D-maltoside (DDM), 500 mM NaCl, 10 mg/mL benzamidine, 10 mg/mL leupeptin, 0.1 mM PMSF, pH 7.4). To obtain functional purified AT₁R, we added telmisartan (10 µM), a long-lasting antagonist of the receptor, before loading the cell lysate to purify it from those nonfunctional ones via Ni sepharose 6 Fast Flow affinity column (Figure S1). After purification, DDM was exchanged to maltose-neopentyl glycol (MNG) by a gradual gradient of DDM:MNG buffer with cholesterol hemisuccinate (CHS, w/v = 0.01%).

Preparation of the single-stranded DNA library

The initial single-stranded DNA (ssDNA) library was obtained from Sangon Biotech, which is composed of 80 nucleotides (nts), including two constant regions (20 nts at the 5' and 3' ends) and a variable region (40 nts) in the middle: 5′-ACCGCCTTGTAGTCTAGCTA-N40-CTGAGTCGCATCGATAGTCT-3′. Biotinylated primer 1 (P1, Biotin-5′-ACCGCCTTGTAGTCTAGCTA-3′) and unmodified primer 2 (P2, 5′-AGACTATCGATGCGACTCAG-3′) were purchased from Sangon Biotech. To obtain the ssDNA sub-library, we performed a two-step procedure (Figure S3): i) Symmetric PCR: the 50-µL reaction system contains 2.0 µL P1 (10 µM), 2.0 µL P2 (10 µM), 0.5 µL initial ssDNA library (10 µM), 1.0 µL dNTP (10 µM, NEB), 0.25 µM Taq DNA polymerase (NEB), 2.5 µM PCR reaction buffer (10×, NEB), and 41.75 µL H₂O. The reaction mixture was preheated at 95°C for 5 min and followed by nine thermal cycles of denaturing at 94°C for 30 s, annealing at 58°C for 30 s, and extending at 72°C for 30 s. After the thermal cycles, another extending at 72°C for 2 min was performed. ii) Asymmetric PCR: the 50-µL reaction system contains 2.0 µL P1 (10 µM), 2.0 µL P2 (0.5 µM), 0.5 µL purified symmetric PCR product (10 µM), 1.0 µL dNTP (10 µM, NEB), 0.25 µM Taq DNA polymerase (NEB), 2.5 µM PCR reaction buffer (10×, NEB), and 41.75 µL H₂O. The reaction mixture was preheated at 95°C for 5 min and followed by twenty-seven thermal cycles of denaturing at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s. After all the thermal cycles, another extension at 72°C for 2 min was performed. The products of symmetric and asymmetric PCR were incubated in phenol/chloroform/isoamyl alcohol (v:v:v = 25:24:1) for 10 min at room temperature (RT), chloroform extracted, ethanol precipitated, and re-suspended in ddH₂O at a final concentration of 10 µM.

In vitro selection of aptamers

DNA aptamers were obtained through an on-column Systematic Evolution of Ligands by Exponential Enrichment (SELEX) procedure against purified AT₁R that was either unliganded or telmisartan-bound.
The affinity chromatographic stationary phase for SELEX was prepared through three steps: i) activated the amino-functionalized APS by N,N'-carbonyldimidazole; ii) the activated APS was modified by ANTA-Ni²⁺; iii) the purified His-tagged AT₁R was immobilized on the APS surface. The ssDNA library in selection buffer (20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 12.5 mM MgCl₂) was heat denatured at 95°C for 10 min, quenched at 4°C for 10 min, kept at RT for 5 min, and supplemented with MNG (w/v = 0.1%) and CHS (w/v = 0.01%). The mobile phases for selection against AT₁R and AT₁R-telmisartan were selection buffers with or without telmisartan (10 µM). The library solution (10.0 µL) was loaded onto the affinity column and the peak with retention time longer than void time was collected for symmetric and asymmetric PCR. In the negative selection during rounds 4, 6, and 8, a control column with immobilized AT₂R was applied. Eleven rounds of selection were performed, and selection pressure was increased by adjusting the ionic strength (concentration of NaCl) as follows: 50 mM for rounds 1–4, 75 mM for rounds 5–8, and 100 mM for rounds 9–11.

We also performed the bead-based SELEX as a control: i) the AT₁R modified APS was incubated with the library solution with or without telmisartan for selection against AT₁R and AT₁R-telmisartan for 30 min at RT, before every round of selection. ii) The APS with bound aptamer was separated from the unbound aptamers through centrifugation. iii) The resulting APS was washed by the selection buffer containing 0.05% Tween 20 for three times to remove the non-specific aptamers. iv) The bound aptamers were then incubated in phenol/chloroform/isoamyl alcohol for 30 min at RT, chloroform extracted, ethanol precipitated, and re-suspended in ddH₂O. Negative selection using the AT₂R-covered APS was also performed in rounds 4, 6, and 8. The concentration of NaCl was also consistent with the on-column selection in rounds 4, 6, and 8, a control column with immobilized AT₂R was applied. Eleven rounds of selection were performed, and selection pressure was increased by adjusting the ionic strength (concentration of NaCl) as follows: 50 mM for rounds 1–4, 75 mM for rounds 5–8, and 100 mM for rounds 9–11.

**Binding affinity measurements by enzyme-linked oligonucleotide assay (ELONA)**

The purified AT₁R in the absence or presence of telmisartan (10 µM) was incubated on a 96-well plate in the immobilization buffer (100 µL) containing 50 mM Na₂CO₃/NaHCO₃ (pH 9.6) with 0.1% MNG and 0.01% CHS at 4°C overnight. The concentration of AT₁R was within the range of 0–20 µM. Phosphate-buffered saline Tween (PBST, 10 mM, pH 7.4, 200 µL each) was used to wash the wells four times. Then bovine serum albumin (BSA, 5%, 4°C incubated for 1 hour) was used as a blocking solution to prevent nonspecific adsorption on polystyrene wells. The negative control was performed by adding immobilization buffer without protein under the same conditions. After washing with PBST, a thermally treated asymmetric PCR product (1.0 µM, 100 µL) in selection buffer was added to each well, incubated at RT for 2 hours, and washed with PBST to remove the unbound aptamers. Streptavidin-horseradish peroxidase (S-HRP) conjugate diluted 1:10,000 in PBS was added to each well (100 µL) incubated at RT for 1 hour, and then washed by PBST. Next, Turbo-3, 3′, 5′-tetramethylbenzidine (TMB, 100 µL) was added and incubated in the dark (8 min). The reaction was stopped by adding 50 µL of H₂O₂ (2.0 M) and the resulting complexes were measured at an absorbance of 450 nm using a MultiSkan Go plate reader (Thermo Scientific).

**Binding affinity measurements for aptamers and AT₁R by isothermal titration calorimetry (ITC)**

MicroCal iTC200 (Malvern, UK) was used to perform the ITC experiments. Running conditions consisted of 19 or 20 injections of 2.0 µL aptamer (100 µM, total volume: 40 µL) to AT₁R solution (10 µM, total volume: 350 µL) at 25°C in the absence and presence of telmisartan. AT₁R and all the aptamers were dissolved in 20 mM Tris-HCl, pH 7.4, 50 mM NaCl, and 12.5 mM MgCl₂. The duration, spacing, and filter period were set as 4 s, 150 s, and 5 s, respectively. In a control titration, signals are observed due to the dilution of the aptamer into the buffer. The first injection was disregarded, and the data of the blank experiment was subtracted. All the ITC data were fitted with the single-site-binding model in the MicroCal Origin7 software.

**X-Ray photoelectron spectroscopy (XPS)**

XPS was performed on a Thermo Fisher Esca Lab 250Xi analyzer using a monochromatic Al Kα source (1486.6 eV). The filament voltage, electric current, entrance slit, and pass energy were set as 14.7 keV, 10 mA, 0.5 mm, and 30 eV, respectively. The XPS spectra were processed using the Avantage software (Thermo Scientific™). The binding energy was calibrated via the C1s peak at 284.8 eV.

**Cryo-field emission scanning electron microscopy (cryo-FESEM)**

The morphology of the microspheres was characterized by a Hitachi SU8000 field-emission scanning electron microscope (Hitachi Ltd., Tokyo, Japan). The microspheres were dispersed on a double-sided tape and the
tape was immobilized on the sample holder. The holder was then placed on a cold stage with a temperature of –150°C. The accelerating voltage and the working distance were 5 kV and 9.5 mm, respectively.

**Fourier-transform infrared spectroscopy (FTIR) characterization**

Nicolet 5700 FTIR (Thermo, Waltham, MA, USA) was used to monitor the functional groups on the microspheres. The microspheres were dried in a vacuum drying oven at 60°C overnight before FTIR analysis. The dried microspheres were mixed with KBr and compressed into a KBr pellet for FTIR analysis. The scanning range was from 4,000 cm⁻¹ to 400 cm⁻¹.

**Two-point immobilization of AT₁R on APS**

The purified AT₁R was immobilized on the amino-functionalized APS as follows: i) we suspended the APS (amino content: 465 μmol/g) in dichloromethane and added ethyl oxalyl monochloride (3.0 eq.) and triethylamine (1.5 eq.) under ice bath. The mixture was stirred for 2 hours and the resulting APS was washed sequentially with dichloromethane and ethanol three times. ii) Then the resulting ester on the APS surface was hydrolyzed in the presence of NaOH (5.0 eq.) using ethanol as the solvent. The reaction was processed at 60°C under stirring for 30 min and the resulting APS was washed with diluted H₂SO₄ and ddH₂O to neutralize the excessive NaOH. iii) The APS was activated by DMTMM (1.2 eq., 4-(4,6-didethoxy-1,3,5-triazin-2-yl)-4-methyl morpholinium chloride) for 2 hours in ddH₂O and then reacted with CS 1 (1.2 eq., 5’-TAG CTG ACT ACA CAA CGG GT-3’) via the interaction between the carboxyl group on APS surface and the aminol group modified at the 3’-end of CS 1. The unreacted CS 1 was removed by ddH₂O. iv) Ang II (3–8) (1.2 eq.) was then reacted with DMTMM (1.2 eq.), and their mixture was added to the CS 1 modified APS and reacted at RT under stirring for 2 hours. The resulting APS was washed sequentially by ddH₂O and 20 mM PBS. v) Apt (1.2 eq, 5’-ACC CGT TGT GTA GTC AGC TAG CCG GAT CAA TGC TCA AGA TCC ATG GAC AGC TCG TCA GCA-3’) was dissolved in the buffer containing 20 mM PBS, 50 mM NaCl, and 12.5 mM MgCl₂, heated to 95°C for 5 min, placed to an ice bath for 10 min, and mixed with the purified AT₁R for 1 hour to obtain the conformational specific receptor. vi) The above mixture was added to the Ang II (3–8) modified APS and the reaction was proceeded at RT for 3 hours to obtain the APS with tethered AT₁R via two-point immobilization.

**Chromatographic analysis**

The chromatographic analysis was performed on a series of Elite EClassical 3100 chromatographic system (Elite, Dalian, China), which consists of a binary pump, a column oven, and an ultraviolet-visible detector. The wavelengths of olmesartan, irbesartan, candesartan, azilsartan, and valsartan were 251 nm, 254 nm, 252 nm, 251 nm, and 227 nm, respectively. The flow rate for all the experiments was 0.2 mL/min. The mobile phase was 20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 12.5 mM MgCl₂. For nonlinear chromatography (NLC, Figure S8), the concentrations were 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0, 2.5, 3.0, and 4.0 mM for olmesartan, for 0.05, 0.075, 0.1, 0.25, 0.5, 1.0, 2.0, 3.0, and 4.0 mM for irbesartan, for 0.05, 0.1, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, and 5.0 mM for candesartan, 0.025, 0.05, 0.1, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, and 4.0 mM for azilsartan, and 0.05, 0.08, 0.125, 0.25, 0.75, 1.0, 2.0, and 4.0 mM for valsartan.

**Molecule simulation**

The starting coordinates of AT₁R (PDB: 4YAY) were downloaded from the Protein Data Bank (http://www.rcsb.org). The missing amino acid in 4YAY structure was added using an online protein structure homology-modelling server (SWISS-MODEL, https://swissmodel.expasy.org/) (Figure S11). The docking study was carried out by AutoDockTools-1.5.6rc3 (The Scripps Research Institute, California, CA, USA). Polar hydrogens and Gasteiger charges were added to the resulting AT₁R structure. The structures of G-Rg1 and RA were generated by ChemDraw Ultra 8.0. The size of the grid box was set as 60 Å × 60 Å × 60 Å with a spacing of 0.475 Å. The grid center coordinate was set at –16.520, 7.370, and 34.522 in X, Y, and Z dimensions, which enclosed the conventional orthosteric binding pocket and the newly discovered allosteric domain of the receptor. Fifty docking results were obtained for each compound with AT₁R and the complex with the lowest binding energy was used for further analysis by Discovery Studio 4.5 (Dassault Systemes BIOVIA, California, CA, USA).

**Protein isolation, electrophoresis, and western blotting**

Total proteins from the cells were extracted using RIPA lysis buffer (Solarbio, Beijing, China) with a complete protease inhibitor according to the manufacturer’s instructions. Proteins were subjected to 6% or
10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes by electroblotting. After blocking in tris-buffered saline containing 0.1% Tween-20 and 5% non-fat dry milk for 2 hours at room temperature, the membranes were incubated with the rabbit polyclonal antibody against AT1R (Cat# ab124505; RRD:AB_10976053, 1:1,000, Abcam, Cambridge, UK) overnight at 4°C. After washing 3 times, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 2 hours at room temperature. Immunoblots were revealed by enhanced chemiluminescence reaction and visualized using a high-performance chemiluminescence film. Images were captured by Clinx ChemiScope 6100 Pro (Shanghai, China), and densitometry analysis was conducted using Clinx Image Analysis Software (Shanghai, China). The expression of the proteins was normalized by the housekeeping protein β-actin.

**Intracellular Ca^{2+} release measurement**

Calcium levels were measured using the Fura-2, AM, Cell Permeant (Yeasen, Shanghai, China) as a calcium fluorescent probe. HBZY-1 cells (100,000 cells/well) were seeded in a 96-well clear bottom black plate. Following serum starvation of the cells, the calcium-sensitive dye was added at a final concentration of 1.0 μM. Fluorescence Microplate Reader (FLx800, BioTek, Winooski, VT, USA) was programmed to add ligands to the cells and monitor the fluorescence before and after the addition of ligands. The concentrations of Ang II (3–8) were in the range of 0.01 pM–100 μM. RA was added in a defined concentration of 30 μM and G-Rg1 was added at final concentrations of 5, 10, 30, and 50 μM. Changes in intracellular calcium were recorded by measuring \( \Delta F/F \) (max-min) and are represented as relative fluorescence units. All the experiments were conducted in triplicate.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Unless otherwise stated, all experimental results are obtained from at least three independent experiments and error bars represent the standard error of the mean (SEM). Nonlinear chromatographic data processing was carried out using Peakfit v4.11 (SPSS Inc., Chicago, IL, USA). The data of RA and G-Rg1 were fitted with the ‘Dose-Response EC50 shift’ and ‘Allosteric EC50 shift’ functions embedded in GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA), respectively. Visualization of the molecular docking results was achieved using Discovery Studio 4.5 (Dassault Systemes BIOVIA, California, CA, USA). Statistical details are all provided in the figure legends.