Supporting Information

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

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Experimental Procedures

1. Materials

Fmoc (9-fluorenylmethoxycarbonyl)-amino acids and Fmoc-Phe-Wang resin (loading 0.7 mmol/g) were from Iris Biotech GmbH (Marktredwitz, Germany). 2-Chlorotrityl chloride resin (loading 1.0–2.0 mmol/g) and *O*-(1H-6-chlorobenzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate (HCTU) were from Chem-Impex (Wood Dale, USA). *N*,*N*-diisopropylethylamine (DIEA) peptide synthesis grade was from Auspep (Melbourne, Australia). Hydrazine hydrate, triisopropylsilane (TIPS), acetonitrile (ACN), sodium 2-mercaptoethanesulfonate (MESNA), tris(2-carboxyethyl)phosphine (TCEP), McCoy's 5A medium and recombinant EGF were from Sigma Aldrich (Sydney, Australia). N,N-dimethylformamide (DMF), trifluoroacetic acid (TFA) and diethyl ether were obtained from Chem-Supply (Gillman, Australia). Trypsin-EDTA 0.25%, Dulbecco's modified Eagle's medium (DMEM) and L-glutamine were from Invitrogen (Mulgrave, Australia). Foetal bovine serum (FBS) was from Scientifix (South Yarra, Australia). The HT-29 cell line was obtained from CellBank Australia (Wentworthville, Australia). HEK-293 (ATCC CRL-1573) human embryonic kidney cells were obtained from American Type Culture Collection (ATCC). Human whole blood was obtained from the Australian Red Cross Blood Service. All other reagents and solvents were obtained from Sigma Aldrich (Sydney, NSW, Australia) in the highest available purity and used without further purification.

2. Chemical synthesis of TFF1 and TFF1 homodimer

2.1 Peptide synthesis

Peptide fragments were synthesised on a Symphony automated peptide synthesiser (Protein Technologies Inc., Tucson, AZ) via Fmoc- solid phase peptide synthesis (SPPS) on 0.1 mmol scale. C-terminal fragment ($TFF1_{32–60}$-OH) with the Cys^{58} free or protected by Acm was assembled on a Phe-Wang resin and N-terminal fragment ($TFF_{11–31}$-$NHNH_2$) on a freshly prepared 2-chlorotrityl hydrazine resin. To prepare the 2-chlorotrityl hydrazine resin, the 2-chlorotrityl resin was swelled in 50% (v/v) DMF/DCM and treated with 10% (v/v) hydrazine hydrate/DMF for 30 min. The unreacted resin was deactivated with 5% (v/v) MeOH/DMF for 10 min and directly used for the next coupling step. Resin loading was determined by quantitative Fmoc release.[1] Amino acid side-chains were protected as follows: Arg(Pbf), Asn/Gln/His(Trt), Asp/Glu(OtBu), Cys(Trt/Acm), Lys/Trp(Boc) and Ser/Thr/Tyr(tBu). Fmoc removal was performed using 30% (v/v) piperidine/DMF. Couplings were achieved using 5 eq, relative to the resin loading, of Fmoc–amino acid/HCTU/ DIPEA (1:1:1) in DMF two times (5 and 10 min). A cocktail of 90% (v/v) TFA / 5% (v/v) TIPS / 5% (v/v) H_2O was used to simultaneously remove side-chain protecting groups and cleave the peptides from the resin. Crude peptides were precipitated and washed with diethyl ether three times, then lyophilised in 50% ACN/0.1% TFA/H_2O and purified by preparative RP-HPLC.

2.2 Native chemical ligation

Only fragments with purity ≥ 90% were used for native chemical ligation (NCL). TFF1 N- and C- terminal fragments were ligated by hydrazide-based NCL.[2] First, the hydrazide peptide (2 mM) was oxidised to an azide by dissolving it in 0.2 M phosphate solution containing 6 M GdnHCl (pH 3) followed by reaction with $NaNO_2$ (10 eq relative to the hydrazide fragment) for 15 min at −15 °C. The C-terminal fragment (1.5 mM) was dissolved in 0.2 M phosphate solution containing 6 M GdnHCl and MESNA (100 eq relative to the N-terminal fragment).[2] The peptide segments were combined, and the pH adjusted to 7.5. Once the reaction was completed (24 h), TCEP was added (75 mM, ~50 eq), the pH lowered with TFA (~pH 2) to quench the reaction and the full-length reduced TFF1 was purified by RP-HPLC.

2.3 Oxidative folding and dimerisation

Full-length reduced TFF1 or TFF1(C^{58}Acm) was dissolved in a minimum amount of 50% ACN/0.1% TFA/H_2O and diluted in 0.1 M ammonium bicarbonate (NH_4HCO_3), pH 8.5 at 25 °C, to a final peptide concentration of 50 μM. The folding of the three disulifde bonds was monitored by analytical RP-HPLC and ESI-MS. Folding was complete after 48 h. The pH was then adjusted to 2 with neat TFA and the oxidised peptide purified by preparative RP-HPLC. TFF1 with the C^{58} free was dissolved in water at a high concentration (1.5 mM; pH ~5) and let dimerise for 48 h.

2.4 RP-HPLC and LC-MS analysis

Peptides were purified using either a preparative C_{18} (Vydac; 10 μm, 2.2 cm ID x 250 mm, flow rate 15 mL/min) or a preparative C_5 (Phenomenex Luna; 10 μm, 21.2 mm ID x 250 mm, flow rate 15 mL/min) column on a Waters 600 HPLC system (Waters Co., Milford, MA) using a gradient of solvent A (0.05% TFA/ H_2O) and B (90% ACN/0.043% TFA/H_2O) according to the peptide's retention times. $TFF1_{1-31}$ was purified using the C_{18} preparative column with a gradient of 10-40% B over 60 min. $TFF1_{32-60}$ and reduced TFF1 were also purified on the C_{18} preparative column with a gradient of 20-50% B over 60 min. Reduced TFF1 was washed with 10% B for 15 min before its purification to remove the salts from the ligation buffer. Folded TFF1 was purified using the C_5 preparative column with a gradient of 10-40% B over 60 min. Analytical reversed-phase (RP) HPLC was carried out using analytical C_3 (Agilent Zorbax SB-C_3, 5 μm, 2.0 x 250 mm, 80 Å) or C_{18} (Phenomenex Jupiter; 5 μm, 2.0 x 250 mm, 300 Å) columns. Peptide masses were determined with a Q-Star Pulsar mass spectrometer (SCIEX, Ontario, Canada) with a Series 1100 solvent delivery system equipped with an auto-injector (Agilent Technologies Inc., Palo Alto, CA) and a Phenomenex Jupiter LC-MS C_{18} column (90 Å, 4 μm, 250 mm x 2 mm). The instrument scanned from 500–1800 m/z.

3. NMR spectroscopy

Peptides were dissolved in 90% H_2O/10% D_2O (~500 μM) and spectra were acquired using a Bruker 600 MHz NMR spectrometer equipped with a cryogenically cooled probe (cryoprobe) at 298 K. NOESY spectra was acquired with mixing time of 200 ms and TOCSY with a spin-lock of 80 ms. Samples were internally referenced to water at 4.76 ppm. Spectra were processed and assigned using TopSpin (Bruker Biospin) and CCPNMR Analysis 2.4.1 (CCPN, University of Cambridge, Cambridge, UK), respectively. NOEs in the NOESY spectra were manually picked and assigned. Secondary shifts were calculated by subtracting the random coil Hα shift from the experimental Hα shifts.[3]

4. Circular dichroism

Stock solutions of TFF1-C^{58}(Acm) and homodimer were prepared in 50% ACN/H_2O at 1 mM concentration. Peptide concentrations for circular dichroism (CD) analysis were 50 μM in 10 mM sodium phosphate buffer (pH 7.4). CD spectra were obtained on a Jasco J-810 spectropolarimeter (Easton, MD, USA). All experiments were carried out in a 0.1 cm quartz cell with 250 μL sample at 25 °C and examined in far UV spectra region (195–260 nm), 20 nm/min scanning speed, 1 nm bandwidth, and 0.5 nm data pitch with 5 scans averaged for each sample. Blank subtraction was performed in the Spectra Management Software followed by smoothing using a binomial algorithm. CD was reported as mean residue ellipticity ($[\theta]$ (mdeg•cm^2•$dmol^{-1}$) = (100 x θ) / (n x c x l), where θ = raw output (mdeg), n = number of peptide bonds, c = concentration (M), l = cuvette path length (cm)). The data were processed and displayed using Prism 7 (GraphPad, La Jolla USA).

5. Scratch wound healing

HT-29 were routinely cultured in McCoy medium supplemented with 10% FBS at 37 ºC in 5% CO_2 until reaching 80–90% confluence. ~135,000 cells/well were grown in IncuCyte® ImageLock 96-well microplates (Essen BioScience) to confluence and serum-starved for 16 h. The cell monolayers were scratched with the IncuCyte® WoundMaker tool and immediately rinsed with phosphate-buffered saline (PBS). Then, the cells were cultured in serum-free medium in the presence of TFF1(C^{58}Acm), TFF1 homodimer, EGF (positive control) or water (vehicle). Photomicrographs were taken every 4 h using an IncuCyte® live-cell imager system (Essen Biosciences, Ann. Arbor, MI). Relative wound density was calculated using the IncuCyte® software and plotted as a function of time. At least three independent experiments were performed in triplicate. Two-way ANOVA followed by Dunnett correction was performed to assess statistical differences between treated cells and vehicle.

6. Cell proliferation

HT-29 cells were seeded in 96-well plates (~6,000 cells/well). The tested compounds were added after 48 h with the cells at 10–25% confluence. The percentage of cell confluence was continuously monitored and measured using the IncuCyte-ZOOM system according to the manufacturer's instructions (Essen Bioscience, Millennium Science, Surrey Hills, NSW, Australia). The results were plotted as the percentage of cell confluence as a function of time. At least three independent experiments were performed in triplicate. Two-way ANOVA followed by Dunnett correction was performed to assess statistical differences between treated cells and vehicle.

7. Cell viability (cytotoxicity)

HEK-293 ATCC CRL-1573 human embryonic kidney cells (~5,000 cells/well), suspended in DMEM media supplemented with 10% FBS, were seeded into 384-well plates in a 20 µL volume. TFF1(C^{58}Acm) and TFF1 homodimer were added to the cells for a final concentration of 0.18–22.7 µM. The cell plates were then incubated for 20 h at 37 ºC, 5% CO_2. Tamoxifen (~1.5-200 µM) was used as a positive control. After the incubation, 5 µL of 100 µM resazurin diluted in PBS was added to each well (final concentration ~11 µM). The plates were then incubated for 3–4 h at 37 ºC, 5% CO_2. The fluorescence intensity (FI) was read using the TECAN Infinite M1000 PRO with excitation/emission 560/590 nm. Cell viability was calculated using the following equation: Cell viability (%) = (FI_{sample} – $FI_{negative}$/$FI_{untreated}$ –$FI_{Negative}$)*100. CC_{50} (concentration at 50% cell viability or conversely cytotoxicity) was calculated using a nonlinear regression analysis of log (concentration) vs normalised cell viability.

8. Haemolysis

Human ethics approval was obtained for use of human blood for haemolysis studies, from the University of Queensland Medical Research Ethics Committee (approval number 2014000031). TFF1(C^{58}Acm) and TFF1 homodimer were serially diluted 2-fold in 0.9% NaCl and plated (25 µL) into a 384-well polypropylene plate (0.2–25 µM final concentration). Whole blood (10 mL/tube) was washed 2–3 times in 3 volumes of 0.9% NaCl, with centrifugation at 500 g, with reduced deceleration, for 10 min between washes. Cells were counted using a Neubauer haemocytometer, then diluted to 1×10^8/mL in 0.9% NaCl. Cells (25 µL/well) were added to the plates containing TFF1. Melittin (0.15–20 µM) was used as a positive control. Plates were sealed and then placed on a plate shaker for 10 min before being incubated for 1 h at 37 ºC without shaking. Following incubation, plates were centrifuged at 1,000 g for 10 min to pellet cells and debris and then 25 µL of the supernatant was transferred into a 384-well flat-bottom polystyrene plate. Absorbance (Abs) was read at 405 nm using a Tecan M1000 Pro monochromator plate reader. The percent of haemolysis was calculated using the following equation: Haemolysis (%) = (Abs_{sample}–$Abs_{negative}$/$Abs_{positive}$–$Abs_{negative}$)*100. HC_{10} and HC_{50} (concentration at 10 and 50% haemolysis, respectively) were calculated using nonlinear regression analysis of log (concentration) *vs*. normalised haemolysis.

9. Binding studies

All investigations followed the tenets of the declaration of Helsinki and were approved by the Ethics Committee of the Medical Faculty of the Otto-von-Guericke University Magdeburg (codes: 01/02 January 2002 and July 2007 and 96/06 October 2006). Human tissue from the gastric corpus was investigated from a patient (MC-383) undergoing gastrectomy because of carcinoma. The formal histopathological review excluded neoplastic changes. Gastric specimens were cut into pieces and homogenised by grinding with a pistil under liquid nitrogen as described.[4] The resulting fine powder was then homogenised with a 5-fold amount (w/v) of buffer (30 mM NaCl, 20 mM Tris-HCl pH 7.0 plus protease inhibitors).[4] Furthermore, a commercial porcine preparation (type III from Sigma-Aldrich; S-mucin) was investigated.[5] 6 mL of gastric extracts (human) or 5 mL of commercial porcine preparation (1 mg/mL) were fractioned with the ÄKTATM-FPLC system (Amersham Biosciences, Freiburg, Germany) *via* a HiLoad 16/600 Superdex 75 prep grade (S75HL) column as described previously.[5-7] Additionally, fractions B5-C2 were investigated from a *X. laevis* gastric extract as described in detail previously.[7] Fractions were separated by non-denaturing agarose gel electrophoresis (AgGE) and these gels were directly blotted.[6, 7] Mucins were detected with the biotinylated GSA-II (2 µg/mL), a lectin from *Griffonia simplicifolia* which recognises αGlcNAc (α1,4-linked N-acetylglucosamine), a conserved carbohydrate motif in gastric mucins.[6, 7] *H. pylori* bacterial culture and preparation of bacterial cell lysates was done as described previously.[8] A chloramphenicol resistance cassette was inserted into the gene *HP0857* of *H. pylori* strain P12 to generate the mutant P12Δ*HP0857*. Bacterial cell lysates were separated by reducing SDS-PAGE containing 15% polyacrylamide and were transferred to nitrocellulose membrane. Before the overlay assay was performed, general protein on the membrane was stained with 0.2% Ponceau S in 3% trichloracetic acid for 2 min and the membrane was rinsed with desalted water until background was destained. TFF1 peptides were labelled with ^{125}I according to iodogen method.[5] Briefly, 6 µg TFF1 homodimer was labelled with 5 µL $Na^{125}I$ (100mCi/mL, 2000 Ci/mmol; Hartmann Analytic GmbH, Braunschweig, Germany). Unreacted $Na^{125}I$ was removed by size exclusion column (Sephadex G10). Overlay assays were performed as described previously for TFF2.[5]

Results and Discussion

1. Synthesis of TFF1 monomer and homodimer

Purification of $TFF1_{1-31}$ and $TFF1_{32-60}$ with the C^{58} free by RP-HPLC provided the fragments in 14% and 24% overall yields, respectively. C_{18}-RP-HPLC purification of full-length reduced $TFF1_{1-60}$ once the ligation was completed had a 37% yield. C_5-RP-HPLC purification of folded TFF1 achieved 24% yield. The overall yield of TFF1 based on a synthetic scale of 0.1 mmol of $TFF1_{1-31}$ was 1%. Synthesis of TFF1(C^{58}Acm) was performed using a combination of SPPS and NCL as described for TFF1 with the C^{58} free (Figure S1). Of note, folding was achieved in 24 h instead of 48 h with slightly improved yields by adding 5 mM reduced glutathione (GSH) and 0.5 mM oxidised glutathione (GSSG) to the oxidation buffer. GSH/GSSG was however not used to accelerate the folding of unprotected Cys^{58} TFF1 because of the propensity for the Cys^{58} to form GSH-adducts.

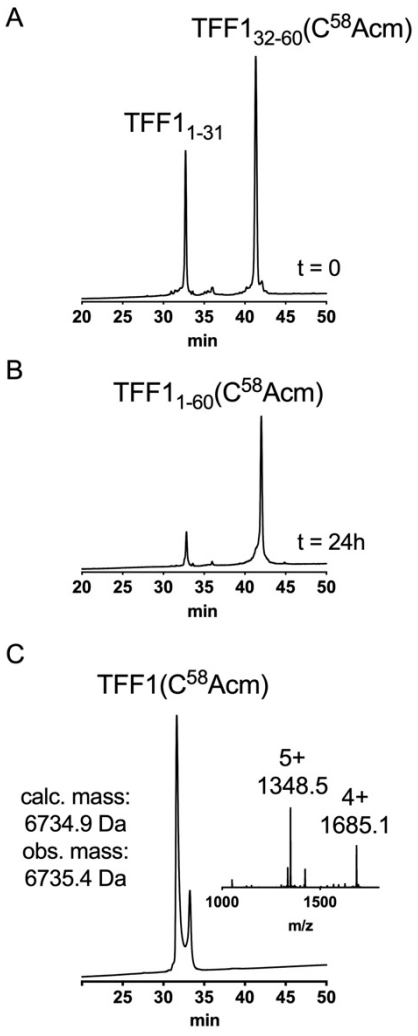

Figure S1. Chemical synthesis of TFF1(C^{58}Acm). (A) Analytical HPLC trace of NCL at 0 h showing the fragments $TFF1_{1-31}$ and $TFF1_{32-60}$(C^{58}Acm). **(B)** Analytical HPLC and MS traces of NCL at 24 h showing the disappearance of the fragments and appearance of full-length reduced TFF1; **(C)** HPLC and MS traces of folded TFF1(C^{58}Acm) after oxidative folding in 0.1 NH_4HCO_3, pH 8.5 for 48 h.

2. Circular dichroism

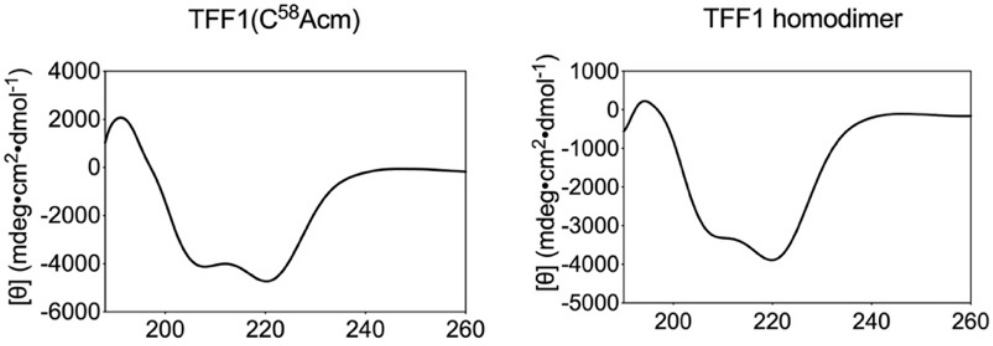

Figure S2. CD spectrum of TFF1(C^{58}Acm) and TFF1 homodimer.

3. Cell migration and proliferation

Effects of TFF1 on cellular processes associated with epithelial restitution including proliferation and migration were investigated. Cell proliferation was quantified based on cell confluence and normalised to the starting cell confluence (~20%). Cell migration was evaluated by scratching a monolayer of cells and allowing the cell to migrate to close the gap. Controls paclitaxel (cell growth inhibitor) or epidermal growth factor (EGF; cell migration and growth inducer) were used as negative and positive controls, respectively. For these bioassays, TFF1(C^{58} Acm) was used to ensure that the monomeric form was being evaluated since an unprotected seventh cysteine residue could induce undesired dimerisation.

Neither TFF1(C^{58}Acm) nor TFF1 homodimer (0.1–10 μM) had any effect on cell migration or proliferation ($p>0.05$), in contrast to the controls paclitaxel or EGF ($p<0.05$) (Figure S3). In previous studies dimerisation of TFF1 was prevented by substitution of C^{58} with a serine residue.[9] Although less potent than its homodimeric counterpart, TFF1(S^{58}) induces cell migration *in vitro*[9] and epithelium repair *in vivo*.[9] NMR characterisation of TFF1(C^{58} Acm) (Figure 2) showed that TFF1(C^{58} Acm) and TFF1(S^{58}) have a similar overall structures, therefore Acm protection of C^{58} is most likely not the reason for the lack of activity of TFF1 monomer. Despite the very similar conditions to the assay previously published,[9] enhancement of cell migration by TFF1 in HT-29 cells could not be reproduced. Unlike the assay reported in the literature, we used the IncuCyte-ZOOM, a state-of-the-art piece of equipment, which uses a wound-making tool rather than manually scratching the cells, which usually produces a wide gap variability.[10] In addition, we measured wound healing based on the relative wound density (%), a robust metric which measures cell density in the wound area relative to the cell density outside of the wound area while in the published work they measured the wound width, i.e. the distance between the edges of the wound. Another difference is that we used McCoy's 5a medium instead of Dulbecco's Modified Eagle Medium (DMEM) since McCoy's 5a is the growth medium recommended by CellBank Australia. This is not the first report of failure to reproduce the TFF1 activity on cell migration of gastrointestinal cell lines with lack of activity of recombinant TFF1 on Caco-2 cells migration having previously been reported.[11] TFF1 also displays no motogenic effects in SW-480, another colonic cancer cell line.[9] Another observation is that TFF1 only displays weak motogenic effects at high concentration ranges (10^{-6}–10^{-7} M) which is atypical for classical peptide ligands such as EGF that activate their receptors in the 10^{-10} M range, raising the possibility of unspecific effects.

TFF1 effect on cellular growth is also controversial. Some studies claim that TFF1 possesses an unusual property of promoting repair only by stimulating cell migration without proliferation.[9, 12, 13] However, there are also reports in the literature of TFF1 inhibiting cell growth.[14, 15] TFF1 inhibited proliferation (1–50 μM) of gastrointestinal cell lines such as IEC-18 (rat intestinal cell), HCT-116 (human colon cancer) and AGS (human gastric cell).[14] Also, TFF1-null mice develop hyperplasia and dysplasia, suggesting that TFF1 inhibits cell proliferation.[15] Herein, the results support the hypothesis that TFF1 does not interfere with cell growth, at least not for the colonic HT-29 cells.

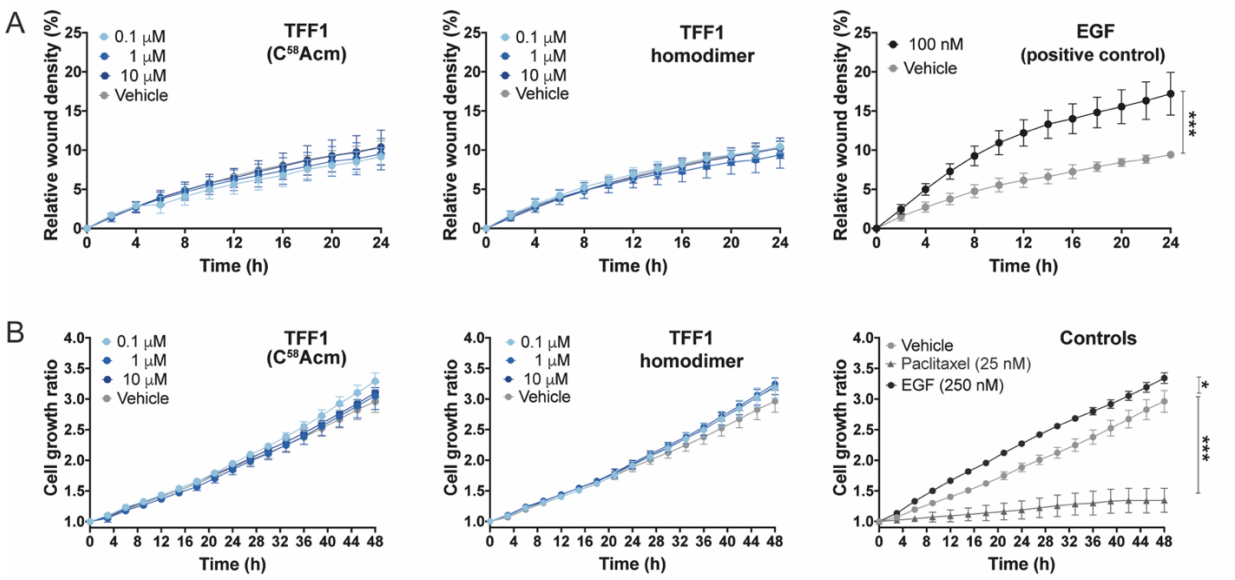

Figure S3. Concentration-dependent effects of TFF1(C^{58}Acm) and TFF1 homodimer on (A) migration and (B) proliferation of HT-29 cells. The peptides were tested from 0.1–10 µM concentration (n=3). Migration and proliferation were monitored and quantified using IncuCyte-Zoom technology. Results are expressed as a percentage of relative wound density (mean ± SEM). EGF and paclitaxel were used as controls and water as vehicle. The curves of the tested compounds were compared to the vehicle curve for statistical significance by two-way ANOVA followed by Dunnett correction. *p<0.05; ***p<0.001.

4. Cytotoxicity and haemolytic effect of TFF1(C^{58}Acm) and homodimer

Cell viability was measured using the indicator dye resazurin which is reduced by a mitochondrial reductase, only present in live cells, to produce a strongly fluorescent colour, proportional to the number of viable cells.[16] HEK-293 (human embryonic kidney 293) cells were used because (1) the kidney is one of the human organs commonly affected by drug toxicity[17] and (2) this cell line is well-characterised and relatively easy to culture, handle and maintain.[18] Haemolytic effects were evaluated by measuring the haemoglobin release as an indicator of erythrocytes lysis following the agent exposure. As expected, neither TFF1(C^{58}Acm) nor homodimer displayed any significant cytotoxic or haemolytic effects at concentrations up to 25 µM (Figure S4).

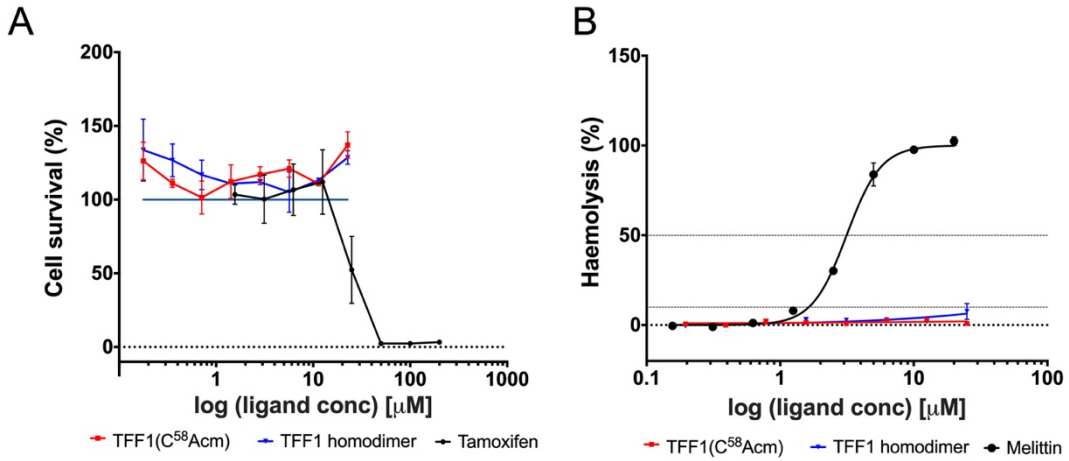

Figure S4. Cytotoxic analysis of TFF1(C^{58}Acm) and TFF1 homodimer on HEK-293 and erythrocyte cells. (A) TFF1 effects on the viability of HEK-293 cells after 20 h. Tamoxifen was used as a positive growth inhibitor control. (B) Haemolytic activity of TFF1 after 1 h. Melittin was used as a positive haemolytic control. Results are presented as mean ± SEM of two independent experiments.

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