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Naturally-occurring TGR5 agonists modulating glucagon-like peptide-1 biosynthesis and secretion

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ABSTRACT

Selective GLP-1 secretagogues represent a novel potential therapy for type 2 diabetes mellitus. This study examined the GLP-1 secretory activity of the ethnomedicinal plant, *Fagonia cretica*, which is postulated to possess anti-diabetic activity. After extraction and fractionation extracts and purified compounds were tested for GLP-1 and GIP secretory activity in pGIP/neo STC-1 cells. Intracellular levels of incretin hormones and their gene expression were also determined. Crude *F. cretica* extracts stimulated both GLP-1 and GIP secretion, increased cellular hormone content, and upregulated gene expression of proglucagon, GIP and prohormone convertase. However, ethyl acetate partitioning significantly enriched GLP-1 secretory activity and this fraction underwent bioactivity-guided fractionation. Three isolated compounds were potent and selective GLP-1 secretagogues: quinovic acid (QA) and two QA derivatives, QA-3β-O-β-D-glycopyranoside and QA-3β-O-β-D-glucopyranosyl-(28 → 1)-β-D-glucopyranosyl ester. All QA compounds activated the TGR5 receptor and increased intracellular incretin levels and gene expression. QA derivatives were more potent GLP-1 secretagogues than QA. This is the first time that QA and its naturally-occurring derivatives have been shown to activate TGR5 and stimulate GLP-1 secretion. These data provide a plausible mechanism for the ethnomedicinal use of *F. cretica* and may assist in the ongoing development of selective GLP-1 agonists.

1. Introduction

Pharmacological interventions targeting the enteroinsular axis are a clinically-proven approach for improving glucose homeostasis in patients with type 2 diabetes mellitus [20]. Clinically approved pharmacological strategies include mimetics and analogues of glucagon-like peptide-1 (GLP-1), but also inhibitors of dipeptidylpeptidase-4 (DPP-4i or gliptins) which act to prevent the physiological breakdown of GLP-1 and its sister hormone glucose-dependent insulinotropic polypeptide (GIP) [7,11,12,20]. A third, more unestablished strategy is the enhancement of postprandial GLP-1 secretion by means of specific secretagogues [25]. The functioning of the enteroinsular axis is markedly affected by the onset of type 2 diabetes. On one hand there is clear evidence of impaired GIP action but not GIP secretion [15,23,34,35]. On the other hand defective or blunted GLP-1 secretion has been reported in type 2 diabetes on some occasions [23,33–35]. Despite these observations of impaired GLP-1 secretion in type 2 diabetes GLP-1-based therapies retain an ability to stimulate insulin secretion from pancreatic beta-cells [15]. One future alternative to GLP-1 analogue/mimetic therapy could involve ways of selectively increasing endogenous GLP-1 secretion. This approach could potentially even be used in combination with gliptin therapies in order to further enhance levels of active endogenous GLP-1.

There is evidence that enhancement of endogenous postprandial GLP-1 secretion is an effective strategy. For example, dosing with the GLP-1 secretagogue L-arginine increases circulating GLP-1 levels in both lean and obese mice, and it substantially improves glucose clearance [5]. GLP-1 receptor signalling appears to be pivotal since improvements in glucose tolerance are significantly less

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effective in GLP-1R$^{-/-}$ mice than wild-type littermates [5]. A number of other dietary-based GLP-1 secretagogues have been reported [4,13,17,22,26] but pharmacologically there is a focus on targeting several intestinal GPCRs believed to be important in modulating gut hormone secretion. These include the bile acid receptor TGR5 [25] and fat-sensing receptors GPR40, GPR119, and GPR120 [38] all of which are present on the surface of enteroendocrine cells.

The present study investigated the incretin hormone secretory activity of the plant *Fagonia cretica* which was identified by routine systematic screening of plant materials. Although there is negligible scientific evidence that *F. cretica* possesses anti-diabetic activity [29] it is noteworthy that it is reportedly used in natural folk/Greco-Arab medicine for the treatment of diabetes [1,3,9]. Medicinal plants have been used to treat diabetes for millennia, and they offer a natural resource of anti-diabetic products for traditional ethnomedical systems in Asia and Africa. The prevalence of type 2 diabetes is rising fastest among developing countries [16] where 75–80% of the population relies on traditional herbal medicines for their primary healthcare [18]. Hence, there is a pressing need for the scientific characterisation of the numerous anti-diabetic medicinal plants described in traditional ethnomedical systems worldwide. There are a number of examples of commonly used clinical anti-diabetic therapies which are based on natural products. For example, the most commonly used anti-diabetic drug metformin is based on the discovery of galegine in the plant *Galega officinalis* [2]. Furthermore, the widely used anti-diabetic drug acarbose (an alpha glucosidase inhibitor) was discovered in a bacterium [32]. The present study sought to identify novel ethnobotanical compounds which preferentially stimulate GLP-1 secretion but not GIP secretion.

2. Materials and methods

2.1. Plant material

An ongoing programme of work involving systematic screening of plant materials for incretin secretory activity led us to focus on *F. cretica* (Synonym *F. indica*). The fresh aerial parts of plants were collected in Pakistan in September 2010. *F. cretica* (locally referred to as Dhamasa) was collected from Mianwali. Plant species identification was carried out by Professor Dr. Rizwana Aleem Qureshi, Department of Plant Sciences, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan. Voucher specimens (HMP-461) were deposited in the "Herbarium of Medicinal Plants of Pakistan", Quaid-i-Azam University, Islamabad, Pakistan.

2.2. Extraction and isolation of F. cretica compounds

The fresh aerial parts of *F. cretica* were rinsed with water, dried and crushed to yield 22 kg (dry weight) of plant material. Crude plant extract (FCC) was prepared by maceration in a methanol–chloroform (1:1) solution for 7 days at room temperature. Extracts were filtered, concentrated with a rotary evaporator (45 °C) under vacuum and the resulting crude was suspended in water. The water suspension was partitioned three times with *n*-hexane to obtain an *n*-hexane fraction (FCN). The residual aqueous suspension was then partitioned with ethyl acetate to obtain an ethyl acetate (FCE) fraction and an aqueous fraction (FCA). Each of the above fractions were concentrated in a rotary evaporator. A total of 3 compounds with GLP-1 secretory activity were isolated from FCE. All 3 compounds were obtained by chromatographic separation on a silica gel column, and their isolation and identification has previously been described in detail [29]. Compounds were identified by a combination of mass spectrometry and NMR spectroscopy (Bruker AVANCE 400 MHz NMR). Chemical structures were confirmed by comparison of their chemical and spectroscopic properties (as previously reported [29]). The compounds were: quinovic acid (QA), quinovic acid-3β-O-β-D-glycopyranoside (dQA), and quinovic acid-3β-O-β-D-glucopyranosyl-(28 → 1)-β-D-glucopyranosyl ester (EdQA). Structures of QA, dQA and EdQA can be found in the Supplementary Appendix (Fig. 1).

2.3. Cells

pGIP/neo STC-1 cells were a gift from Dr. B. Wice (Washington University of St. Louis) [28] with permission from Dr D. Hanahan (University of California, San Francisco, CA). These cells secrete both GLP-1 and GIP and are responsive to nutrient stimulation and are potentially a very useful model for studying the secretory responses of both incretin hormones [10,14,26]. DMEM culture medium containing 4.5 g/l with L-glutamine, without sodium pyruvate (Life Technologies, Paisley, UK) and supplemented with 10% foetal bovine serum, 100 U/ml penicillin, 100 mg/l streptomycin and geneticin—G418, 400 μg/ml purchased from Sigma (Dorset, UK). pGIP/neo STC-1 cells were cultured in culture medium and incubated in a 5% CO_2 humidified atmosphere at 37 °C. Cells underwent passage upon reaching 80–90% confluence and were used for studies between passage numbers 15–50.

2.4. GLP-1 and GIP Secretion

Cells were seeded into 12-well plates (2×10^6 per well) and cultured overnight at 37 °C in a humidified atmosphere of 5% CO_2. Medium was removed and cells washed 3 times with HEPES buffer (20 mM HEPES, 10 mM glucose, 140 nM NaCl, 4.5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$) then allowed to incubate for 1 h in HEPES buffer prior to adding fresh HEPES buffer (vehicle control) or HEPES buffer supplemented with test agents. Cells were incubated for 3 h after which the vehicle control and test agents were removed, centrifuged and stored at −80 °C prior to ELISA assays. GLP-1 and GIP ELISA kits were purchased from Millipore (Billerica, MA, USA). GLP-1 assays detected only active forms of GLP-1 (7–36 amide and 7–37) whilst GIP assays detected total GIP (GIP (1–42) and GIP (3–42)).

2.5. Cellular GLP-1 and GIP content

Cellular GLP-1 and GIP peptide levels were determined in pGIP/neo STC-1 cells after incubations with plant extract/compounds. Initially cells (2×10^6 per well) were seeded into 12 well plates and cultured overnight at 37 °C in a humidified atmosphere of 5% CO_2. Medium was removed and replaced with 1 ml of medium supplemented with 50 μM of the plant extract/compound. Following incubation the medium was removed and cells were washed with 1 ml of HEPES buffer and GLP-1 and GIP were extracted by the addition of acid/ethanol (1.5% HCl (v/v): 75% ethanol (v/v): 23.5% H_2O (v/v)) and incubated overnight at 4 °C. Acid/ethanol solutions were removed, centrifuged ($2000 \times g$, 5 min) to remove cellular debris, and the ethanol evaporated off using a SpeedVac sample concentrator (Genevac, Ipswich, UK). Samples were reconstituted in buffer and stored at −80 °C prior to measurement by ELISA.

2.6. Real-time PCR

Cells (4×10^6 per well) were seeded into 6-well culture plates with 1 ml of culture medium and allowed to attach overnight (24 h; 37 °C; 5% CO_2). Medium was removed, the cells were washed with HEPES buffer and then underwent pre-incubation with HEPES buffer (60 min). Buffer was aspirated off and cells were incubated