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Cell active and functionally-relevant small-molecule agonists of calcitonin receptor

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ABSTRACT

The natural calcitonin (CT) receptor and its peptide agonists are considered validated targets for drug discovery. A small molecule agonist, SUN-B8155, has previously been shown to efficiently activate cellular CTR. Herein, we report the synthesis of a series of compounds (S8155 1-9) derived from SUN-B8155, and investigate the structural-functional relationship, bias properties and their cellular activity profile. We discover that the *N*-hydroxyl group from the pyridone ring is required for G protein activity and its affinity to the CT receptor. Among the compounds studied, S8155-7 exhibits improved G protein activity while S8155-4 displays a significant β-arrestin-2 signaling bias. Finally, we show that both S8155-4 and S8155-7 inhibit tumour cell invasion through CTR activation. These two compounds are anticipated to find extensive applications in chemical biology research as well drug development efforts targeting CT receptor.

1. Introduction

CTR is the most ancient member of the class B GPCR family and is widely expressed in many tissues of the human body [1–3]. It is known that the CTR has multiple functions, including but not limited to maintaining the quiescent state of muscle stem cells [4], limiting bone loss, promoting osteoclast survival [5–7], regulating brain function, and contributing to cancer progression [8–14]. Therefore, it is not surprising that CTR has been an important therapeutic target for the treatment of osteoporosis, Paget's disease, hypercalcaemia of malignancy, and cancer [3,15].

The natural CTR peptide agonists derived from both human and salmon calcitonin have been used clinically to treat osteoporosis and Paget's disease [15,16]. Recent studies have also developed mimetic calcitonin peptides, including KBP-042, KBP-088 and KBP-089, which not only preserved the high efficacy of salmon calcitonin but also showed increased tolerability in rats [15–19]. However, due to the cost and to potential side effects with unclear mechanisms, these peptide-based therapies still experience limited use [16]. Alternatively, small-molecule agonists of CTR with defined signalling pathways, which may have a longer half-life in the body than peptide agonists, have broader application potentials. To date, only one small-molecule agonist SUN-B8155 was reported to effectively activate CTR [20]. CTR activates both G proteins and β-arrestin-2 signalling [21–36]. It was proposed that the functions of inhibition of cancer progression and limiting bone loss mediated by CTR are dependent on Gs signalling [11,10,15]. However, functional selectivity of different downstream effectors, such as arrestins downstream of CTR have not been elucidated yet, partially

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due to the lack of functionally-selective CTR agonists.

Recently, development of biased agonists with functional selectivity, which signal with different efficacies to a particular receptor's multiple downstream pathways (e.g. individual G protein subtype or β-arrestin pathways) are useful tools for dissection of specific signaling pathways[29,30,49–52]. These biased ligands may also possess better therapeutic potential because they can avoid "harmful" effect selectively, compared to "balanced agonists" [46,47]. Therefore, development of small molecules eliciting biased CTR functions will be valuable tools in studying selective CTR downstream functions. However, the β-arrestin activity of the only known CTR small-molecule agonist SUN-B8155 has not been fully characterized [20].

In the present study, we synthesized a series of compounds (S8155-1 to S8155-9) based on the chemical structure of SUN-B8155, and investigated the bias property using SUN-B8155 as control. Compound S8155-7 displayed a Gs biased property and at least five SUN-B8155 derivatives exhibited β-arrestin-2 biased signalling properties. Cellular transwell studies demonstrated that compounds SUN-B8155, S8155-4 and S8155-7 inhibited breast cancer cells (MCF-7) migration via activation of CTR.

2. Materials and methods

2.1. Chemical synthesis

2.1.1. Synthetic procedure for 5-acetyl-1,6-dihydroxy-4-methyl-2(1H)-pyridone (1)

Hydroxylamine hydrochloride (9 g, 130 mmol), water and acetonitrile (2:1 v/v, 6 mL) were added to a 250 mL three-necked flask. At 0 °C, triethylamine (60 mL, 260 mL) was slowly added to the reaction mixture, and then diketene (20 mL, 260 mmol) dissolved in a mixture of water and acetonitrile (2:1 v/v, 12 mL) was added dropwise to the reaction solution while the temperature was maintained below 5 °C. After the completion of addition of diketene solution, the reaction mixture was stirred at 0 °C for 30 min, and then allowed to stand at ambient temperature for 1 h. 6 N HCl aqueous solution was slowly added to the reaction mixture to adjust the pH to 7 that resulted in formation of a white suspension. The precipitated solid was filtered and washed with water, and the crude product was recrystallized from ethanol to obtain pale yellow solid **1** (11.9 g, 50% yield). Mp 195–197 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 5.81 (s, 1H), 2.56 (s, 3H), 2.32 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 194.3, 165.1, 157.9, 149.1, 107.6, 104.7, 28.0, 23.3.

2.1.2. General procedure for synthesizing SUN-B8155 and its derivatives (S8155-1, -2, -4-9)

A mixture of **1** (300 mg, 1.6 mmol) and substituted aniline (1.6 mmol) in EtOH (12 mL) was heated at 80 °C for 3 h. After the reaction was completed, the reaction solution was cooled to ambient temperature, resulting in precipitates. The solid was obtained by suction filtration, washed with cold ethanol and air-dried. **SUN-B8155** (R = 2-NH_2): 75% yield, pale yellow crystal. Mp 196–198 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 14.36 (s, 1H), 10.06 (s, 1H), 7.12–7.07 (m, 1H), 7.00 (dd, J = 7.8 Hz, 1.5 Hz, 1H), 6.82 (dd, J = 8.1, 1.2 Hz, 1H), 6.65–6.60 (m, 1H), 5.65 (s, 1H), 5.26 (s, 2H), 2.37 (s, 6H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 170.7, 164.3, 158.8, 148.6, 144.0, 128.8, 127.1, 121.4, 116.3, 115.8, 109.3, 99.2, 25.3, 20.2. HRMS (ESI, positive) Calcd for $C_{14}H_{15}N_3NaO_3$ $[M+Na]^+$ 296.1006, found: 296.1007.

S8155-1 (R = 4-F): 40% yield, pale yellow solid. Mp 215–216 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 14.72 (s, 1H), 10.05 (s, 1H), 7.34–7.23 (m, 4H), 5.61 (s, 1H), 2.35 (s, 3H), 2.28 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 169.1, 164.4, 162.6, 159.3, 159.0, 148.6, 133.2, 128.0, 127.9, 116.6, 116.3, 99.3, 25.2, 20.7. HRMS (ESI, positive) Calcd for $C_{14}H_{13}FN_2O_3H$ $[M+H]^+$ 277.0983, found: 277.0984.

S8155-2 (R = 3-NH_2)**:** 63% yield, pale yellow solid. Mp 214–216 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 14.77 (s, 1H), 10.10 (s, 1H), 7.12 (s, 1H), 6.57 (d, J = 6.0 Hz, 1H), 6.43 (s, 2H), 5.68 (s, 1H), 5.43 (s, 2H), 2.48 (s, 3H), 2.37 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 168.8, 164.3, 158.9, 150.0, 148.6, 137.3, 129.9, 113.0, 112.6, 110.1, 109.8, 98.9, 25.2, 20.7. HRMS (ESI, positive) Calcd for $C_{14}H_{15}N_3O_3H$ $[M+H]^+$ 274.1186, found: 274.1184.

S8155-4 (R = 4-NH_2)**:** 63% yield, pale yellow solid. Mp 251–253 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 14.62 (s, 1H), 10.04 (s, 1H), 6.95 (d, J = 8.7 Hz, 2H), 6.63 (d, J = 8.7 Hz, 2H), 5.63 (s, 1H), 5.42 (s, 2H), 2.43 (s, 3H), 2.34 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 168.9, 164.0, 158.8, 148.5, 148.4, 126.3, 124.5, 114.0, 109.3, 98.7, 25.2, 20.6; HRMS (ESI, positive) Calcd for $C_{14}H_{15}N_3NaO_3$ $[M+Na]^+$ 296.1006, found: 296.1004.

S8155-5 (R = 2-MeO)**:** 76% yield, pale yellow solid. Mp 238–240 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 14.72 (s, 1H), 10.09 (s, 1H), 7.38–7.31 (m, 2H), 7.21–7.19 (m, 1H), 7.08–7.05 (m, 1H), 5.67 (d, J = 0.6 Hz, 1H), 3.84 (s, 3H), 2.43 (s, 3H), 2.37 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 169.1, 164.4, 158.9, 153.0, 148.4, 128.9, 127.0, 125.2, 120.6, 112.3, 110.0, 99.3, 55.9, 25.3, 20.7. HRMS (ESI, positive) Calcd for $C_{15}H_{16}N_2NaO_4$ $[M+Na]^+$ 311.1002, found: 311.1004.

S8155-6 (R = 3-Cl)**:** 67% yield, pale yellow solid. Mp 204–206 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 14.84 (s, 1H), 9.24 (s, 1H), 7.41–7.31 (m, 2H), 7.21 (s, 1H), 7.11 (d, J = 7.5 Hz, 1H), 5.81 (s, 1H), 2.46 (s, 3H), 2.37 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 168.8, 162.1, 158.1, 148.3, 137.9, 135.4, 130.8, 128.3, 126.2, 124.3, 110.3, 100.0, 26.2, 21.2. HRMS (ESI, positive) Calcd for $C_{14}H_{13}ClN_2NaO_3$ $[M+Na]^+$ 315.0507, found: 315.0508.

S8155-7 (R = 2-Me)**:** 45% yield, pale yellow solid. Mp 197–199 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 14.74 (s, 1H), 9.24 (s, 1H), 7.33–7.26 (m, 3H), 7.15–7.12 (m, 1H), 5.85 (s, 1H), 2.42 (s, 3H), 2.41 (s, 3H), 2.30 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 169.7, 161.9, 158.0, 148.3, 135.7, 134.0, 131.4, 128.6, 127.0, 126.7, 109.4, 99.5, 26.2, 21.0, 18.1. HRMS (ESI, positive) Calcd for $C_{15}H_{16}N_2NaO_3$ $[M+Na]^+$ 295.1053, found: 295.1053.

S8155-8 (R = 3-Br)**:** 50% yield, pale yellow solid. Mp 227–229 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 14.86 (s, 1H), 10.14 (s, 1H), 7.62–7.57 (m, 2H), 7.45 (t, J = 7.8 Hz, 1H), 7.35 (d, J = 7.8 Hz, 1H), 5.71 (s, 1H), 2.47 (s, 3H), 2.36 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 168.6, 164.4, 158.9, 148.5, 138.5, 131.3, 130.2, 128.5, 124.9, 122.0, 110.6, 99.7, 25.1, 20.9. HRMS (ESI, positive) Calcd for $C_{14}H_{13}BrN_2O_3H$ $[M+H]^+$ 337.0182, found: 337.0180.

S8155-9: 71% yield, pale yellow solid. Mp 231–233 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 15.24 (s, 1H), 10.23 (s, 1H), 8.08–7.90 (m, 3H), 7.66–7.58 (m, 4H), 5.75 (s, 1H), 2.42 (s, 3H), 2.39 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 169.9, 164.8, 158.9, 148.6, 133.8, 132.8, 128.6, 128.4, 128.1, 127.7, 127.0, 125.7, 124.3, 121.9, 110.3, 99.6, 25.2, 20.9. HRMS (ESI, positive) Calcd for $C_{18}H_{16}N_2NaO_3$ $[M+Na]^+$ 331.1053, found: 331.1049.

2.1.3. Synthetic procedure for S8155-3

A 50 mL round-bottom flask was charged with **1** (300 mg, 1.6 mmol), Raney nickel (30 mg), and methanol (15 mL), and the reaction was carried out at ambient temperature and under H_2 atmosphere for 12 h. After the reaction was completed, the catalyst was removed by filtration, and the crude product was recrystallized to afford 5-acetyl-6-hydroxy-4-methyl-2(1*H*)-pyridone (**2**) (207 mg, 80% yield) as white crystal. ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.07 (s, 1H), 5.85 (s, 1H), 2.70 (s, 3H), 2.49 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 195.0, 169.3, 161.5, 151.9, 110.4, 103.8, 28.1, 23.5.

To a 50 mL round bottom flask was added **2** (196 mg, 1.3 mmol), *O*-phenylenediamine (138 mg, 1.3 mmol) and ethanol (10 mL), and the mixture was heated to reflux for 3 h. After the disappearance of the starting material, the reaction solution was cooled to ambient temperature, and the resulting solid (213 mg, 60% yield) was obtained by suction filtration, washing and air-drying.

S8155-3: Mp 230–232 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 14.56 (s, 1H), 10.75 (s, 1H), 7.10–7.04 (m, 1H), 6.98–6.95 (m, 1H), 6.82–6.80

(m, 1H), 6.64–6.58 (m, 1H), 5.52 (s, 1H), 5.23 (s, 2H), 2.35 (s, 3H), 2.33 (s, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 169.8, 168.6, 163.1, 152.1, 143.9, 128.6, 127.2, 121.6, 116.2, 115.7, 110.9, 99.2, 25.4, 19.9. HRMS (ESI, positive) Calcd for $C_{14}H_{15}N_3O_2H$ $[M+H]^+$ 258.1237, found: 258.1241.

2.2. Constructs

Wild-type human CTR was generated by DNA synthesis and modified to include a C-terminal YFP tag in a mammalian expression vector pcDNA3.1. Plasmid encoding β-arrestin-2 was a generous gift from Dr. R.J Lefkowitz at Duke University. The GloSensor plasmid was obtained from Promega. All constructs were verified by DNA sequencing.

2.3. Measurements of cAMP accumulation

HEK293 cells were co-transfected with CTR-YFP and GloSensor using PEI (Polysciences) according to the manufacturer's instructions. Twenty-four hours later, transfected cells were seeded into 96-well plates at a density of 30,000 cells per well. For measurement of intracellular cAMP accumulation, cells were incubated with GloSensor cAMP reagent for two hours and then stimulated with different CTR ligands. The cAMP signal was measured by the plate reader Mithras LB940 (2013, Berthold Technologies).

2.4. BRET assay

For measurement of β-arrestin-2 recruitment, HEK293 cells were co-transfected with plasmids encoding CTR-YFP and Luc-β-arrestin-2 for 48 h. After 12 h of starvation (with low glucose medium culture), cells were harvested and washed at least three times with PBS, and then cells were stimulated with different CTR ligands for 10 min at 37 °C. Subsequently, transfected cells were incubated with Coelenterazine h (Promega S2011, final concentration of 5 μM) at rt, and two different light emissions were used for measurement (480 nm for luciferase and 530 nm for yellow fluorescent protein). All the BRET measurements were performed by Mithras LB940 (2013, Berthold Technologies), and the signal was determined by calculating the ratio of light intensity emitted by yellow fluorescent protein over the intensity emitted by luciferase.

2.5. Bias property quantification

The bias of CTR ligands are quantified according to previous studies (shown as Eq. (1) at the end of the paragraph) [40,48], Both in cAMP assay and arrestin recruitment BRET assay, concentration-dependent response curves were used to determine the EC50 and Emax using Graphpad. The P1 and P2 denote signaling through GS or β-arrestin-2 activity respectively. "*lig*" is the abbreviation used for "ligand", which denotes the 9 SUN8155 derivatives. "*ref*" is the abbreviation used for "reference", which denotes the compound SUN-B8155. β is the bias factor, which is calculated as the logarithm of the ratio of intrinsic relative activities for a ligand at two different assays compared with a reference agonist. A bias factor of 1 between two pathways means that a ligand is 10 times better for one pathway over the other pathway compared with the reference balanced agonist [40].

$$\beta = \left(\left(\frac{E\max,P1\, EC50,\ P2}{EC50,\ P1\, E\max,P2} \right) lig \left(\frac{E\max,P2\, EC50,\ P1}{EC50,\ P2\, E\max,P1} \right) ref \right) \quad (1)$$

2.6. Transwell® assay

Transwell assays were performed by using transwell chambers with an 8.0 μm membrane. Briefly, MCF-7 cells were transfected with CTR-YFP using PEI (Polysciences) according to the manufacturer's instructions. After 24 h, transfected cells were added to the upper chamber and cultured in 10% FBS, while the lower chamber was supplied with DMEM containing 10% FBS and different CTR ligands. After 48 h, cells on the lower surface of membrane were fixed and stained with crystal violet. For each well, cell counting was performed in ten randomly selected fields with absorbance measured at 490 nm. Each experiment was performed in triplicate and repeated at least three times. sCT is the agonist of CTR and SCT 8-32 is the antagonist of CTR, both of which were used at a concentration of 1 μM. SUN-B8155, S81554 and S8155-7 were used at a concentration of 10 μM.

2.7. Statistics

All data were present as mean ± SEM. The EC50 values for cAMP assays and BRET assays were calculated using Graphpad Prism 6 software. The differences between groups in Transwell® assays were analyzed using one-way analysis of variance (one-way ANOVA). P-values less than 0.05 were statistically significant.

3. Results and discussion

3.1. Synthesis of the control compound SUN-B8155 and its derivatives

The synthetic routes for synthesizing SUN-B8155 and its derivatives are described in Fig. 1. Hydroxylamine hydrochloride reacted with two equivalents of diketene in the presence of triethylamine, resulting in the formation of 5-acetyl-1, 6-dihydroxy-4-methyl-2(1*H*)-pyridone (**1**) in only 26% yield [37,38]. When a mixture of water and acetonitrile (2:1 v/v) was used as solvent, the reaction yield was increased to 50%. Different bases (such as pyridine, LiOH, NaOH, K_2CO_3, etc.) have been used to replace triethylamine, but none improved the yield. Ketone **1** and the substituted anilines were heated at reflux in EtOH [39] to afford SUN-B8155 and its derivatives (S8155-1, S8155-2, S8155-4 to S8155-9), respectively. Catalytic reduction of **1** with Raney nickel in MeOH gave 5-acetyl-6-hydroxy-4-methyl-2(1*H*)-pyridone (**2**) in 80% yield. Under the same reaction conditions used for the preparation of SUN-B8155 and its derivatives, **2** was reacted with one equivalent amount of *O*-phenylenediamine to generate S8155-3 in 60% yield (Fig. 1). The structural characterization and purity of all the compounds described above were confirmed by their spectral properties (Supplemental file). Detailed information can be seen in "2.1 Chemical synthesis" in the section "Materials and Methods".

3.2. Biological characterization of SUN-B8155 and its derivatives

Calcitonin, a 32-amino acid peptide hormone secreted mainly from the thyroid gland, plays an important role in maintaining bone homeostasis [42-45]. Previous research showed that SUN-B8155 is a pyridine derivative that mimic the biological activities of CT, acting via the CTR [20]. SUN-B8155 elevates cAMP accumulation in human and rat cells and this effect was blocked by CTR antagonist (sCT 8-32). Here, we determined the ability of SUN-B8155 to activate Gs protein signalling and the recruitment of β-arrestin-2 in CTR-overexpressing HEK293 cells. The EC_{50} value of SUN-B8155 for eliciting intracellular cAMP accumulation levels through human CTR was determined to be 17.3 ± 2 μM, whereas the EC_{50} of SUN-B8155 for recruitment of β-arrestin-2 was 80.7 ± 26.5 μM (Table 1 and Fig. 2). Therefore, the synthesised SUN-B8155 elicits both Gs and β-arrestin-2 mediated signalling downstream of CTR activation.

We then measured the ability of SUN-B8155 derivatives (S8155-1 to S8155-9) to promote cAMP accumulation and β-arrestin-2 recruitment in CTR-transfected cells and compared them to that of SUN-B8155 (Table 1 and Fig. 3). Firstly, elimination of the N-hydroxyl group from the pyridone ring abolished the activity of S8155-3 in cAMP accumulation and decreased the EC_{50} of β-arrestin-2 recruitment by at least 6-fold (Fig. 2B and Table 1). This result indicated that an H-bond or polar

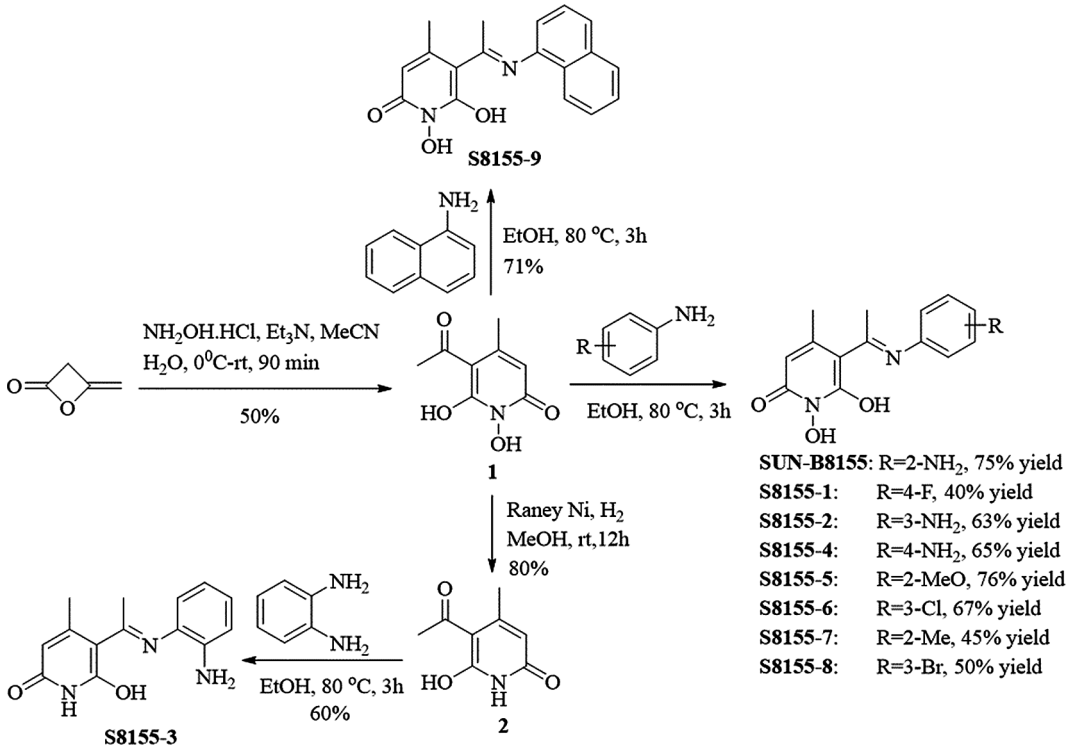

Fig. 1. Synthetic route for SUN-B8155 and its derivatives.

interaction of SUN-B8155 derivatives formed by N-OH with CTR is a key interaction in the mediation of both the binding of the compound to the receptor and its activation of the Gs signalling pathway.

Secondly, moving the NH_2 group of SUN-B8155 from the ortho to meta or para positions in S8155-2 or S8155-4, respectively, greatly improved their EC_{50} in recruiting β-arrestin-2, but has no significant effect on their EC_{50} in cAMP accumulation. In particular, S8155-4 has an EC_{50} of 7.6 ± 2.5 μM and only 10% of the Rmax in cAMP accumulation compared to SUN-B8155. Consistently, the β values for S8155-2 and S8155-4 are calculated as −1.38 and −2.14 respectively, according to Eq. (1). This result suggested that the property of S8155-4 activating the β-arrestin-2 pathway is 100 folds stronger than the Gs pathway, taking SUN-B8155 as the balanced ligand.

Thirdly, among the nine newly synthesised SUN-B8155 analogues, S8155-7 is the only compound showing slightly increased Gs bias property compared to SUN-B8155, with a β value of 0.21 as calculated by Eq. (1) [32,40,41]. It also exhibits an increase in its EC50 value by approximately 1.7-fold without significant effect on Rmax. It is possible that this could be due to the effect of substitution of the NH_2 group of the phenyl ring of SUN-B8155 by the CH_3 group; which may result in increased hydrophobic interactions within the receptor site.

Taken together, by synthesizing and screening the nine SUN-B8155 derivatives, we have acquired a compound with slightly improved Gs signalling (S8155-7), and a compound with significant β-arrestin-2 biased properties (S8155-4). How the differences in chemical structures determine the bias properties of S8155-4 and S8155-7 still waits for further structural elucidations.

3.3. Effects of SUN-B8155 derivatives on tumour cell invasion

Previous studies report that calcitonin and its analogues are involved in cancer cell growth. For example, MCF-7 and other several cancer cell lines contain specific high-affinity receptors for calcitonin and a calcitonin-responsive adenylate cyclase, which have been characterized with the aid of salmon, human calcitonin and their analogues [10,15]. In T 47D breast cancer cell line, low doses of salmon calcitonin

Table 1
Bias properties of the 9 derivatives of SUN-B8155.

Ligand	Gs		β-arrestin-2		β	Bias
	Rmax (%)	EC50 (μM)	Rmax (%)	EC50 (μM)		
SUN-B8155	100.00	17.30 ± 2.05	100.00	80.71 ± 26.59	0.000	
S8155-1	103.83 ± 38.94	477.48 ± 56.58	114.45 ± 28.49	324.29 ± 106.81	−0.879	β-arrestin-2
S8155-2	99.50 ± 21.16	112.45 ± 13.32	136.93 ± 34.09	29.78 ± 9.81	−1.385	β-arrestin-2
S8155-3	–	–	108.32 ± 39.01	538.01 ± 177.25	–	β-arrestin-2
S8155-4	10.13 ± 1.71	25.60 ± 3.03	89.28 ± 22.23	7.59 ± 2.50	−2.142	β-arrestin-2
S8155-5	90.90 ± 6.75	40.83 ± 4.84	81.91 ± 23.84	85.23 ± 28.07	−0.304	β-arrestin-2
S8155-6	39.19 ± 3.90	58.65 ± 6.95	75.69 ± 15.65	65.38 ± 21.53	−0.908	β-arrestin-2
S8155-7	97.14 ± 4.09	11.59 ± 1.37	65.59 ± 9.41	59.24 ± 19.51	0.210	Gs
S8155-8	38.91 ± 6.51	56.57 ± 6.70	138.70 ± 55.15	105.33 ± 30.78	−0.951	β-arrestin-2
S8155-9	101.24 ± 20.87	18.51 ± 2.19	96.50 ± 63.55	41.08 ± 13.53	−0.302	β-arrestin-2

Annotation. Rmax (%) represents the ratio of each derivative's Emax to that of SUN-B8155. β is called bias factor. This bias factor is an estimate for the molecular efficacy of Gs pathway or β-arrestin-2 pathway on a logarithmic scale. If $\beta > 0.000$, it means the bias property of the Gs pathway; if $\beta < 0.000$, it indicates the bias property of the β-arrestin-2 pathway. "–" is a null value which is not be able to be determined.

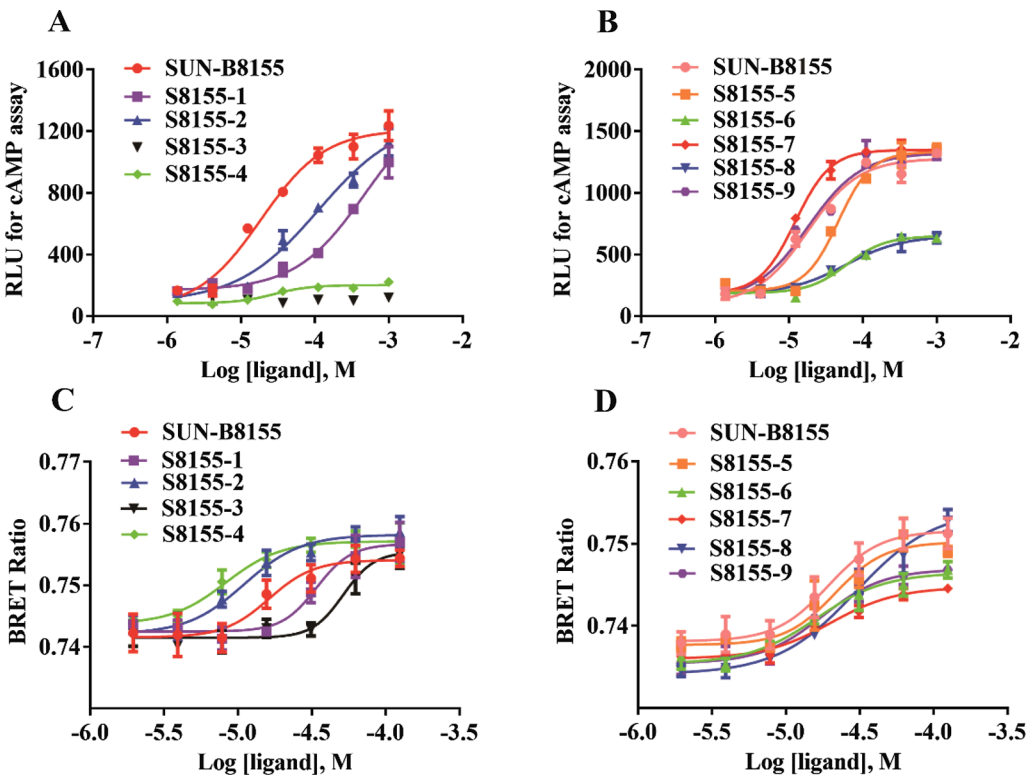

Fig. 2. Measurement of cAMP accumulation and β-arrestin-2 recruitment in response to S8155 1-9 stimulation. (A-B) Effects of S8155 1-9 on cAMP accumulation in HEK293 cells co-transfected with CTR-YFP and GloSensor. "RLU" mean relative light unit which measured the level of cAMP accumulation in living cells. (C-D) Effects of S8155 1-9 on β-arrestin-2 recruitment in HEK293 cells co-transfected with CTR-YFP and Luc-β-arrestin-2. Data are shown as mean ± SEM and representative of 3 individual experiments. The BRET Ratio was determined by dividing the intensity of acceptor (YFP) luminescence emission at 530 nm by the intensity of donor (luciferase) luminescence emission at 480 nm.

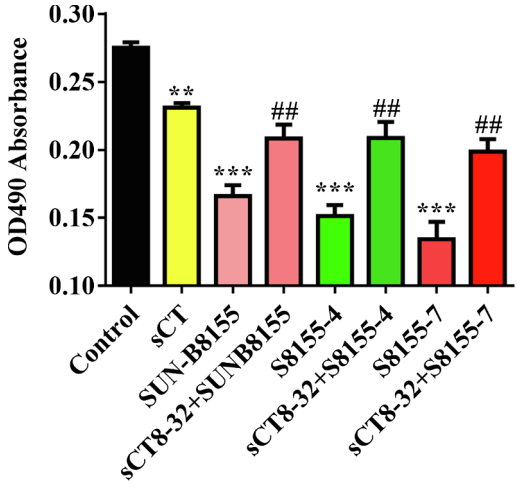

Fig. 3. Effects of different CTR ligands on tumor cell invasion. Transwell assays were performed on MCF-7 cells to detect the effects of different CTR ligands. sCT, salmon calcitonin (1 μM); sCT 8-32 (1 μM); SUN-B8155 and S8155 derivatives were applied at a concentration of 10 μM. $^{**}p < 0.005$, $^{***}p < 0.0001$, inhibition of tumor cell migration by different ligands were compared with that of vehicle. $^{\#\#}p < 0.005$, inhibition of tumor cell migration by treatment with sCT8-32 was compared with no treatment.

initially stimulated cell growth and then followed by an inhibitory effect for cell proliferation and migration, which occurred during the log phase of growth [11]. In DU145 prostate cancer cells, calcitonin induced the inhibition of mitogen-activated kinase and prevented prostate cancer progressing [12]. However, how activation of different CTR downstream effectors participated in the function of CTR in tumor inhibition in these different cellular contexts remain elusive.

Here, we have acquired the S8155-7, a compound that slightly activates more Gs signalling downstream of CTR, as well as a compound S8155-4 with significant β-arrestin-2 bias. These compounds could serve as tools for CTR functional studies. We then used these tools to study the biased signalling of CTR in contribution to its inhibition role in cancer progression in MCF-7 cells. When we treated MCF-7 cells with 1 μM sCT, the tumour invasion ability significantly decreased in the Transwell® assay (Fig. 3). The application of 10 μM SUN-B8155 has a much stronger effect on the invasion of MCF-7, as does the application of 10 μM S8155-4 or 10 μM S8155-7. Importantly, the application of the CTR antagonist sCT (8-32) significantly blocked the ability of SUN-B8155, S8155-4 and S8155-7 to inhibit MCF-7 cell migration (Fig. 3). Therefore, we speculate that the effects of these compounds on MCF-7 cell invasion are partially due to the activation of CTR. However, further in vivo studies are still needed to explain the detailed mechanism of how Gs or β-arrestin-2 mediated pathways contribute to cancer cell migration inhibition downstream of CTR.

4. Conclusion

SUN-B8155 activated both the Gs and arrestin pathways through its engagement with CTR, which is dependent on the hydroxyl group attaching to the pyridine amide of SUN-B8155. Analogues of SUN-B8155 can act as agonists of CTR through the Gs or arrestin pathway at different levels. S8155-7 improved Gs signalling, whereas S8155-4 is β-arrestin-2-biased. S8155-4 and S8155-7 inhibited breast cancer cell migration through CTR agonism.

Our research provides useful chemical tools for studying CTR-mediated signaling pathways. Future work will involve investigating effects of these compounds in different CTR-mediated patho-physiological processes as well as structural determination of the CTR in complex with these small chemical probes. This will facilitate therapeutic development targeting CT receptors.

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Author contributions

Professors JP S, X C and Y Z planned the research. S Z, YM W and XY J finished the synthesis of the 9 derivatives of SUN-B8155 under the guidance of professor X C. SC G and Z G performed the experiment to test the function of the 9 derivatives as agonist to CTR under the guidance of professor JP S and X Y. Professor Y Z gave guidance to analyze the data. SC G, Z G and C Y wrote the paper. Professor X C helped polish the manuscript.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2020.103596.

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