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Discovery of the cyclotide caripe 11 as a ligand of the cholecystokinin-2 receptor

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The cholecystokinin-2 receptor (CCK₂R) is a G protein-coupled receptor (GPCR) that is expressed in peripheral tissues and the central nervous system and constitutes a promising target for drug development in several diseases, such as gastrointestinal cancer. The search for ligands of this receptor over the past years mainly resulted in the discovery of a set of distinct synthetic small molecule chemicals. Here, we carried out a pharmacological screening of cyclotide-containing plant extracts using HEK293 cells transiently-expressing mouse CCK₂R, and inositol phosphate (IP1) production as a readout. Our data demonstrated that cyclotide-enriched plant extracts from *Oldenlandia affinis*, *Viola tricolor* and *Carapichea ipeacavanha* activate the CCK₂R as measured by the production of IP1. These findings prompted the isolation of a representative cyclotide, namely caripe 11 from *C. ipeacavanha* for detailed pharmacological analysis. Caripe 11 is a partial agonist of the CCK₂R ($E_{\max} = 71\%$) with a moderate potency of 8.5 μM , in comparison to the endogenous full agonist cholecystokinin-8 (CCK-8; $EC_{50} = 11.5 \text{ nM}$). The partial agonism of caripe 11 is further characterized by an increase on basal activity (at low concentrations) and a dextral-shift of the potency of CCK-8 (at higher concentrations) following its co-incubation with the cyclotide. Therefore, cyclotides such as caripe 11 may be explored in the future for the design and development of cyclotide-based ligands or imaging probes targeting the CCK₂R and related peptide GPCRs.

The use of plants has attracted wide interest in drug discovery due to the existence of many different and chemically diverse bioactive molecules¹. Natural products play a significant role in the search for novel cures of cancer, cardiovascular diseases, infectious diseases, and immune disorders¹. One class of natural products, namely ribosomally synthesized and post-translationally modified peptides (RiPP) are increasingly being recognized for their potential as drug lead molecules or chemical probes^{2,3}. They are produced in bacteria, archaea, and eukaryotes and since they are true gene products, their biosynthetic precursor sequences are accessible via mining of genomes and transcriptomes.

Their extensive structural variety combined with restricted conformational flexibility—frequently achieved by disulfide bond formation and cyclization—is considered to lead to improved affinity and selectivity for target proteins as well as increased metabolic and chemical stability². As compared to larger proteins and antibody therapeutics, peptides are small in size, have the ability to penetrate cells and tissues, and their synthesis is more cost-effective^{4,5}. Currently, there are over 60 approved peptide drugs on the pharmaceutical market and considerable more in the development pipeline³.

An interesting group of peptides for pharmaceutical applications are plant-derived cyclotides. They belong to the large group of RiPPs^{2,6}, that are endogenously expressed for instance in the plant families of *Cucurbitaceae*, *Rubiaceae*, *Solanaceae*, *Fabaceae*, *Violaceae*, and *Poaceae*⁷. These peptides are about 30 amino acids in size, and they comprise a head-to-tail cyclized backbone stabilized by three disulfide bonds arranged in a knotted topology. Importantly, these unique structural features render them extremely stable in particular against chemical and enzymatic degradation⁸. Intrinsically, cyclotides exhibit various biological activities, including anti-HIV⁹, immunosuppressive¹⁰, protease inhibitory^{11,12}, uterotonic¹³ and cytotoxicity against cancer cells^{14,15}. Besides, cyclotides are amenable to molecular grafting, which allows to introduce an ‘artificial’ peptide sequence into the cyclotide backbone and create a chimeric molecule with novel biological properties¹⁶. For instance grafted

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cyclotide molecules can bind to and activate G protein-coupled receptors (GPCRs)¹⁷, inhibit serine-proteases¹⁸, inactivate vascular endothelial growth factor¹⁹, and stimulate angiogenesis²⁰. Therefore, cyclotides represent an ideal scaffold for drug discovery with potential for the development as imaging probes or drug lead candidates²¹.

In recent years, numerous studies have unveiled the potential of nature-derived peptides as an extensive source for GPCR ligand design^{22,23}. These peptide GPCR ligands have been isolated from various organisms including plants²². Accordingly, over 50 peptides targeting GPCRs have been approved as drugs²⁴. Cyclotides are a rich source for GPCR ligand discovery, and they have previously been demonstrated to modulate the oxytocin/vasopressin V_{1a} receptors²⁵, the corticotropin-releasing factor type 1 receptor⁶, and the κ -opioid receptor²⁶. Here, we explored yet another GPCR target of cyclotides namely the cholecystokinin-2 receptor (CCK₂R). This receptor belongs to an important neuroendocrine system comprising the peptide hormones cholecystokinin (CCK) and gastrin, which mediate their physiological actions through two closely related receptors, i.e. the cholecystokinin-1 receptor (CCK₁R) and the CCK₂R (also referred to as CCK_AR and CCK_BR)^{27,28}. Both receptors are known to be involved in various physiological processes, including the regulation of food intake, increasing pancreatic enzyme secretion and delaying gastric emptying²⁸. Importantly, the CCK₂R has been suggested to participate in tumor development and progression^{27,28}. It is often overexpressed in cancer tissue, in particular in gastrointestinal stromal tumors, medullary thyroid cancers, small cell lung carcinomas and insulinomas tumors²⁹. Therefore, it is not surprising that the CCK₂R has been explored as possible drug target for cancer treatment, since reducing the intrinsic activity or blocking of the receptor has yielded promising results in human studies³⁰. In this study, we (i) screened three cyclotide-enriched plant extracts of *Oldenlandia affinis*, *Viola tricolor*, and *Carapichea ipeacacuanha* for modulation of CCK₂R signaling, (ii) isolated a particular cyclotide (caripe 11) from *C. ipeacacuanha*⁶ and (iii) characterized its pharmacodynamic properties in HEK293 cells overexpressing the CCK₂R.

Materials and methods

Plant material. Plant specimen of *C. ipeacacuanha* (Brot.) L.Andersson and *V. tricolor* L. were purchased as powdered material from Alfred Galke GmbH (Germany; catalogue no. 66804 and 13804, respectively). *O. affinis* DC. was grown from seeds (derived from glasshouse grown plants) obtained as a gift from D. Craik (Australia)²⁵.

Peptide extraction and purification. Dried and powdered *C. ipeacacuanha*, *O. affinis*, and *V. tricolor* were extracted using dichloromethane:methanol (1:1, v/v) in the ratio of 1:10 (w/v) under permanent stirring at 25 °C for 18–24 h. After filtration, 0.5 volume of ddH₂O was added to the extract and the aqueous methanol phase was separated by liquid–liquid extraction in a separation funnel. The lyophilized extract was dissolved in ddH₂O, and peptides were pre-purified in batch using C₁₈ silica resin (40–60 μ m, ZEOprep 60, ZEOCHEM, Switzerland) activated and equilibrated with methanol and solvent A (0.1% trifluoroacetic acid, TFA in ddH₂O, v/v), respectively. The resin was washed with 30% solvent B (90% of acetonitrile, ACN, 9.9% of ddH₂O and 0.1% of TFA, v/v/v) and eluted with 80% solvent B. The eluate was lyophilized and stored at -20 °C until further use. Native cyclotide (caripe 11) was purified by reversed-phase high performance liquid chromatography (RP-HPLC) as previously described^{6,25,26}. The purity was assessed by analytical RP-HPLC column (250 \times 4.6 mm, 5 μ m, 100 Å; Kromasil) at a flow rate of 1 mL/min with a gradient of 5–65% solvent B and by using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) as previously described^{16,25,26}.

Peptide quantification. Peptide quantification was carried out by measuring absorbance at 280 nm using a nanodrop instrument and using Beer-Lambert equation. The molar extinction coefficient (ϵ) for each peptide was determined according to the equation: $\epsilon_{280} = nC \cdot 120 + nW \cdot 5690 + nY \cdot 1280$ [M⁻¹ cm⁻¹], where n is the number of residues.

Cloning of a CCK₂R-encoding gene and expression vector preparation. A tagged ORF clone encoding the mouse CCK₂R was purchased from the OriGene (CAT#: MR222564; Germany). The restriction sites of NheI and XhoI endonucleases were introduced into the CCK₂R cDNA and amplified using the forward primer: 5'-aaaaaagctagcATGGATCTGCTCAAGCTGAACCG-3' and the reverse primer: 5'-aaaaaactcgagGCCAGGCCCCAGCGT-3' (restriction sites are underlined; receptor specific sequence in capital letters). The amplified and digested PCR product was cloned into the pEGFP-N1 plasmid and transfected into competent *E. coli* XL1 cells. Following selection of positive-transfected bacteria, the plasmid was prepared and extracted using the NucleoBond Midi kit (Macherey–Nagel, Germany), quantified using a nanodrop protocol and its sequence confirmed by DNA sequencing. This plasmid produced a receptor with a C-terminal GFP tag; adding a stop codon to the reverse primer, yielded an untagged receptor, which was used for control studies (data not shown).

Cell culture and transfection. Human embryonic kidney (HEK293) cells (Ref.³¹) were maintained in a fresh Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μ g/mL streptomycin in a humidified atmosphere of 5% CO₂ at 37 °C. 2 mL of cell suspension was poured into each well of a 6-well plate and incubated overnight at 37 °C. After reaching to a confluency of 70–80%, the cells were transfected with a plasmid encoding EGFP-tagged CCK₂R using the jetPRIME transfection reagent according to manufacturer's instructions (Polyplus-transfection, USA).

Cell viability assays. Effect on cell viability of plant extract was measured against the HEK293 cell line using the Cell Counting Kit-8 (VitaScientific, USA). Briefly, 100 μ L of medium containing 1×10^4 cells was seeded in each well of a 96-well plate and incubated at 37 °C for 24 h. Afterwards, 10 μ L of various concentration of extract (0–300 μ g/mL) or caripe 11 (0.3, 1, 3, 10, 30, 100 μ M) was added into each well and then incubated

at 37 °C for 2 h. Finally, 10 µL of the kit reagent (final concentration of 10%, v/v) was added into each well and incubated at 37 °C for 3 h. Absorbance was measured at 450 nm using the FlexStation 3 multi-mode microplate reader (Molecular Devices, USA). Triton X-100 and medium were used as positive and negative controls, respectively. The cell viability (CV) percentage was calculated using the following equation: $CV (\%) = (A_S/A_C) \times 100$, whereas A_S and A_C are related to the absorbance of sample and negative control at 450 nm.

Inositol-1-phosphate (IP1) accumulation assay. The receptor-mediated activation of Gq-dependent signaling was measured using a homogenous time-resolved fluorescence (HTRF) based inositol-1-phosphate (IP1) assay kit (Cisbio, France). Approximately $3\text{--}6 \times 10^4$ HEK293 cells expressing the CCK₂R were seeded into each well of a 96- or 384-well plate and incubated overnight at 37 °C. Afterwards, the medium was removed, and the cells were stimulated with ‘stimulation buffer’ at 37 °C for 15–30 min followed by incubation with various concentrations of extracts [100 and 300 µg/mL for Caripe (*C. ipecacuanha* extract), 100 µg/mL for Oaff (*O. affinis* extract), and 300 µg/mL for Vitri (*V. tricolor* extract)] and caripe 11 (3, 10 and 30 µM), alone or in combination with 70 nM (EC₈₀) of CCK-8 endogenous agonist. Antagonists YM-022 and LY225910 (500 nM or 1 µM, both Sigma -Aldrich, Austria) were preincubated at 37 °C for 30 min. After adding IP1-d2 conjugate and anti-IP1 Eu Cryptate according to manufacturers’ instructions, the plate was incubated at room temperature for 3 h. The HTRF measurement was carried out using the FlexStation 3 multi-mode microplate reader with excitation at 330 nm and emissions at 620 and 665 nm.

Data analysis. All experiments were performed in triplicate, analyzed using the GraphPad prism software (GraphPad Software, USA) and expressed as mean ± SD (standard deviation). Pharmacological data of concentration response curves were normalized to the maximal response of CCK-8 detected at the highest concentration. The potency (EC₅₀) and maximum efficacy (E_{max}) were calculated from concentration response curves fitted to the three-parameter non-linear regression with a top and bottom constrained to 100% (for CCK-8) and 0, respectively. Single concentration efficacy data were normalized to baseline (assay buffer) and data were expressed as IP1 formation (fold difference over baseline).

Ethics statement. The study was carried out in accordance with relevant guidelines and regulation.

Results

Preparation and analysis of cyclotide-containing extracts. For this study we chose three representative cyclotide producing plants, namely *O. affinis* (Oaff), *V. tricolor* (Vitri) and *C. ipecacuanha* (Caripe). The cyclotides elute late in RP chromatography due to their hydrophobic surface properties and a molecular mass between m/z 2700–3500 in MALDI-TOF MS⁶. The initial aqueous extracts of Oaff, Vitri and Caripe were prepared by maceration and pre-purified with C₁₈ solid-phase extraction to remove polar compounds. Afterward, the presence of cyclotides in these extracts was confirmed and analyzed using RP-HPLC and MALDI-TOF MS (Fig. 1). Cyclotides were assigned by molecular weight according to previous studies^{6,31–33}. Accordingly, the major cyclotides (based on their relative peak intensity in MALDI-TOF MS) determined in the Caripe extract were caripe 13 (monoisotopic [M + H]⁺: 3237.15 Da), caripe 8 (3238.12 Da), caripe 7 (3254.08 Da), caripe 11 (3282.12 Da), caripe 12 (3288.06 Da), and caripe 10 (3302.05 Da); in the Oaff extract there were kalata B1 (2890.73 Da), kalata B2 (2953.65 Da), and kalata B7 (3069.79 Da) and in the Vitri extract there were vigno 5 (2858.93 Da), kalata S (2876.67 Da), kalata B1 (2890.66 Da), vigno 4 (2904.66 Da) and vitri 2 (3138.85 Da), respectively (Table 1). Next, we opted to determine the GPCR-modulating activity of cyclotide extracts at the CCK₂ receptor. Since the extracts still contain many peptides and ‘contaminations’ of small molecules, we first examined suitable concentrations to be used for the pharmacological experiments by performing cell viability assays.

Cell viability assays of cyclotide extracts. The viability of HEK cells to be used for the IP1 second messenger accumulation experiments was determined with extracts of Caripe, Oaff and Vitri. Accordingly, Caripe had no major influence on viability against HEK293 cells up to 100 µg/mL with > 65% of cells remaining viable at 300 µg/mL (Fig. 2a). In addition, the Oaff extract showed no effect at concentrations of < 100 µg/mL, while increasing concentrations of up to 300 µg/mL led to a decrease in viability after 2 h of incubation (Fig. 2b). The Vitri extract exhibited no major effects on viability up to concentrations of 300 µg/mL (Fig. 2c). Therefore, concentrations of up to 300 µg/mL of Caripe and Vitri extracts, and 100 µg/mL of Oaff extract were chosen for further analysis in the IP1 assay.

Modulating effects of cyclotide plant extracts at CCK₂R. Cyclotide-containing extracts of Caripe, Oaff and Vitri were screened for their pharmacological activity at the CCK₂R to detect agonism and antagonism using a commercially available IP1 second messenger assay and HEK293 cells transiently expressing EGFP-tagged CCK₂R. The pharmacological effect of Caripe extract was determined with two concentrations of 100 and 300 µg/mL, respectively, whereas one concentration was used for the Oaff (100 µg/mL) and Vitri (300 µg/mL) extracts. The functionality of the receptor was validated by CCK-8, the endogenous CCK₂R peptide ligand. As expected, CCK-8 produced a concentration-dependent increase in IP1 (Fig. 3), an effect that was absent in non-transfected HEK293 cells (data not shown). All three plant extracts produced a moderate accumulation of IP1 (Fig. 3): Caripe at 300 µg/mL (0.16 fold difference over baseline), Oaff at 100 µg/mL (0.15) and Vitri at 300 µg/mL (0.17) had a comparable efficacy at IP production as the endogenous agonist CCK-8 at 1 and 3 nM (0.09 and 0.31), respectively. These results indicate the presence of molecules in these extracts that are capable of activating

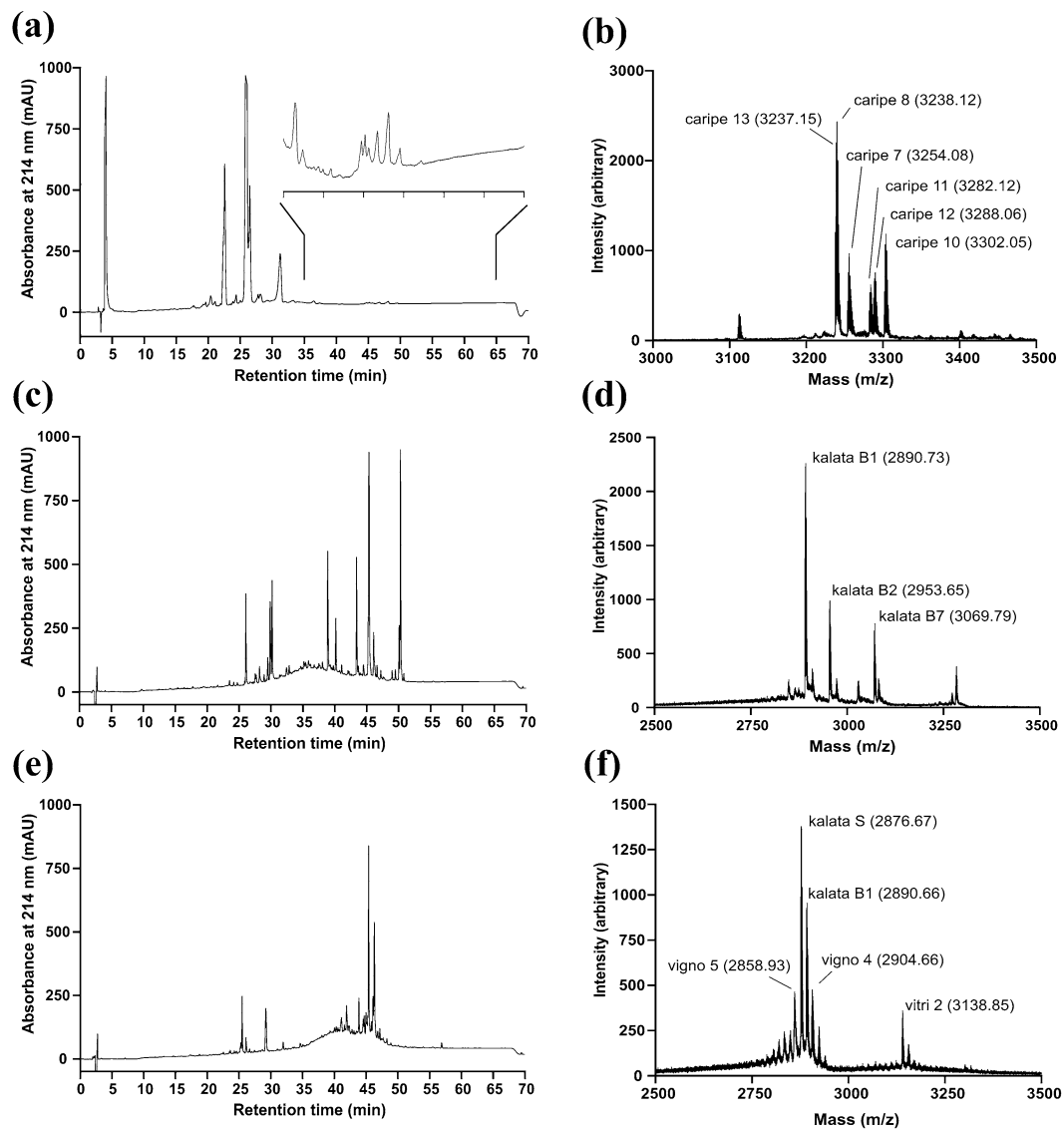


Figure 1. Analytical profile of cyclotide-containing plant extracts. Analytical RP-HPLC chromatograms of the extracts of *C. ipecacuanha* (a) with magnification of the cyclotide eluting region (inset), *O. affinis* (c), and *V. tricolor* (e). MALDI-TOF MS of the extracts of *C. ipecacuanha* (b), *O. affinis* (d), and *V. tricolor* (f), including labelling of identified major cyclotides according to their monoisotopic $[M+H]^+$ ions (in parentheses) and common name (according to the CyBase⁵⁵).

the CCK₂R. Given its ethnopharmacological importance as former phyto-medicine, a representative extract of Caripe (also known as the ‘syrup of ipecac’³⁴) was chosen for isolation of a purified cyclotide and further analysis to determine the pharmacological effect of a cyclotide at the CCK₂R.

Purification of caripe 11. Previously, we have demonstrated the pharmacology-guided isolation of cyclotides from *C. ipecacuanha* and other cyclotide-containing plants^{6,33}. *C. ipecacuanha* is known to contain several cyclotides with closely related sequences (Table 1)^{6,33}. Therefore, we isolated a representative cyclotide, namely caripe 11, to determine its properties to modulate CCK₂R signaling. The cyclotide was purified using preparative RP-HPLC⁶ and its purity and identity were determined using analytical RP-HPLC and MALDI-TOF MS, respectively, which yielded the cyclotide caripe 11 with 99.8% purity and a molecular weight of 3281.3 Da (Fig. 4). For cell-based assays, the concentration of a caripe 11 solution was calculated using the absorbance at 280 nm (extinction coefficient: 2000 M⁻¹ cm⁻¹). Furthermore, we determined its effect on cell viability; concentrations of ≤ 30 μM of caripe 11 did not have a pronounced effect, but the concentration of 100 μM slightly decreased HEK cell viability (Fig. 2d). Therefore, concentrations of up to 30 μM of caripe 11 were considered as appropriate for further analysis.

Cyclotide	Amino acid sequence	Molecular weight (Da)*
kalata B7	GLPVCGETCTLGTCTY---TQGCCTCSWPICKRN	3069.27
kalata B2	GLPVCGETCFGGTCN---TPGCSCTWPICTRD	2953.14
vigno 5	GLPLCGETCVGGTCN---TPGCSCGWPVCVRN	2858.15
vigno 4	GLPLCGETCVGGTCN---TPACSCSWPVCVRN	2904.16
kalata B1	GLPVCGETCVGGTCN---TPGCTCSWPFVCTRN	2890.14
kalata S	GLPVCGETCVGGTCN---TPGCSCSWPFVCTRN	2876.13
caripe 7	G-IPCGESCVFIPCTVTALLGCSCKNKVCYRN	3253.47
vitri 2	GSIPCGESCVWIPCI-SGIAGCSCSNKVCYLN	3138.32
caripe 13	G-IPCGESCVFIPCF-TSVFGCSCKDKVCYRN	3237.37
caripe 8	GVIPCGESCVFIPCI-TAAIGCSCKKKVCYRN	3237.51
caripe 11	GVIPCGESCVFIPCI-STVIGCSCKKKVCYRN	3281.53
caripe 10	GVIPCGESCVFIPCF-STVIGCSCKNKVCYRN	3301.47
caripe 12	GVIPCGESCVFIPCF-SSVIGCSCKNKVCYRN	3287.45

Table 1. Sequence alignment and molecular weight of cyclotides analyzed in plant extracts from *O. affinis*, *V. tricolor*, and *C. ipecacuanha*. *Molecular weight is provided as monoisotopic mass taken from CyBase⁵⁵.

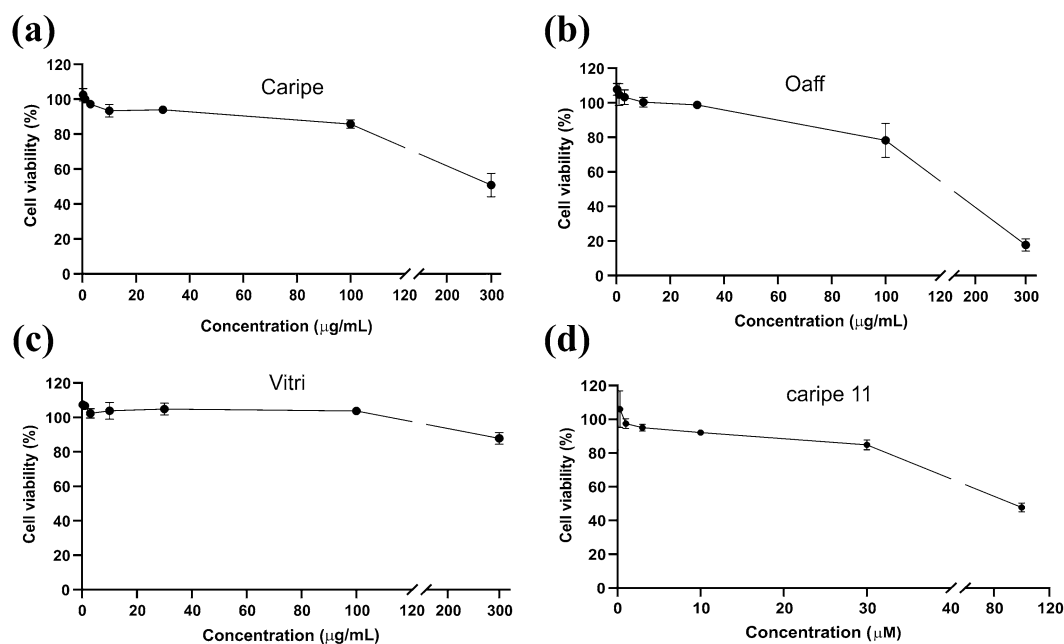


Figure 2. Cytotoxicity of plant extracts and purified cyclotides. Viability of HEK293 cells was determined using the cell counting kit-8 after 2 h incubation with varying concentrations (as indicated in the graphs) of (a) extracts of *C. ipecacuanha* (Caripe), (b) *O. affinis* (Oaff), (c) *V. tricolor* (Vitri), as well as (d) purified caripe 11. Cell viability was calculated as percentage using an equation described in the Materials and Methods section using absorbance at 450 nm. Data represent technical triplicates ($n = 2$) and were expressed as mean \pm SD.

Caripe 11 is a partial agonist of the CCK₂R. Partial agonists of GPCRs are defined as ligands that trigger submaximal efficacy at the receptor as compared to full agonists³⁵. To determine the activity of caripe 11 we measured concentration–response curves of the CCK-8 control agonist and caripe 11 using HEK293 cells transiently expressing CCK₂R. By definition, CCK-8 is an endogenous peptide ligand that per se fully activates the receptor. Our results demonstrate that CCK-8 activates the receptor with a potency (EC_{50}) of 11.5 nM. On the other hand, caripe 11 activates the receptor with a submaximal efficacy ($E_{\text{max}} = 71\%$) and a potency of 8.5 μM compared to CCK-8 (Fig. 5a and Table 2). This suggests that caripe 11 is a partial agonist of the CCK₂R with moderate potency. To confirm the pharmacological mechanism of caripe 11, we determined the effects of three concentrations of caripe 11 upon pre-treatment of cells with an EC_{80} concentration of CCK-8. As expected, caripe 11 concentration-dependently decreased the EC_{80} effect of CCK-8 when both ligands were co-incubated. Accordingly, caripe 11 (30 μM) was able to reduce the EC_{80} effect (70 nM) of CCK-8 by $\sim 20\%$ (Fig. S1a). Furthermore, in the concentration–response curve the basal activity of CCK-8 was increased (0 to 17%) upon co-

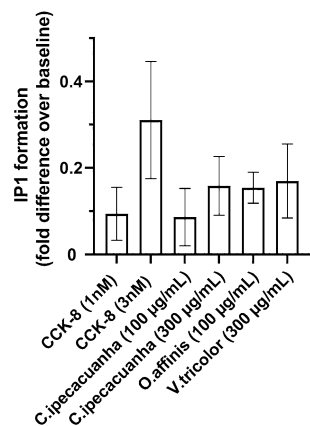


Figure 3. Pharmacological activity of the plant extracts at the CCK₂R. Agonist efficacy of the plant extracts of *C. ipecacuanha* (Caripe), *O. affinis* (Oaff), and *V. tricolor* (Vitri) were measured by IP1 quantification (fold difference over baseline). Receptor activation was presented as ‘fold difference over baseline’, and data were expressed as mean \pm SD (n = 4–5, except Caripe 300 μ g/mL is n = 2). Stimulation buffer (not shown) and CCK-8 peptide (1 and 3 nM, n = 3–4) were used as negative and positive controls, respectively.

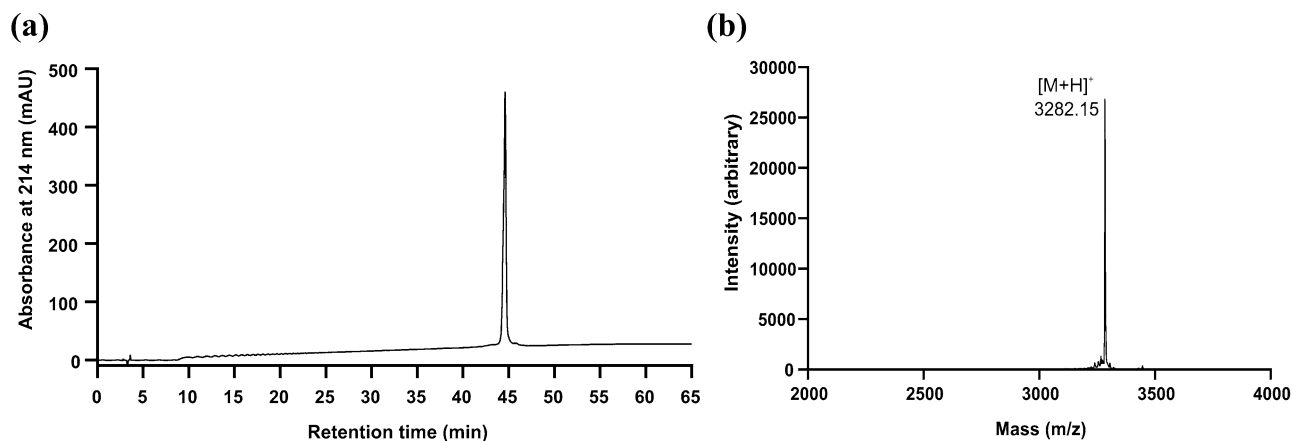


Figure 4. Analytical profile of native caripe 11 cyclotide isolated from *C. ipecacuanha*. The quality control using RP-HPLC (a) and MALDI TOF MS (b) with yielded 99.8% purity, whereas labeled m/z refers to monoisotopic $[M+H]^+$ ion.

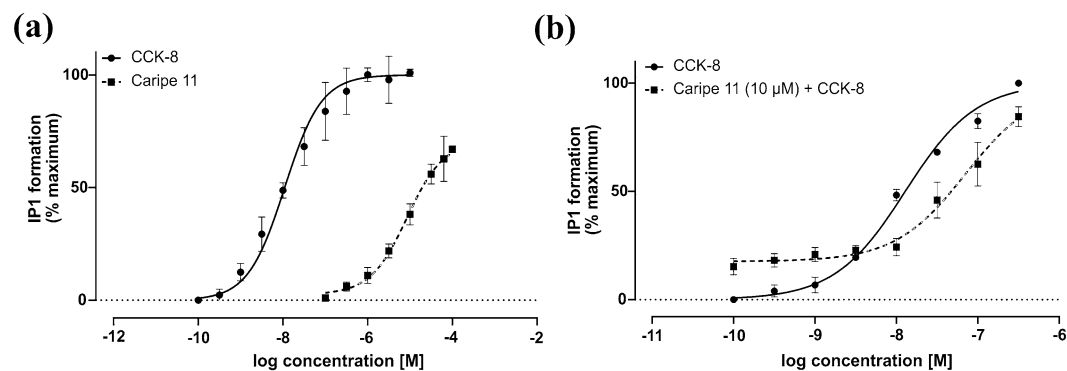


Figure 5. Partial agonist activity of caripe 11 at the CCK₂R. (a) Concentration–response curves of the effect of CCK-8 and caripe 11 upon activation of the receptor via measurement of the intracellular formation of IP1. Potency and efficacy values are listed in Table 2. (b) Concentration–response curves of CCK-8 alone and in combination with caripe 11 (10 μ M). Stimulation buffer and CCK-8 peptide were used as negative and positive controls with 0 and 100% efficacy, respectively. Each experiment was carried out in technical triplicate and three (n = 3) biological repeats. All data are shown as mean \pm SD.

CCK-8			caripe 11		
EC ₅₀	logEC ₅₀	E _{max}	EC ₅₀	logEC ₅₀	E _{max}
11.5 ± 1.5 nM	- 7.9 ± 0.06	100	8.5 ± 1.7 μM	-5.1 ± 0.08	71 ± 6.9

Table 2. Potency (EC₅₀) and maximum efficacy (E_{max}) of CCK-8 and caripe 11 at the CCK₂R. Data are shown as mean ± SD based of three biological measurements (n = 3).

treatment with caripe 11 (10 μM) (Fig. 5b), a phenomenon commonly observed for partial agonists³⁶. Moreover, the curve of CCK-8 in combination with caripe 11 was shifted to the right; this led to a nearly sixfold decrease in the potency of CCK-8 from an EC_{50 CCK-8} of 12.9 nM to an EC_{50 CCK-8+caripe11} of 71 nM (Fig. 5b). These characteristics suggest that caripe 11 is a partial agonist of the CCK₂R.

Discussion

Cyclotides are cyclic plant peptides comprising a unique structural topology that is currently being explored in drug discovery and development. Cyclotides and many other nature-derived peptides occupy a chemical space that is different from small molecules, and therefore they may be able to interact with proteins otherwise difficult to target by small molecules. The use of plants in traditional medicine for the discovery of new pharmaceuticals and lead compounds is one of the central dogmas of ethnopharmacology and pharmacognosy^{1,25}. The prototypical cyclotide plant *O. affinis*, known for its traditional use in childbirth and post-partum care, was the source for the first nature-derived cyclotide/GPCR ligand, kalata B7, which acts as a partial agonist at the oxytocin- and vasopressin V_{1a}-receptors²⁵. Meanwhile a number of different cyclotide GPCR ligands has been discovered and synthesized²³, and hence in this study we aimed at increasing the repertoire of GPCR-modulating cyclotides by exploring the CCK₂R.

GPCRs are one of the largest group of membrane proteins in the human body with over 800 unique receptor sequences known to date³⁷. The CCK₂R is a class A GPCR that is relevant in many physiological and pathological processes, e.g. it is involved in several metabolic and gastric disorders³⁰, as well as cancer²⁹. Based on the therapeutic potential of CCK₂R and therefore the need for new ligands targeting this receptor, in this study we screened cyclotide-containing extracts of *O. affinis*, *C. ipecacuanha*, and *V. tricolor* to explore their ability to modulate CCK₂R signaling. The content and nature of cyclotides in these extracts were analyzed and confirmed by RP-HPLC and MALDI-TOF MS. In line with the defining criteria of cyclotides, peptides identified in the three plant extracts were late-eluting in RP-HPLC and exhibited a molecular mass between 2700 and 3500 Da³⁸. We pharmacologically screened these cyclotides-enriched extracts in a functional IP1 assay using HEK293 cells overexpressing the mouse CCK₂R. All three tested extracts exhibited the ability to modulate Gq-dependent CCK₂R signaling. To demonstrate specificity of these effects we tried co-treatment with two competitive human CCK₂R inhibitors, YM-022³⁹ and LY225910⁴⁰. Unfortunately, it was not possible to block the agonist effects of the extracts (data not shown). Reasons for this are discussed in the following: (i) the antagonists were specifically designed to the human receptor; however, in our study we used the mouse receptor. Not surprisingly, LY225910 did not displace the effects of endogenous ligand CCK-8; to demonstrate an antagonistic effect of YM-022 we had to use high concentrations (> 500 nM) despite its reported picomolar affinity (Fig. S1b). (ii) Some compounds in the extract (which contain up to hundreds of peptides and other molecules)³⁸ may interfere with the activity of the antagonist, and (iii) cyclotides may form a stable complex with the receptor that cannot easily be displaced by the antagonist; this phenomenon has been observed for other GPCRs previously (summarized in Ref.⁴¹). For instance, partial agonists of the adenosine receptor such as LUF7746 bind covalently and cannot be displaced by antagonists^{42,43}, and the same is true for the cannabinoid 1 receptor ligand AM841⁴⁴.

To identify cyclotides responsible for the modulation of CCK₂R signaling, we next isolated several cyclotides from a representative cyclotide extract of *C. ipecacuanha*. At least one of these peptides, namely caripe 11 exhibited the ability to partially activate CCK₂R. This partial agonism of caripe 11 was further analyzed by co-incubation of the cyclotide with CCK-8 and caripe 11, which led to a dextral shift of the CCK-8 concentration–response curve (i.e., decrease of CCK-8 potency). These findings are in line with our previous studies that identified kalata B7 cyclotide to be a partial agonist of oxytocin and vasopressin receptors²⁵. Furthermore, caripe cyclotides, first reported by Koehbach et al.³³, have been demonstrated to function as antagonists at the corticotropin-releasing factor type 1 receptor⁶ and agonists of the κ-opioid receptor²⁶.

Because partial agonists of GPCRs trigger submaximal effector coupling and thus induce less receptor desensitization as compared to full agonists, they provide opportunities to develop pharmacotherapies with improved side effect profiles⁴⁵. A prime example is salmeterol, a partial agonist of β₂-adrenoceptor⁴⁶ in clinical use for treatment of asthma and chronic obstructive pulmonary disease⁴⁷. Furthermore, buprenorphine displays partial agonist activity at the μ-opioid receptor yet exerts pharmacological effects similar to an antagonist⁴⁸. In fact, these characteristics of buprenorphine make it an attractive compound for clinical use in pain management⁴⁹ and opioid dependence⁵⁰.

The unique features of cyclotides have led to their use in the design of cyclotide-based drugs with improved pharmacological properties. Due to their capability to accommodate structural variations, cyclotides have been extensively utilized as molecular scaffolds to design new molecules and ligands with interesting biological features by applying ‘molecular grafting’⁵¹. For instance, Camarero et al. designed and synthesized a cyclotide-based antagonist of the HIV-1 viral replication that is able to target the chemokine receptor CXCR4⁵². In addition, the grafted cyclotide MCo-CVX-5c was used as a template for designing and synthesizing MCo-CVX-6D (the Lys

residue in loop 1 has been conjugated to DOTA) that exhibited affinity at CXCR4 in the sub-nanomolar range⁵³. These studies demonstrated feasibility for grafting GPCR-binding peptide motifs into the cyclotide framework. The grafted CXCR4 cyclotides are important and stable cancer imaging tools⁵³. Accordingly, the affinity of the cyclotide caripe 11, identified in this current study, may be exploited as a scaffold for molecular grafting to design CCK₂R ligands with improved pharmacological properties, e.g., enhanced potency/affinity and stability. Given the therapeutic potential of CCK₂R in the treatment of cancer^{27,29}, for instance, by grafting of the endogenous CCK-8 sequence into the cyclotide scaffold, and the conjugation of an imaging reagent could yield a high-affinity cancer imaging probe. In this study, we did not determine activity of caripe 11 at the related CCK₁R, and therefore we cannot address receptor-subtype selectivity. Proof-of-principle for such a grafting application, and possibly to investigate receptor selectivity will have to be provided in future studies.

At a more general level, our work provides yet another example that cyclotides are capable of modulating GPCR signaling²³. Their functional diversity, structural plasticity and high stability make them suitable scaffolds to develop new GPCR-targeting ligands with unique pharmacological properties²³. In fact, cyclotides are amenable to molecular grafting, which facilitates the engineering of chemical probes and ligands of GPCRs^{23,53,54}. Here, we discovered for the first time a cyclotide ligand of the CCK₂R that may for instance be utilized as a stable labelled ligand in imaging applications, as a gut-stable probe, or as a scaffold for designing stabilized peptide ligands of the CCK₂R. Thus, our study helped to increase the rich diversity of cyclotides as ligands of GPCR and points to their potential use as starting points for the design of cyclotide-based ligands targeting CCK₂R to treat human illnesses such as cancer.

Conclusion

GPCRs remain privileged drug targets. The CCK₂R is an example of a GPCR with therapeutic potential for the treatment of gastrointestinal disorders including cancer. Cyclotides are nature-derived peptides that represent an emerging class of GPCR modulators. In this study, we demonstrated a cyclotide that modulates CCK₂R signaling as a partial agonist. Therefore cyclotides may be utilized as templates for designing new GPCR ligands with unique pharmacological properties²⁵.

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Author contributions

C.W.G. has designed research. M.S.T., B.R., E.M. and C.T. have performed experiments. B.R., E.M. and E.C. contributed new reagents and analytical tools. M.S.T., E.C., A.M., A.A., A.N. and C.W.G. analyzed data. M.S.T., B.R., E.M. and C.W.G. prepared the manuscript with the help from all other authors. All authors approved the final version of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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