The steroid glycoside H.g.-12 from *Hoodia gordonii* activates the human bitter receptor TAS2R14 and induces CCK release from HuTu-80 cells.

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Running Head: Bitter taste sensing and CCK release in the gut

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ABSTRACT

Steroid glycosides extracted from the succulent plant *Hoodia gordonii* are suggested to have appetite suppressant effects both in animals and humans. Yet, the mechanisms underlying the putative satiety action of *Hoodia* steroid glycosides are not fully understood. We found that H.g.-12, a steroid glycoside purified from *Hoodia gordonii* extract, initiated cholecystokinin (CCK) secretion both *ex vivo* in rat intestine and *in vitro* in the human enteroendocrine (EC) cell line HuTu-80. CCK is known to exert central effects on appetite suppression via the vagus nerve which afferents terminate in the gut wall. Recent data show that G-protein-coupled receptors signalling bitter taste (T2Rs) are expressed in both rodent and human gastrointestinal tract. It was further demonstrated that bitter sensing is functional in mouse STC-1 enteroendocrine cells and leads to CCK secretion via increased intracellular Ca\(^{2+}\) concentrations. Based on the bitter taste of *Hoodia gordonii* purified extracts, we assessed whether H.g.-12 could activate human bitter receptors. The steroid glycoside activated selectively TAS2R7 and TAS2R14, both heterologously expressed in HEK 293 cells. Removing an essential structural feature from the steroid glycoside inhibited H.g.-12-induced Ca\(^{2+}\) increase in TAS2R14-expressing HEK cells and abolished H.g.-12-induced CCK secretion from human EC cells. Similarly, a non-specific bitter receptor antagonist abolished H.g.-12-induced CCK secretion in HuTu-80 cells.

These results point to a potential route of action by which components of *Hoodia* might influence appetite control. Our data also provide additional evidence that bitter taste sensing mechanisms are coupled to hormone release from enteroendocrine cells in the intestine. Moreover, we identified a natural agonist of TAS2R7 and TAS2R14 for further studies on the role of bitter receptors in satiety control and food intake.

Key words: nutrient sensing; satiety; HuTu-80; CCK\(_{1}\)R;
INTRODUCTION

Hoodia gordonii (Masson) Sweet ex Decne is a perennial, succulent plant species from the Apocynacaea (previously Asclepiadaceae) family that is indigenous to arid regions of South Africa, Botswana and Namibia. Initial exploratory research with several species of Hoodia showed that Hoodia gordonii extract contained a mixture of steroid glycosides (20, 29, 34) that decreased food intake and body weight in animals (34). The two major steroid glycosides H.g.-12 and H.g.-20 present in Hoodia gordonii were purified and shown to exhibit food intake suppressive effects in rats, strongly indicating that H.g.-12 and H.g.-20 are at least two of the pharmacologically active components in Hoodia gordonii (34). However, the mechanisms of action by which the steroid glycosides might influence energy intake and appetite control are not fully understood. So far, only one study investigating potential mechanistic effects of one of the Hoodia gordonii steroid glycosides has been published. Here it was hypothesised that H.g.-12 has a modulating effect on ATP production and its content in hypothalamic neurons after intracerebroventricular injection and after in vitro exposure of foetal hypothalamic neurons to the compound (24). However, it is not clear that the primary bioactivity of Hoodia components is exerted post-absorptive. Furthermore, bioavailability studies must demonstrate that following ingestion, H.g.-12 has access to the brain tested regions in vivo and that concentrations of the active form of the molecule are likely to be achieved at the site of action.

Based on the bitter taste of Hoodia gordonii extracts, we hypothesised that the putative effects of Hoodia gordonii could in part be mediated by luminal sensing of its steroid glycosides in the small intestine. Recent scientific evidence indeed suggests the existence of functional taste-sensing mechanisms in the intestine resembling those found in the neuroepithelium of the tongue (11). Various members of the T2R family, along with components of the taste signalling pathway such as α-gustducin, have been shown to be expressed in enteroendocrine...
(EC) cell lines and pancreas, as well as in the gastric and intestinal mucosa in rodents (8, 18, 21, 35, 36) and also in human colonic tissue (21, 30, 31). Data from these studies suggest that bitter taste receptors expressed in the small intestinal tract are involved in sensing of food components. In addition, some *in vitro* and *in vivo* animal studies showed that sweet and bitter compounds known to mediate gustatory signals in the oral cavity elicit gut hormone secretion from EC cells and lead to a neural activation in the brain (16, 17, 19).

The TAS2R family contains approximately 25 receptors as identified by genomic sequencing (1, 6, 9, 27). Agonists for almost all human TAS2Rs have been identified (2, 3, 26, 28, 32); all agonists identified so far have been bitter tasting compounds (5, 26). Based on the presence of both bitter taste receptors and taste transduction proteins in EC cells, it has been postulated that TAS2Rs may be involved in regulation of food intake via initiation of secretion of satiety hormones such as glucagon like peptide-1 (GLP-1), peptide YY (PYY) and cholecystokinin (CCK). *In vitro*, bitter tastants like denatonium benzoate (DB) and phenylthiocarbamide (PTC) cause an increase in intracellular calcium followed by cholecystokinin (CCK) secretion as shown in the rodent EC cell line STC-1 (8). Ofloxacin, a bitter ligand for TAS2R9, elicits a GLP-1 secretion from human enteroendocrine NCI-H716 cells (11). In rats, an intragastric infusion of TAS2R agonists was reported to excite neurons in the nucleus tractus solitarius (NTS), to condition flavour aversions and to delay gastric emptying (14, 16, 17). NTS activation is followed by reflex activation of vagal efferent neurons, which itself results in changes in gastrointestinal functions, including inhibition of gastrointestinal motility (15). However, to date, there is only one study reporting changes in GI physiology in response to bitter compounds in rats (21), and in humans, the only study available failed to show effects of an intragastric infusion of the bitter tastants quinine and naringin on gastric emptying (23). Direct effects of these bitter compounds on hormone secretion in EC models have not been shown yet.
Here, we demonstrate that the *Hoodia gordonii* steroid glycoside H.g.-12 elicits CCK secretion both *ex vivo* in rat intestine and *in vitro* in cultured human EC cells. We also investigated whether this hormone secretion could be mediated via intestinal bitter receptors, and demonstrated that H.g.-12 selectively activates TAS2R7 and TAS2R14 out of 25 TAS2Rs. Finally, bitter receptor activation and subsequent satiety hormone secretion *in vitro* were linked to essential structural features of H.g.-12.
MATERIALS AND METHODS

Materials. HuTu-80 cells (ATCC, HTB40) were obtained from the American Type Culture Collection (WZ). HEK293 cells were obtained from Invitrogen (Breda, The Netherlands). A Chinese hamster ovary cell line (CHO) functionally expressing rat CCK₁R (CHO-CCK₁-R) was kindly provided by Dr. R. Smeets (University of Nijmegen, The Netherlands). Other reagents used in this study were purchased from Sigma Aldrich unless indicated differently. The *Hoodia gordonii* steroid glycoside H.g.-12 was purified from a *Hoodia gordonii* extract and had a chemical purity of 92%. (Russell, P.J., Swindells, C.; Chemical characterization of *Hoodia gordonii* extract; in preparation). Removal of the tigloyl group from the steroid core by alkaline cleavage was used to generate detiglated H.g.-12; acid cleavage was used to generate the aglycon of H.g.-12 and combination of both processes led to the detiglated-aglycon of H.g.-12. Chemical structures are presented in Figure 1.

A synthetic compound library containing 10,000 different compounds was obtained from Specs, The Netherlands. The compounds were dissolved in DMSO at a 20mM stock concentration.

Cell Culture conditions. HuTu-80 human duodenal cells were grown in minimum essential Eagle’s medium (MEM) containing 10% FBS, antibiotics (100U·ml⁻¹ penicillin, 100μg·ml⁻¹ streptomycin) and 100μg·ml⁻¹ L-glutamine in plastic flasks or Poly-Lysin-coated 24 wells plates. Cells were used until passage 32.

HEK293 cells (human embryonic kidney cells) expressing each of the 25 bitter receptors were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 4.5g·l⁻¹ glucose and L-glutamine and supplemented with 10% tetracycline-free FBS (Cambrex), blasticidin (5μg/ml), geneticin (400μg/ml) and hygromycin (100μg/ml) in plastic flasks or Poly-Lysin-coated 96 wells plates. Cells were used until passage 25.
CHO-CCK1R cells were grown in DMEM and Ham’s F12 medium 1:1 (DMEM-F12) with 15mM HEPES and L-glutamine supplemented with 10% FBS (Gibco, Paisley, UK), 500μg · ml⁻¹ penicillin, 500U·ml⁻¹ streptomycin (Cambrex, Baltimore, USA), and 500μg · ml⁻¹ geneticin (Gibco, Paisley, UK). As negative control cells, native CHO-K1 cells (ATCC, CCL61) were used and grown in the same medium without geneticin. Cells were used until passage 25. All cells were grown and maintained at 37°C / 5%CO₂.

**Cloning and expression of human TAS2 receptors in HEK293 cells.**

For functional expression of the human bitter taste receptors, HEK293 cells (Invitrogen) stably expressing the chimeric G-protein α-subunit Go16-gust44 (cloned into pcDNA4 (Invitrogen, San Diego, CA)) and one of each 25 bitter receptor genes (cloned into pcDNA5/FRT (Invitrogen, San Diego, CA)), were used. To improve receptor membrane targeting, each bitter receptor gene contained the first 45 amino acids of rat somatostatin receptor type 3 at its amino terminus. Cells were maintained in DMEM and 10% tetracycline-free FBS (Cambrex) supplemented with blasticidin (5 μg/ml), geneticin (400 μg/ml) and hygromycin (100 μg/ml).

**Activation measurement of TAS2R via monitoring of intracellular calcium concentrations**

[Ca²⁺]ᵢ, using a fluorescent plate reader. Activation of human TAS2 receptors was measured by monitoring variations in intracellular Ca²⁺ concentrations [Ca²⁺]ᵢ, using the FlexStation II 384 (Molecular Devices). TAS2R-expressing cells and non-transfected cells were seeded in supplemented DMEM into Poly-Lysin-coated 96-well plates (black wall, clear bottom, Greiner) at a density of 1.10⁵ cells/ml, 100μl/well and cultured overnight. The following day, transcription of the receptors was induced by adding 0.25μg·ml⁻¹ doxycyclin. Cells were induced for 24h and then loaded with the calcium-sensitive fluorescent dye Fluo-4AM (Molecular Probes), following suppliers guidelines. Dye loading medium composition was the following: 0.01% Pluronic F-127 (Molecular Probes), 0.5mM Probenecide (Sigma) and
2.5µM Fluo-4AM in Tyrode’s buffer pH 7.4 (140mM NaCl, 5mM KCl, 10mM Glucose, 1mM MgCl₂·6H₂O, 1mM CaCl₂, 20mM Heps). Compounds to test were prepared in parallel in Tyrode’s buffer in a 96 wells plate (V96 Microwell, Nunc) and automatically pipetted onto the cells during the assay. Measurements were carried out for 90s with an interval time of about 1.6s, giving 55 data points per measurement. To obtain a baseline, fluorescence signals (excitation 485nm/emission 520nm) were measured for 20s prior to the addition of compounds on the cells (80µl, injection speed of 100µl/s). After agonist addition, the fluorescence signals were measured for an additional 70s at 37°C. The fluorescence values (ΔF) were calculated by subtracting the maximum fluorescence from the average fluorescence of the first 10 time points (baseline). Non-induced cells were measured in parallel as a negative control.

**TAS2R antagonists screening from chemical libraries.** A commercially available library (Specs, The Netherlands) consisting of 10,000 chemical compounds was screened for TAS2R antagonists. Around 3000 compounds (final concentration 100µM) were co-incubated with 0.25mM of activating ligand on TAS2R-expressing HEK cells for identification of receptor blockers.

**CCK secretion studies in HuTu-80 cells.** For each secretion study, HuTu-80 cells were seeded at 2.10⁵ cells/ml, 0.5ml/well in Poly-Lysin-coated 24 wells plate 48h prior to the experiment. HBSS buffer (Gibco) supplemented with 10mM Hepes (Sigma) was used for the secretion studies. Test compounds were prepared in HBSS buffer and incubated on the cells (0.5ml/well) for 2h at 37°C in a humidified incubator at 5%CO₂. Medium (0.5ml) was collected and centrifuged at 4°C for 5min at 1000g to remove cell debris and the supernatants were stored at -20°C until further analysis. A 100mM stock solution for H.g.-12 was prepared in DMSO; final concentration of DMSO never exceeded 0.5% on the cells.
**Determination of CCK concentrations using a CCK receptor-1 activation assay by monitoring \([Ca^{2+}]_i\).** Samples derived from CCK secretion studies in HuTu-80 cells and rat gut rings were tested for CCK in a CCK-receptor 1 (CCK\(_1\)R) activation assay as described previously (12). This method has been shown to display higher sensitivity and lower variability than an ELISA (12). In brief, CHO-K1 and CHO-CCK\(_1\)-R cells (100 µl/well) were seeded into Poly-Lysin-coated 96 wells microtiter plate (black wall, clear bottom, Greiner) at a density of 3 \(10^5\) and 4 \(10^5\) cells/ml, respectively, and cultured overnight. Cells were loaded with Fluo-4AM as described above. Supernatants from the secretion studies were thawed from -20°C and prepared in parallel in a 96 wells plate (V96 Microwell, Nunc). The Flexstation measurement settings were identical as those used for the HEK cells expressing human bitter taste receptors. CCK\(_1\)R activation was measured by monitoring fluorescence signals in CCK\(_1\)R-expressing CHO cells after the addition of the supernatants derived from CCK-release experiments as described above. Each experiment was conducted in parallel using wild type CHO cells to assess any non-specific signal. Calibration was performed using sulphated CCK-8 as a standard.

**Animals and tissue preparation.** The use of animals was conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Technical University of Munich. Male Sprague Dawley rats (Charles River Laboratories, Germany) weighting 240-260g (n=6) were used for this study. Rats were maintained on a regular laboratory chow and were fasted overnight previous to the experiment. Water was supplied *ad libitum* before all experimental procedures. Rats were anesthetized with ether (Sigma) and sacrificed by cervical dislocation. Median laparotomy was performed and the entire small intestine was carefully removed by cutting along the mesenteric border. Duodenum was defined as a 10cm section downstream the pyloric sphincter, ileum as a 10cm section upstream the caecum. The tissue in-between was defined as jejunum.
Hormone secretion studies using rat intestinal rings. The protocol used by Jang et al. (19) was adapted to everted rat intestine. All steps prior to incubation were performed on ice. Briefly, luminal contents were gently removed using a fresh-made Krebs buffer saturated with 95%O$_2$-5%CO$_2$ (pH 6.5, all mM: 119 NaCl; 4.7 KCl; 2.5 CaCl$_2$·2H$_2$O; 1.2 MgSO$_4$·7H$_2$O; 1.2 KH$_2$PO$_4$; 25 NaHCO$_3$; 20 MES). Duodenum and ileum segments were everted using a customized metallic rod. Rings of 0.5cm were prepared and briefly incubated in Krebs supplemented with 0.5mM DTT (dithiothreitol) in order to prevent excessive mucus production. The gut rings were then randomly transferred in 200µl of effectors (triplicates) prepared in Krebs buffer containing dipeptidyl peptidase (DPP)-IV inhibitor (20μl·ml$^{-1}$; Millipore) in a 96 wells plate. A stock solution of H.g.-12 was prepared in DMSO (100mM), and freshly diluted in Krebs buffer on the day of the experiment. Final concentration of DMSO during incubation never exceeded 0.5%. The gut rings were incubated 30min at 37°C in a shaking incubator (60-100rpm), after what the total content of each well was centrifuged at 4°C for 15min at 5000g. CCK-8S levels were measured in the supernatants using the CCK$_1$R activation assay. PYY (total) and GLP-1 (active) levels were measured using a Milliplex MAP rat-gut-hormone kit (RGT-88K, Millipore, Billerica, MA, USA) read on the Luminex 100 IS system (Luminex corporation, Austin, TX, USA).

Polymerase chain reaction of TAS2R7 and TAS2R14 in HuTu-80 enteroendocrine cells and human duodenum and jejunum. Polymerase chain reactions (PCR; Lightcycler, Roche Applied Science, Mannheim, Germany) were performed using HuTu-80 cells and human adult normal RNA for duodenum (R1234101-50) and jejunum (R1234230-50) purchased from BioChain (Hayward, USA) from 4 different male donors. Total RNA from HuTu-80 cells was isolated using the RNeasy mini kit (Qiagen) and cDNAs were synthesized by a ThermoScript RT-PCR system (Invitrogen). Specific PCR primers for TAS2Rs and GAPDH (to control the absence of genomic contamination in the probes) were designed using the
Lightcycler Probe design software version 1.0 (Roche applied science) and synthetised by Eurofins (Eurofins MWG Operon, Ebersberg, Germany). Primers sequences were as follows: hTAS2R7 (amplicon size = 215bp); forward primer 5'-ATTGTTCTTAGCAGTTGGAG-3'; reverse primer 5'-CTTTACCAGTGCGATAGAC-3'; hTAS2R14 (amplicon size = 344bp); forward primer 5'-AAGACTTTGGCAGTTCTGATT-3'; reverse primer 5'-GAGTGACATGAAGGATAAGC-3'; hGAPDH (amplicon size = 215bp); forward primer 5'-CATCGCTCAGACACCA-3'; reverse primer 5'-AGCTTCCCCCTTCTCAC-3'; The sequence specificities of the primers were verified with NCI BLAST. In addition, RT-PCR on cDNAs from TAS2R7 and TAS2R14 expressing HEK cells were performed to test the primers reliability. PCR products were separated on agarose gel and stained with ethidium bromide. Gel images were recorded by a digital camera.

**Data processing and statistical analysis.** Data in the figures are presented as Mean ± SEM. GraphPad Prism version 4.01 for Windows (GraphPad Software, San Diego, California, USA) was used for statistical analysis. Differences in the potency of the agonists/effectors towards receptor activation/hormone secretion were analysed using one-way ANOVA followed by the appropriate multiple comparison test. Differences were considered to be significant at $P<0.05$. Receptor activation data (TAS2Rs, CCK1R) are expressed in DeltaF values. All *in vitro* experiments were performed at least in duplicate. Hormone secretion data from the rat gut rings studies are expressed in fold changes of the control (Krebs buffer).
RESULTS

H.g.-12 elicits a significant CCK-8S secretion in a rat *ex vivo* model.

A study with rat gut rings was conducted to assess whether H.g.-12 could elicit gut satiety hormone secretion. When duodenal tissue was exposed to 0.5mM of H.g.-12, a significant 1.4±0.2-fold increase in CCK-8S secretion was observed (Figure 2, *P*<0.001). Here as a positive control, a protein hydrolysate (4%) was shown to significantly increase CCK secretion around 1.7±0.2-fold (*P*<0.001). In ileal tissue specimens, H.g.-12 tended to elicit a 1.6±0.8-fold GLP-1 secretion (*P>*0.05; not shown) and a 1.5±0.4-fold increased PYY output (*P>*0.05, not shown).

H.g.-12 induces CCK release from the human EC cell line HuTu-80.

To investigate whether the steroid glycoside H.g.-12 could elicit hormone secretion from human EC cells, HuTu-80 cells were exposed to increasing concentrations of H.g.-12 for 2 hours. H.g.-12-mediated CCK secretion into the medium was demonstrated by increased CCK receptor-1 activation in a receptor cell line. A significant mean 3-fold increase (*P*<0.001) in CCK$_1$R activation as compared to the control cells was observed (Figure 3 A). Whereas H.g.-12 exhibited potent effects on CCK secretion, removal of the tigloyl group in the detiglated and detiglated-aglycon forms completely abolished the CCK secretion from HuTu-80 cells (Figure 3 B) and the aglycon form was not able to significantly induce hormone secretion from the cells. H.g.-12 effects on CCK secretion in HuTu cells did not exhibit a clear dose-dependency. Concentrations of 30µM and below failed to elicit a significant CCK output, whereas concentrations ranging from 60µM to 500µM displayed similar effects on CCK$_1$R activation. In order to determine whether the effect of H.g.-12 on CCK secretion in HuTu-80 cells is specific or a more generic effect, we assessed whether bitter tastants like denatonium benzoate (DB), phenylthiocarbamide (PTC) or quinine could as well elicit CCK secretion with H.g.-12 as a positive control. Whereas 0.25mM H.g.-12 in a robust manner increased 3-
fold CCK release, DB, PTC and quinine at concentrations up to 10mM failed to cause a
stimulation of CCK release in HuTu-80 cells (data not shown), thus demonstrating the
selectivity of H.g.-12.

**H.g.-12 selectively activates the human bitter receptors TAS2R7 and TAS2R14.**

Purified *Hoodia gordonii* extracts taste bitter. To identify whether the *Hoodia gordonii*
steroid glycoside H.g.-12 selectively activates one or more human TAS2 bitter receptors, a
screening of 25 human bitter receptors each expressed heterologously in HEK 293 cells was
conducted (Figure 4). Monitoring $[\text{Ca}^{2+}]_{i}$ revealed that 0.25mM H.g.-12 specifically activated
hTAS2R7 (wells E4, E10) and hTAS2R14 (wells B5, B11) whilst receptors TAS2R38 (well
D11) and TAS2R43 (well A6) displayed a non-specific response in induced cells that also
failed to show a dose-dependency. In contrast, the increase in $[\text{Ca}^{2+}]_{i}$ for cells expressing
TAS2R7 and TAS2R14 was dose-dependent for both H.g.-12 and its aglycon at
cconcentrations ranging from 30$\mu$M to 250$\mu$M (Figure 5 A and B). The absence of a plateau at
high compound concentration, that would allow calculation of an EC$_{50}$ value, is due to the
poor solubility of H.g.-12 and its aglycon in buffer at concentrations above 250$\mu$M.

**Structural determinants of H.g.-12-induced activation of TAS2R7 and TAS2R14 and
CCK secretion from EC cells.**

The common structural elements in all *Hoodia gordonii* steroid glycosides are the steroid
core, the tigloyl group and the chain of (deoxy and/or methoxy-) sugars (20). In order to
understand which structures of H.g.-12 are essential for TAS2R activation, we tested H.g.-12,
its aglycon, the detiglated compound and the detiglated aglycon on both TAS2R7 and
TAS2R14 (Figure 5 C and D). Whereas the removal of the tigloyl group abolished the
activation of both TAS2R7 and TAS2R14, the removal of the sugar residues did not
significantly alter TAS2R7 activation. The aglycon even appeared to be a better agonist for
TAS2R14 when compared to H.g.-12 (Figure 5 D; $P<0.001$). Similarly to detiglated H.g.-12,
The detiglated aglycon did not exhibit any effect on \([\text{Ca}^{2+}]\) in TAS2R7 and TAS2R14 expressing HEK cells (data not shown).

To further investigate the role of the tigloyl group on TAS2R7 and TAS2R14 activation, we tested a number of structural unrelated compounds containing a tigloyl-function such as geranyl-tiglate, citronellyl-tiglate and phenethyl-tiglate. None of the compounds activated either TAS2R7 or TAS2R14 (data not shown).

A bitter receptor inhibitor was identified by screening of a chemical library with 10,000 compounds against the receptors TAS2R14, TAS2R16 and TAS2R39. Co-incubation with the lead candidate 03A3 (100 \(\mu\text{M}\)) in the presence of the activating ligand (0.25 mM) caused a significant inhibition of \([\text{Ca}^{2+}]\) in TAS2R expressing cells, up to 35% in the case of H.g.-12-induced TAS2R14 activation (Figure 7A). Similarly, co-incubation of HuTu-80 cells with 03A3 (100 \(\mu\text{M}\)) and H.g.-12 (0.25 mM) abolished CCK secretion from EC cells (Figure 7B). However, the compound 03A3 also inhibited other TAS2Rs, suggesting that it is not specific for TAS2R14.

**TAS2R14 but not TAS2R7 is expressed in HuTu-80 cells and human proximal intestine.**

PCR experiments were conducted in the enteroendocrine cell model as well as in human proximal small intestine to establish the expression pattern of the two TAS2Rs activated by H.g.-12. Presence of the TAS2R14 transcript was found in the HuTu-80 enteroendocrine cell line of duodenal origin as well as in human normal duodenum and jejunum (Figure 6). The presence of a TAS2R7 transcript could be detected neither in HuTu-80 cells nor in human proximal intestinal tissues whereas in the positive control, TAS2R7 expressing HEK cells, the transcript was detected (data not shown).
**DISCUSSION**

In this paper, we report for the first time that the steroid glycoside H.g.-12 isolated from *Hoodia gordonii* can elicit a CCK release from rat *ex vivo* tissue preparations and from a human EC cell line. In addition, we have established that H.g.-12 is a natural and specific agonist for the human bitter receptors TAS2R7 and TAS2R14 but not for any of the other human bitter receptors. We demonstrated that TAS2R14 but not TAS2R7 is expressed in the human EC cell line HuTu-80 as well as in human proximal intestinal tissues. The tigloyl group in the steroid molecule was found in different experimental models to be an essential structural feature needed for both bitter receptor activation and hormone secretion. Thus, by demonstrating specificity of solely TAS2R7 and TAS2R14 activation and employing derivatives of H.g.-12 in both TAS2R14 over-expressing cells and in human EC cells, we have supportive, but not conclusive evidence that CCK secretion from HuTu-80 cells is mediated via TAS2R14. Our experiments emphasize the notion that the steroid glycoside H.g.-12 could via bitter receptor activation in endocrine cells cause CCK-8 release. CCK-8 has central effects on appetite suppression (7, 33); thus, bitter receptor activation by *Hoodia gordonii* steroid glycosides in the intestine might be a part of the mechanistic route of its effects in suppressing food intake.

Rat tissues exposed *ex vivo* to H.g.-12 responded with a significantly increased CCK secretion. This tissue model takes into account the morphology in which enteroendocrine cells are surrounded by enterocytes and an intact enteric nervous system. Although the finding that H.g.-12 induced CCK secretion in this system is considered to be physiological, future studies have to confirm that H.g.-12 and other *Hoodia* components increase CCK *in vivo* to plasma concentrations which could explain the effects of *Hoodia* extracts on food intake. Furthermore, it remains speculative whether the effect of H.g.-12 on CCK secretion in rat
tissues is mediated by bitter receptor activation. It has been shown that rT2R6, the rat orthologue gene to TAS2R7 (70% of sequence identity), is expressed in gastric and duodenal mucosa (35, 36), but for TAS2R14 no close orthologue has yet been found in rats. Thus, whether CCK secretion in rat duodenum depends on the TAS2R7 orthologue and whether this receptor is activated in a similar manner as the human receptor is currently not known. However, we expect some differences between human and rodent bitter receptor activation by Hoodia gordonii steroid glycosides based on the observations that in rodents only Hoodia gordonii steroid glycosides with at least two sugar moieties have an appetite suppressant effect (34). In our cell systems, the aglycon possessed similar activities with respect to TAS2R7 and TAS2R14 activation.

By employing the human enteroendocrine cell line HuTu-80, we demonstrated that H.g.-12 can increase CCK secretion by 2- to 3 times although with an unusual dose-dependency. It is likely that in this endocrine cell line that possesses a wide range of different receptors and signalling pathways, H.g.-12 effects at low concentrations are masked and a threshold concentration is needed for final stimulus-secretion coupling. However, H.g.-12-induced stimulation in CCK secretion from HuTu-80 cells was specific as other bitter tasting compounds such as denatonium benzoate, PTC and quinine, even at concentrations as high as 10mM did not induce CCK secretion. Although PTC was shown previously to increase [Ca²⁺], in HuTu-80 cells (31), it failed to cause a CCK output in our experiments. In this respect, the capability of H.g.-12 to induce a CCK release from HuTu-80 cells is an original feature amongst the various bitter tastants.

The structure-specificity in the ligands that cause CCK release was demonstrated by the removal of the tigloyl group (detiglated and detiglated-aglycon forms) that abolished the capability for stimulation of CCK secretion. Removal of the sugar moieties from H.g.-12
(aglycon) decreased its stimulation of CCK secretion from HuTu cells, in contrast to its increased capability to activate TAS2R14 in HEK cells. We assume that this is related to a lower receptor density on HuTu cells and a much higher hydrophobicity of the aglycon with increased diffusion and removal by the cells over the time of incubation. This problem could not be overcome by increasing concentrations as those used are already at the limits of solubility.

The H.g.-12 steroid glycoside from *Hoodia Gordonii* extract is identified as a new agonist for both TAS2R7 and TAS2R14. TAS2R7 displays a quite broad agonist spectrum with currently 9 ligands known (28, 32) that are all structurally unrelated to H.g.-12. As for TAS2R14, 33 compounds have been identified as agonists, yet without any obvious common structural motif in the ligands (3, 28). This makes TAS2R14 one of the broadest human bitter receptors in terms of agonist spectrum along with TAS2R10 and TAS2R46, of which all three tend to display a slight preference for natural compounds (28).

We have observed that removal of the tigloyl group from H.g.-12 abolishes its interaction with both tested TAS2 receptors, thus mirroring the situation in HuTu-80 cells where removal of the tigloyl group abolished CCK secretion. Although the compound 03A3 was shown to inhibit TAS2R14 and also to markedly reduce hormone secretion in response to H.g.-12 activation, it also interacted with some other bitter receptors.

Tiglic acid alone and three structurally unrelated compounds containing a tigloyl group failed to activate TAS2R7 or TAS2R14. This demonstrates that the tigloyl function as such is not the sole structural feature needed for TAS2R activation. Quantitative structure-activity relationship studies are currently not possible due to a very limited availability of H.g.-12-related structures.
We observed that removal of the glycoside groups (aglycon) in H.g.-12 increased the selectivity towards receptor TAS2R14. Using a simulated gastric and small intestinal digestion model, it was recently suggested that H.g.-12 is partly deglycosylated during gastrointestinal digestion (25). In the same study, the aglycon molecule was shown to cross both the intestinal and blood brain barriers by passive diffusion. However, further studies would have to demonstrate that the aglycon is not extensively metabolised during intestinal absorption and/or first liver passage before post-absorptive systemic effects are proposed. This is, however, conceptually different from the pre-absorptive effects that H.g.-12 can have by interacting with luminal-facing taste receptors in the gastrointestinal tract.

We identified the transcript for TAS2R14 in HuTu-80 cells as well as in human duodenal and jejunal tissue samples. In contrast, the TAS2R7 transcript was not detectable with the probes employed in the cell and tissue samples. Our data confirm recent findings on the presence of TAS2R14 but not TAS2R7 in HuTu-80 cells together with 13 other TAS2Rs (31). Although TAS2R7 and TAS2R14 are both found in the neuroepithelium of the tongue (4), they could not be detected in human colon (31) and TAS2R7 could also not be identified in human ileal NCI-H716 cells or human caecum samples (11). Concerning the proximal intestine, this is to our knowledge the first report demonstrating the expression of TAS2R14 in human duodenum and jejunum. However, detailed cellular and sub-cellular distribution of TAS2R14 proteins in intestinal tissues, combined with co-localisation data for gut hormones are needed before more profound suggestions for the physiological relevance of TAS2R14 activation can be made.

In conclusion, we provide supportive evidence for a mechanism through which the proposed appetite-suppressant properties of Hoodia gordonii extract may partly originate from its main
steroid glycoside H.g.-12. We have shown that H.g.-12 elicits *in vitro* CCK release from enteroendocrine cultured cells, most probably through the activation of one subtype of human bitter receptors. The same mechanism could possibly be involved in CCK secretion from rat duodenal tissues *ex vivo*. CCK is known to induce satiety in rodents (13, 33) and in humans (10, 22, 23). In addition, T2R agonists administered into the stomach of rats by oral gavage were found to increase c-Fos gene expression and c-Fos positive neuron-numbers in the nucleus of the solitary tract (NTS) and CCK₁ receptors located on vagal afferent terminals in the gut wall are thought to form this gut-brain axis (16, 17). The vagal afferent terminals found in the lamina propria are in close vicinity to the enteroendocrine cells, suggesting that the transformation of the chemosensor input signals into neuronal signals could take place at this location. Combined with a possible direct interaction of CCK secreted from the intestine with the CCK₂ receptor in the brain, the inhibition of food intake associated with *Hoodia gordonii* consumption in rats and putatively in humans could result, in part, from this intestine to brain communication route.
Acknowledgements

The author’s responsibilities were as follows: BLN, RG and MF conceived and designed the experiments; RG established the TAS2R stable expressing HEK cell lines; BLN and RG performed the experiments; BLN analysed the data and wrote the manuscript; BLN, MF and HD edited the paper.

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References


Figure Captions

Figure 1. Chemical structures of H.g.-12, its aglycon, detiglated and detiglated aglycon. Removal of the tigloyl group from the steroid core by alkaline cleavage was used to generate detiglated H.g.-12; acid cleavage was used to generate the aglycon of H.g.-12 and combination of both processes led to the detiglated-aglycon of H.g.-12.

Figure 2. H.g.-12 induced CCK secretion in the rat intestine ex vivo model. Duodenum sections (0.5cm) prepared from everted rat intestine were incubated with 0.5mM H.g.-12 and 4% PEP (positive control, protein hydrolysate) for 30 minutes at 37°C. After centrifugation (4°C) of the samples, CCK-8S levels were measured in the supernatants using the CCK1R activation assay. The data are averages of triplicate determinations from n=6 animals and are expressed as control fold (Krebs buffer); ***P<0.001 (one way ANOVA + Bonferroni’s multiple comparison test).

Figure 3. H.g.-12 structure-activity relationship on CCK secretion from HuTu-80 cells. Indirect determination of CCK-8S levels in HuTu-80 supernatants using the CCK1R activation assay. A: Cells were incubated for 2 hours with increasing concentrations of H.g.-12 (0.03-0.5mM). The data are averages of triplicate determinations from three independent experiments; ***P<0.001 (one way ANOVA + Bonferroni’s multiple comparison test). B: Cells were incubated for 2 hours with 0.25mM of H.g.-12, detiglated, aglycon or detiglated-aglycon. The data are averages of triplicate determinations from three independent experiments; ***P<0.001 (one way ANOVA + Bonferroni’s multiple comparison test). All data are reported in changes of FLUO-4-AM fluorescence (Delta F).

Figure 4. Screening of hTAS2Rs transfected HEK 293T cells for activation by H.g.-12. Activation of hTAS2Rs by H.g.-12 (0.25mM) was measured over 90s in TAS2R-expressing HEK cells by monitoring of variations in FLUO-4-AM fluorescence (Delta F) induced by changes in [Ca2+]. Non-doxycyclin-induced HEK cells were used as controls. Receptor
identity is shown for each well of the 96 wells plate. Columns 1-3 and 7-9 are control cells, columns 4-6 and 9-12 are induced cells (all duplicates).

**Figure 5. H.g.-12 structure-activity relationship on TAS2R7 and TAS2R14 activation.**

**A and B:** Changes in $[Ca^{2+}]$ in TAS2R7 and TAS2R14 expressing HEK cells activated by H.g.-12 and its aglycon (0.03-0.25mM). Data are averages of duplicate determinations from a representative experiment. **C and D:** $[Ca^{2+}]$ answer in TAS2R7 and TAS2R14 expressing HEK cells to either 0.25mM of H.g.-12, detiglated, or aglycon. The data are averages of triplicate determinations from three independent experiments; ***$P<0.001$ (one way ANOVA + Bonferroni’s multiple comparison test). All data are reported in changes of FLUO-4-AM fluorescence (Delta F).

**Figure 6. TAS2R14 is expressed in HuTu-80 human enteroendocrine cells and human proximal intestine.** Expression of human taste receptor TAS2R14 in HuTu-80 enteroendocrine cells ($Hu$) and human normal duodenum ($D$) and jejunum ($J$). PCR was performed using the specific primers listed in methods to detect the expression of TAS2R14 (predicted amplicon size 344bp). PCR for GAPDH was performed to verify the absence of genomic contamination in the probes. $L$: DNA sizing ladder; $W$: PCR performed without DNA as a negative control.

**Figure 7. Inhibition of H.g.-12 induced activation of TAS2R14 and CCK secretion in HuTu-80 cells by compound 03A3.** **A:** $[Ca^{2+}]$ answer in TAS2R14 expressing HEK cells to 0.25mM H.g.-12 alone (positive control) or co-incubated with compound 03A3 (25-50-100$\mu$M). $[Ca^{2+}]$ were determined using FLUO-4-AM and are reported in Delta F. The data are averages of triplicate determinations from two independent experiments; *$P<0.05$ (one way ANOVA + Dunnett’s multiple comparison test). **B:** Indirect determination of CCK-8S levels in HuTu-80 supernatants using the CCK$_1$R activation assay. Cells were incubated for 2 hours with 0.25mM H.g.-12 alone (positive control), or co-incubated with compound 03A3.
(50-100µM). The data are averages of triplicate determinations from two independent experiments; ***$P<0.001$ (one way ANOVA + Bonferroni’s multiple comparison test).
Figure 1.
Figure 2.

Fold increase in CCK8S secretion (CHO-CCK1R activation)
Figure 3

(A) Graph showing the effect of H.g.-12 (mM) on DeltaF CCK1R. The graph includes a curve with points marked at 0.03, 0.06, 0.125, 0.25, and 0.5 mM, with *** indicating significance.

(B) Bar graph comparing Delta F CCK1R among different treatments: buffer, H.g.-12, detiglated, aglycon, and detig-agly. The bars are labeled with NS for non-significance.
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Figure 4.
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Figure 6.

344bp amplicon
Figure 7.