Chemical synthesis of human trefoil factor 1 (TFF1) and its homodimer provides novel insights into their mechanisms of action†

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TFF1 is a key peptide for gastrointestinal protection and repair. Its molecular mechanism of action remains poorly understood with synthetic intractability a recognised bottleneck. Here we describe the synthesis of TFF1 and its homodimer and their interactions with mucins and Helicobacter pylori. Synthetic access to TFF1 is an important milestone for probe and therapeutic development.

TFF1 is a member of the trefoil factor family (TFF) of gastrointestinal peptides, well-known for their role in protecting and repairing the gastrointestinal tract.1–3 A substantial body of evidence suggests TFF1’s role in gastric protection.1–5 TFF1 is conserved three-loop containing TFF domain.1,17 A free cysteine residue (CysVII) is located outside the TFF domain and enables homo- and heterodimerisation via an intermolecular disulfide bond (e.g. TFF1–gastrokine 2).5,18,19 The molecular mechanisms through which TFF1 protects the gastrointestinal mucosa are not fully understood,10,11 but could involve enhancement of cell migration due to its motogenic20 and anti-apoptotic21 effects (‘restitution’),22 a scavenger function for extracellular reactive oxygen/nitrogen species,2,17 and/or mucin interactions.3,5

Lack of synthetic access to TFF1 and associated probes is the main bottleneck hindering progress on identification of its mode of action on a molecular level. Recombinant expression and purification of TFF1 is challenging,18,23 and synthesis of TFF1 monomer was attempted in the past, but no correctly folded TFF1 was obtained nor characterised.24 Reliable access to TFF1 peptides through chemical synthesis would facilitate large scale production, library design for structure–activity relationship (SAR) studies and site-specific chemical modifications for molecular probes, setting the stage for therapeutic development. We thus set out to develop a synthetic strategy for the efficient production of bioactive TFF1 and its analogues.

Attempts to directly assemble TFF1 via solid phase peptide synthesis (SPPS) were unsuccessful and prompted us to switch to a two-fragment ligation strategy (Fig. 1). TFF1 was split between Gly31 and Cys32 which was chosen based on fragment size and favoured kinetics for native chemical ligation (NCL) (ligation proceeds faster at sterically less hindered thioester positions).25,26 Traditionally, thioester fragments are synthesised by tert-butyloxy carbonyl (Boc)-SPPS because the thioester is not stable under the basic conditions and nucleophiles required for N²-Fmoc deprotection.27 Attempts to synthesise the N-terminal segment containing the thioester by manual Boc-SPPS were however unsuccessful, resulting in poor crude quality and low yield. Thus, a Fmoc-SPPS compatible strategy that uses a C-terminal hydrazide as a thioester surrogate was pursued (Fig. 1). TFF1c–d was produced with a C-terminal hydrazide that was converted to a thioester prior to ligation with TFF1a–b to form full-length reduced TFF1c–d (Fig. 2A and B). TFF1c–d was then folded at pH 8.5 (50 μM) for 48 hours, forming...
three disulfide bonds. TFF1 displayed a two-peak analytical HPLC profile with identical mass (Fig. 2C). Both peaks were collected and each re-run, yielding the same two-peak profile, confirming a single molecular entity. Such multi-peak conformational HPLC peak profiles are not uncommon with peptides and proteins and have also been observed with TFF3.

Homodimerisation of TFF1 was achieved through disulfide bond formation in water at high concentration (1.5 mM; pH 5, 48 h). TFF1 homodimer also displayed a two-peak HPLC profile (Fig. 2D) with identical mass. Since TFF1 dimerises slowly in water, we also synthesised TFF1 with CysVII protected (TFF1(C58Acm)) to ensure proper differentiation between TFF1 monomer and homodimer for our functional studies (Fig. S1, ESI†) and to have the option to unprotect CysVII when needed for dimer formation or conjugation to reporter tags. In previous studies with recombinant TFF1, undesired homodimerisation was achieved through a CysVII to Ser replacement.

NMR spectroscopy confirmed the correct fold of both TFF1 and TFF1 homodimer. Hα chemical shifts were assigned using total correlated spectroscopy (TOCSY) and nuclear Overhauser effect spectroscopy (NOESY) and compared to the chemical shifts of recombinant TFF1 (BMRB: 4933) and TFF1 homodimer (BMRB: 4930). Synthetic and recombinant TFF1 analogues displayed good overlap of the secondary Hα chemical shifts (deviation of the Hα chemical shifts from random coil values) confirming the same overall fold and 3D structure (Fig. 3). Circular dichroism (CD) experiments confirmed the presence of an α-helix characterised by negative bands at 208 nm and 222 nm and a positive band at 193 nm (Fig. S2, ESI†), corresponding well with the presence of negative secondary Hα chemical shifts indicative of an α-helix between position Pro24–Lys30 in loop 2 (Fig. 3).

Interaction of TFF1 (C58Acm) and TFF1 homodimer with carbohydrates was tested in different mucin binding assays since TFF1 has been associated with lectin activities. Both peptides were labelled with 125I and then used in binding studies against purified mucin fractions from the stomach of human, pig, and frog (Xenopus laevis) (Fig. 4).

In these overlay assays, only TFF1 homodimer bound to these mucins. Attempts to bind TFF1 (C58Acm) to mucin preparations failed. This indicates that dimerisation of TFF1 is essential for mucin interaction. This is in agreement with other observations that have also highlighted dimerisation as a critical factor for the protective function of TFF1.
(boiled with 1% β-mercaptoethanol) (Fig. 4B), suggesting lectin-like binding to the carbohydrate moieties and not to its protein structure.\(^{36–38}\) Binding to frog mucins demonstrated that the gastric mucin sugar epitope recognised by TFF1 is evolutionary conserved (Fig. 4C). GSA-II, a lectin from *Griffonia simplicifolia* that recognises α-GlcNAc (α\(_1,4\)-linked N-acetylglucosamine; a conserved carbohydrate motif in gastric mucins), confirmed the presence of mucins since it recognises αGlcNAc (α\(_1,4\)-linked N-acetylglucosamine), a conserved carbohydrate motif in gastric mucins.

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TFF1 has also been implicated in cell migration and proliferation.\(^{3,20,21}\) However, independent confirmation, target identification and in-depth pharmacology remain sparse for these studies.\(^1\) With multi-milligrams of TFF1 at hand, our goal was to validate some of these studies in well-controlled experiments. We carried out cell migration and proliferation assays with HT-29 (human colorectal adenocarcinoma) cells using a state-of-the-art IncuCyte \(^{6}\) system. In these studies, neither TFF1 nor TFF1 homodimer had any effect on cell migration or proliferation at concentrations of 0.1–10 μM (Fig. S3, ESI †). We also performed toxicity assays with TFF1 and its homodimer. Neither peptide...
displayed any cytotoxic or haemolytic effects at concentrations up to 25 μM [Fig. S4, ESI†].

In conclusion, we developed a synthetic strategy for TFF1, its homodimer and analogues. TFF1 homodimer bound to gastric mucins supporting the consensus that TFF1 elicits its protective action in the gut via lectin-like mucin cross-linking. We could not reproduce TFF1 activity in well-controlled HT-29 cell migration or proliferation assays (see ESI† for further discussion). Chemical access to TFF1 and its analogues represents an important milestone for the TFF field since it enables the production of homogenous material in addition to regioselective incorporation of chemical modifications that will facilitate molecular probe development for more in-depth mechanistic and target validation studies as well as therapeutic development. This in turn will advance our understanding of TFF1’s physiology and therapeutic potential.

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Conflicts of interest

There are no conflicts to declare.

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