Potential role of the bitter taste receptor T2R14 in the prolonged survival and enhanced chemoresponsiveness induced by apigenin

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Abstract. Bitter taste receptors (T2Rs) are G protein-coupled receptors originally detected in the gustatory system. More recently, T2Rs have been shown to be expressed in extra-oral cells eliciting non-gustatory functions. Emerging evidence has suggested a potential role for T2R signaling in diverse pathophysiological conditions, including cancer. The aim of the present study was to evaluate the expression of T2R14 in pancreatic ductal adenocarcinoma (PDAC) and to assess its involvement in the anticancer effects induced by apigenin, a natural ligand of T2R14. For this purpose, T2R14 expression was explored in PDAC tumor tissue and tumor-derived cell lines. Using the cell lines expressing the highest levels of T2R14, its effects on chemoresponsiveness and migration upon activation with apigenin were investigated *in vitro*. To the best of our knowledge, the present study was the first to confirm the expression of the T2R family member T2R14 in PDAC. Patients with relatively high levels of T2R14 expression exhibited significantly prolonged overall survival compared with that of patients with low T2R14 expression. Furthermore, novel functions for apigenin were revealed; notably, apigenin was shown to elicit cytotoxic, anti-migratory and chemosensitizing effects to 5-fluoruracil (5-FU) and to 5-FU, leucovorin, irinotecan and oxaliplatin in pancreatic cancer cells. In conclusion, the present study extended the evidence for the anticancer effects of apigenin and strongly indicated the functional relevance of T2R14 in PDAC, even though their respective underlying pathways appear to be independent of each other.

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal malignancies, accounting for ~8% of all cancer-related deaths in the United States (1). Despite recent advances in cancer treatment, the prognosis of patients with PDAC remains poor, with a 5-year overall survival rate of <10% (1). This poor prognosis is mainly attributed to an aggressive tumor biology and diagnosis at advanced stages when curative treatment is no longer an option. The vast majority of patients with PDAC will receive chemotherapy during the course of multimodality treatment; either as a neoadjuvant treatment for borderline resectable tumors, as an adjuvant treatment following surgical resection or as definitive chemotherapy in a palliative setting (2,3). However, the strong chemoresistance of PDAC remains a major obstacle to its cure (4). Therefore, agents enhancing the efficacy of systemic treatment are urgently required to improve patient outcome. Clinical standard chemotherapeutic regimes include gemcitabine, 5-fluoruracil (5-FU), or the combination of 5-FU, leucovorin, irinotecan and oxaliplatin (FOLFIRINOX) (5). FOLFIRINOX has gained increasing attention due to its superior response rates compared with previously established chemotherapeutic regimens (6).

Natural compounds have always been a major resource for drug development. In this context, the group of bitter compounds has emerged as potential antineoplastic agents (7-9); however,

the mechanisms underlying these anticancer effects remain to be elucidated. Notably, since all of these bitter compounds are able to activate bitter taste receptors (T2Rs), these receptors have emerged as a novel focus of research (10). T2Rs are G protein-coupled receptors that were initially identified in the oral cavity where they mediate bitter taste (10-12). However, in recent years, functional expression of T2Rs has been reported extra-orally and T2Rs have been shown to be involved in a wide range of biological functions, including antineoplastic effects (13,14).

The first T2R identified in PDAC tissue was T2R38. In 2016, our previous study reported its expression in pancreatic cancer tumor tissue and tumor derived-cell lines. Upon activation, T2R38 was shown to modulate key transcription factors and to induce overexpression of the multi-drug resistance (MDR) protein ABCB1 (15). MDR proteins are major contributors to chemoresistance by shuttling drugs, including chemotherapeutics, outside of the cell (16). In a subsequent study, another T2R, T2R10, was also revealed to be functionally expressed in pancreatic adenocarcinoma. T2R10 was shown to be partially involved in caffeine-induced chemosensitization in tumor-derived cell lines (17). Increased susceptibility to chemotherapeutics has been reported to be associated with inhibition of AKT activation and down-regulation of ABCG2 (17), both of which serve key roles in the induction of chemoresistance (18-21). Therefore, different T2R subtypes may have opposing roles in the modulation of response to chemotherapy.

T2R14 is a broadly tuned T2R (12), which has been identified as an important modulator of innate immune responses in various cell types (22-24). In 2014, Singh *et al* (25) identified T2R14 expression in normal and breast cancer cells. This receptor was also shown to confer antiproliferative and anti-migratory effects on highly metastatic breast cancer cells upon activation (26). More recently, T2R14 was reported to be differentially expressed in various solid tumor subtypes. Elevated T2R14 gene expression has been shown to be associated with prolonged survival in non-papillary bladder cancer, but with worse survival in patients with esophageal and adrenocortical adenocarcinoma (27). To date, to the best of our knowledge, there are no reported studies on T2R14 expression at the protein level or its functional role in PDAC. A well-known ligand for T2R14 is apigenin. Besides signaling via T2R14, apigenin has been described to activate another T2R family member, T2R39 (28). It is a natural flavonoid found in most vegetables and fruits (29-31). In 1986, Birt *et al* (32) first reported that apigenin could confer anticancer effects. Since then, numerous studies have revealed that apigenin can exhibit antiproliferative effects and can act in synergy with certain anticancer agents in various tumor types (33,34). In PDAC, apigenin has been reported to inhibit cell proliferation in human tumor cells (35,36). The chemosensitizing effects of apigenin in PDAC have to date only been reported in the context of the single chemotherapeutic agent gemcitabine (37,38). Although there is evidence of potential antitumor mechanisms involving apigenin and T2R14, to the best of our knowledge, these have not yet been investigated and reported. Therefore, the present study aimed to investigate T2R14 in PDAC and to explore whether the chemosensitizing effects induced by its agonist apigenin are mediated via T2R14.

Materials and methods

Chemicals, reagents and antibodies. Apigenin (MilliporeSigma) was dissolved in dimethyl sulfoxide (DMSO; MilliporeSigma). Gemcitabine (Hexal AG), 5-FU (medac GmbH), oxaliplatin (Accord Healthcare GmbH) and leucovorin (TEVA GmbH) were dissolved in phosphate-buffered saline (PBS; MilliporeSigma). SN-38 (irinotecan; Selleck Chemicals), the active metabolite of irinotecan, was dissolved in DMSO. For immunocytochemistry, immunohistochemistry and flow cytometry, the following antibodies were used: T2R14 polyclonal antibody (cat. no. OSR00161W), rabbit IgG isotype control (cat. no. 31235), goat anti-rabbit IgG (H+L) highly cross-adsorbed secondary antibody, Alexa Fluor™ 568 (cat. no. A-11036) and goat anti-rabbit IgG (H+L) cross-adsorbed secondary antibody, Alexa Fluor 488 (cat. no. A-11008) (all from Invitrogen; Thermo Fisher Scientific, Inc.) and Envision Flex+Rabbit Linker (cat. no. DM825; lot no. 20079633; Dako; Agilent Technologies, Inc.).

Cell culture. All listed cell lines were obtained from American Type Culture Collection. The PDAC cell lines BxPC-3, MiaPaCa-2, PANC-1, SU.86.86, T3M4 and the breast cancer cell line MCF-7 were cultured in RPMI 1640 (MilliporeSigma) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin (Gibco; Thermo Fisher Scientific, Inc.). 293T cells were cultured in DMEM (MilliporeSigma) supplemented with 10% FBS and 1% penicillin-streptomycin. The normal human pancreatic cell line HPDE was cultured in keratinocyte serum-free medium supplemented with 30 μg/ml bovine pituitary extract, 200 pg/ml epidermal growth factor and 1% antibiotic-antimycotic (10,000 U/ml penicillin, 10,000 μg/ml streptomycin and 25 μg/ml Amphotericin B; cat. no. 15240062; Gibco) (all from Thermo Fisher Scientific, Inc.). All cells were maintained in a humidified atmosphere containing 5% CO_2 at 37˚C. Cell culture flasks, dishes, cell scraper and serological pipettes were obtained by Sarstedt AG & Co. KG. Inc. Accutase™ (PAN-Biotech GmbH) was used for detaching cells.

Gene expression analysis. The data of T2R14 mRNA expression in PDAC tumors of 75 patients, aged between 41 and 83 years were obtained from the database of the Heidelberg Institute of Personalized Oncology (HIPO) biobank. Tumor samples with reads per kilobase million (rpkm) ≥1 for T2R14 were defined as T2R14 positive. Kaplan-Meier analysis and log-rank test were used to assess survival between patients with relatively high (≥5.8 rpkm; n=48) and low (<5.8 rpkm; n=37) mRNA expression levels of T2R14.

Patient and biopsies. Pancreatic tissue samples were obtained from the databank of the Institute of Pathology, University Medical Center Mainz (Mainz, Germany) in accordance with the regulations of the tissue bank. The histological examination of formalin-fixed, paraffin-embedded and hematoxylin and eosin-stained pancreatic tissue sections was performed during clinical routine diagnostics at the Pathology Department, University Medical Center Mainz, by board-certified pathologists, and the diagnosis was

established according to the criteria of the World Health Organization Classification of Tumors of the digestive system (39). From this cohort, a tissue microarray (TMA) was created. Briefly, representative areas were selected by two independent pathologists for each patient and two array spots were included for each of the following areas: Normal ducts, low-grade pancreatic intraepithelial neoplasia (PanIN), high grade PanIN, tumor center, tumor periphery, and if available, lymph node metastasis. The core diameter was 1 mm for each core. Tissue slides were cut at 3 μm and immunohistochemistry was conducted. Patient demographics and clinical data are summarized in Table I.

Immunohistochemistry. After deparaffinization and rehydration of the tissue in xylene and a descending alcohol series, the tissues were incubated with anti-human T2R14 antibody (1:300) for 1 h at room temperature. The antigen retrieval was performed with Dako EnVision™ Flex Target Retrieval Solution high pH (pH 9.0; Dako; Agilent Technologies, Inc.) at 95˚C for 20 min. For permeabilization, the Dako EnVision™ Flex (20X) Wash Buffer (1:20; cat. no. DM831; lot no. 20058883; Dako; Agilent Technologies, Inc.) was used for 10 min at room temperature. To block non-specific binding, the DakoEnvion™ Flex Peroxidase Blocking Reagent (cat. no. DM821; lot no. 20062978; Dako; Agilent Technologies, Inc.) was used for 5 min at room temperature. As a secondary antibody, the EnVision™ Flex+Rabbit Linker (cat. no. DM825; lot no. 20079633; Dako; Agilent Technologies, Inc.) was used for 15 min at room temperature, followed by a color reaction with Dako EnVision™ Flex Substrate buffer (cat. no. DM823; lot no. 20062842; Dako; Agilent Technologies, Inc.) and Dako EnVision™ Flex DAB+ Chromogen (cat. no. DM827; lot no. 20065471; Dako; Agilent Technologies, Inc.) according to the manufacturer's protocol (one drop per 1,000 μl) twice for 5 min. Subsequently, the tissues were counterstained with hematoxylin (Dako; Agilent Technologies, Inc.) for 5 min at room temperature. The presence of T2R14 was evaluated using the established Allred immunoreactive scoring system (40) giving a range of 0-8 (0, negative; 2-3, low; 4-6, medium; 7-8, high). In brief, the Allred score is the sum of the proportion of positive cells score (0, absent; 1, <1% positive cells; 2, 1-10% positive cells; 3, 11-33% positive cells; 4, 34-66% positive cells; 5, ≥ 67% positive cells) and the staining intensity score (0, absent; 1, mild reaction; 2, moderate reaction; 3, intense reaction). T2R14 expression was stained in PDAC tumor tissues (n=102) and visually assessed by analysis of areas in the tumor periphery and center using Olympus BX51 light microscope (Olympus Corporation).

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from BxPC-3, MiaPaCa-2, PANC-1, SU.86.86, T3M4, MCF-7 and HPDE cell lines with TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.), and the purity and quantity of collected RNA was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop; Thermo Fisher Scientific, Inc.). For cDNA synthesis, 500 ng (1 μl) RNA, 2 μl 10X RT buffer, 0.8 μl dNTP mix (100 mM), 2 μl 10X RT random primers, 1 μl MultiScribe reverse transcriptase (50 U/μl) and 0.5 μl RNAse inhibitor (20 U/μl) (all obtained from Applied Biosystems; Thermo Fisher Scientific, Inc.) and 12.7 μl DEPC H_2O (MilliporeSigma) were used. cDNA synthesis was performed using a C1000 Touch™ thermal cycler (temperature protocol: 25˚C for 10 min, 37˚C for 120 min and 85˚C for 5 min). T2R14 and HPRT (internal control) cDNA was quantified using StepOnePlus™ Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The final reaction volume included 2.5 μl PCR master mix (TaqMan™ Fast Advanced Master Mix; cat. no. 4444558; Applied Biosystems; Thermo Fisher Scientific, Inc.), 0.25 μl predesigned TaqMan™ Gene Expression Assays for T2R14 (Assay ID: Hs00256800_s1; cat. no. 4331182; Applied Biosystems; Thermo Fisher Scientific, Inc.) or HPRT (Assay ID: Hs02800695_m1; cat. no. 4331182; Applied Biosystems; Thermo Fisher Scientific, Inc.), 1.25 μl DEPC H_2O and 1 μl cDNA. The qPCR reaction consisted of 20 sec of initial denaturation at 95˚C (holding stage) followed by 40 cycles with 1 sec at 95˚C for denaturation, and 20 sec at 60˚C for annealing and extension (cycling stage). Expression was analyzed using StepOne™ Real-Time PCR v2.2 software (Applied Biosystems; Thermo Fisher Scientific, Inc.). T2R14 gene expression was normalized to HPRT expression using the $2^{-\Delta\Delta Cq}$ method (41). For quality control, reactions were performed using water instead of DNA. The breast cancer cell line MCF-7 was used as positive control since it is known to express T2R14 (42).

Table I. Patient data for immunohistochemistry analysis.

Diagnosis	Value
Sex, female/male	49/53
Age, years (mean; median)	37-82 (67; 69)
Tumor size[a]	
pT1	10
pT2	64
pT3	28
Lymph node metastases[a]	
pN0	34
pN1	38
pN2	30
Distant metastases	
pM0	15
pM1	4
No data	83
Histological grading[a]	
G1	5
G2	61
G3	35
G4	1
Resection margin[a]	
R0	79
R1	20
No data	3

[a]Pathological evaluation according to the guidelines of the Union for International Cancer Control 2017 (56).

Flow cytometry. Before BxPC-3, MiaPaCa-2, PANC-1, SU.86.86, T3M4, MCF-7 and HPDE cell lines were subjected to flow cytometry, they were grown to 80-90% confluence, harvested with Accutase, and then washed and resuspended in PBS. Initially, the cells were pre-incubated with Zombie UV™ Fixable Viability Kit (1:200; BioLegend, Inc.) and Fc Blocking Reagent (1:100; BD Biosciences) in 100 μl PBS for 15 min. After washing with PBS, the cells were incubated with T2R14 polyclonal antibody or rabbit IgG isotype control primary antibodies (both 1:100) in 100 μl PBS-1% FBS for 30 min. After further washing with PBS-1% FBS, the cells were incubated with secondary fluorochrome-conjugated goat anti-rabbit IgG (H+L) highly cross-adsorbed secondary antibody, Alexa Fluor 568 (1:1,000) in 100 μl PBS-1% FBS for 15 min in the dark. All incubation steps were performed at 4˚C. Finally, the cells were washed in PBS-1% FBS, resuspended in 100 μl PBS-1% FBS and analyzed with the BD LSRFortessa™ flow cytometer (BD Biosciences). Results were analyzed with FlowJo 10.7.1 software (FlowJo LLC). The difference in mean fluorescence intensity (MFI) between the isotype control and T2R14 primary antibody was used to determine expression. For visualization of MFI differences between populations with deviating sample numbers, peak heights of the overlaid curves were scaled to their mode=100%. In the knockdown experiments, the MFI of the short hairpin RNA (shRNA) control was defined as 100%. Knockdown efficacy was expressed as percentage decrease of MFI.

Immunocytochemistry. BxPC-3, MiaPaCa-2, PANC-1, SU.86.86, T3M4, MCF-7 and HPDE cell lines were seeded on coverslips in 24-well culture plates and examined at 80-90% confluence. First, cells were washed with PBS and fixed for 10 min at room temperature using 4% paraformaldehyde (Morphisto GmbH). For permeabilization, the cells were treated with 1% bovine serum albumin (BSA)/PBS-Triton X-100 (all from MilliporeSigma). After blocking with 5% BSA for 1 h at room temperature, the cells were incubated overnight at 4˚C with T2R14 primary antibody or rabbit IgG isotype control (both 1:1,000). The following day, cells were washed repeatedly with PBS and were then incubated with secondary fluorochrome-conjugated antibodies for 2 h at room temperature. For wild-type cells, Alexa Fluor 488-conjugated antibodies (1:1,000) and Texas Red™-X Phalloidin (1:500; Thermo Fisher Scientific, Inc.) were used. For knockdown cell lines, Alexa Fluor 568-conjugated antibodies (1:1,000) were used. The cells were finally washed in PBS repeatedly. The nuclei were stained using fluoroshield DAPI mounting medium (MilliporeSigma). Image acquisition was performed on a Leica TCS SP5 confocal microscope (Leica Microsystems GmbH).

shRNA transduction. shRNA-mediated T2R14 knockdown was established in PANC-1 and SU.86.86 cells. Third generation T2R14 pGFP-C-shLenti shRNA vectors (cat. no. 50840) and non-targeting pGFP-C-shLenti shRNA control vectors (cat. no. TR30021) were purchased from OriGene Technologies, Inc. The following four vectors were tested: TL301238A: 5'-TTTGGTGCTGCTTCTTGTGAC TTCGGTCT-3', TL301238B: 5'-TCACTGCTTTGGCAA TCTCTCGAATTAGC-3', TL301238C: 5'-ATCGCAAGA AGATGCAGCACACTGTCAA-3', TL301238D: 5'-TCT CTGTCAGTGCTACTGTGGCTGAGGTA-3'. For PANC-1 cells, the vector TL301238A and for SU.86.86 cells, another vector TL301238B was found to work most efficiently and used for all functional experiments. Lentiviral particles were produced in $5x10^6$ 293T cells using the calcium phosphate transfection method (CAPHOS Calcium Phosphate Transfection Kit; MilliporeSigma) with 15 μg of one of the four third generation T2R14 shRNAs (TL301238A-D) or non-targeting shRNA pGFP-C-shLenti shRNA vector plasmids, 10 μg Gag/Pol- (pMDLg/pRRE; Addgene, Inc.), 5 μg Rev-(pRSV-Rev; Addgene, Inc.) and 2 μg Envelope-(phCMV-VSV-G; Addgene, Inc.) plasmids. Transfected 293T cells were incubated for 8 h in a humidified atmosphere containing 5% CO_2 at 37˚C before the medium was changed. The supernatant was then collected after every 12 h and filtered through a 0.22 μm syringe filter. Success of transfection was estimated using Carl Zeiss™ Axiovert 40 CFL light microscope with fluorescence light source HXP 120 (both from Carl Zeiss AG). Titration of lenti-vectors and calculation of multiplicity of infection ratio were not performed as no repetitive transduction was intended. Instead, $5x10^6$ PANC-1 and SU.86.86 cells were transduced with half of the supernatant (5 ml each) that was collected after 12 h incubation. The transduction of PANC-1 and SU.86.86 cells with lentiviral particles was accomplished for 48 h in a humidified atmosphere containing 5% CO_2 at 37˚C. Success of transduction was estimated using Carl Zeiss™ Axiovert 40 CFL light microscope with fluorescence light source HXP 120 (both from Carl Zeiss AG). Post-transduction, both cell lines were cultured in RPMI 1640 medium supplemented with 10% FBS, 1% penicillin-streptomycin. For selection and maintenance of stably transfected cell lines, 1.4 μg/ml puromycin (MilliporeSigma) was added according to previously performed puromycin titration. Knockdown efficacy was measured using RT-qPCR and flow cytometry in the first 3 weeks after transduction. All treatments were carried out at 37˚C in a humidified atmosphere containing 5% CO_2. Cells transduced with the scrambled shRNA are referred to as the control-shRNA group and those transduced with the T2R14 shRNA are referred to as the T2R14-shRNA group.

Drug treatment. For cell viability tests, $5x10^3$ PANC-1 cells and $2x10^3$ SU.86.86 cells were seeded in 96-well plates. For FOLFIRINOX treatment, all of the component drugs were mixed and diluted in PBS. The final concentration of 75 μM 5-FU, 100 μM leucovorin, 60 μM oxaliplatin and 0.8 μM irinotecan was regarded as 100X. Prior to MTS assays, cells were treated according to the schemes shown in Fig. S1A and B and cultured in total for 96 h in a humidified atmosphere containing 5% CO_2 at 37˚C. To assess the cytotoxicity of apigenin treatment alone, a single dose of apigenin was administered 24 h after seeding (Fig. S1A). For chemotherapeutic response experiments, cells were pretreated with apigenin 12 h after seeding; 24 h after seeding, the medium was refreshed and another dose of apigenin plus different dosages of either gemcitabine (5-50 nM for SU.86.86 and 1-1,000 nM for PANC-1), 5-FU (0.3-50 μM) or FOLFIRINOX (0.1-10X) was administered. The final concentration of apigenin never exceeded 10 μM (Fig. S1B). Control cells for FOLFIRINOX

were treated with DMSO and for all other drugs with PBS, according to the respective solvent used.

Chemosensitivity assays. To assess cytotoxicity and viability, the half maximal inhibitory concentration (IC_{50}) values of apigenin, gemcitabine, 5-FU, FOLFIRINOX and combination treatments were determined using the MTS cell viability assay. Cell viability was measured after 96 h of cell culture and respective treatments as illustrated in Fig. S1A and B. Media were completely replaced with 100 μl fresh medium. Using a Repeater® M4-Multi-Dispenser Pipette (Eppendorf AG) 20 μl MTS CellTiter 96 Aqueous One Solution (Promega Corp.) was added to each well. After 2 h of incubation at 37˚C absorbance was measured at 490 and 630 nm using FLUOstar® Omega Multi-Mode Microplate Reader (BMG Labtech). Cell viability was defined as the difference in absorbance at 490 nm and background at 630 nm. For normalization, absorbance values obtained on day 0 were defined as 0% viability, and absorbance values obtained from untreated cells on day 3 were defined as 100% viability. In order to assess the effects of apigenin on the responsiveness to the aforementioned chemotherapeutic drugs, this normalization was done separately for the control (PBS) and apigenin treatment group. The coefficient of drug interaction (CDI) was used to assess the synergy of apigenin and the aforementioned chemotherapeutic drugs as previously described (43). The CDI was calculated as follows: CDI=AB/(A x B) where AB is the ratio of number of living tumor cells after the combined treatment of apigenin and chemotherapeutic drug. A is the ratio of number of living tumor cells after the single treatment with apigenin and B the ratio of number of living tumor cells after the single treatment with one of the chemotherapeutic drugs. In this method, a CDI value >1, =1 or <1 suggests that drugs are antagonistic, additive or synergistic, respectively.

Migration assay. A total of $1x10^5$ cells were seeded in 100 μl serum-free media in the upper compartment of 6.5 mm Transwell inserts in a 24-well plate (Corning, Inc.). For PANC-1 cells, inserts with 5.0 μm pores were used; for SU.86.86 cells, inserts with 8.0 μm pores were used. After 15 min of pre-incubation, 600 μl media supplemented with 10% calf serum containing 10, 30 or 50 μM apigenin was added to the lower compartment. The cells were incubated in a humidified atmosphere containing 5% CO_2 at 37˚C for 24 h. Non-migrated cells in the upper compartment were carefully removed with a cotton swab. The migrated cells were then fixed with 4% paraformaldehyde for 10 min at room temperature and stained with 1% crystal violet solution for 20 min at room temperature (MilliporeSigma). The inserts were washed several times with PBS to remove excess dye and finally dried completely. The mean number of migrated cells was counted in seven random spots at x200 magnification using a Carl Zeiss™ Axio Scope. A1 transmitted-light microscope and images were acquired with Zeiss Axiocam MRc (both from Carl Zeiss AG).

Statistical analysis. IC_{50} doses were calculated using GraphPad Prism Software 5.0 (GraphPad Software Inc.). Other statistical analyses were carried out with GraphPad Prism Software and SPSS software (version 26; IBM Corp.). For all *in vitro* studies at least three independent experimental repeats were carried out. The statistical significance of data was obtained from viability assays was tested by running extra-sum-of-squares F tests, and data from migration assays were assessed using one-way ANOVA followed by Dunnett post-hoc-tests. Statistical analysis of expression data was performed using and Kaplan-Meier curves and log-rank test were used to assess survival data. For the univariate survival analysis, the median T2R14 expression level was used as the cut-off. $P<0.05$ was considered to indicate a statistically significant difference.

Results

T2R14 is expressed in human pancreatic cancer tissue and tumor-derived cell lines. The analysis of RNA sequencing data derived from the HIPO databank revealed that T2R14 mRNA was detected in all tested human pancreatic cancer tissue samples. PDAC tumors with >1 rpkm were considered T2R14 positive (44,45). Overall, the expression level was found to be low (median, 5.8 rpkm) (Fig. 1A). Patients with relatively high T2R14 expression (≥5.8 rpkm) exhibited a significantly prolonged survival rate compared with that of patients with low T2R14 expression (<5.8 rpkm) (652 days vs. 429 days; $P=0.03$; Fig. 1B).

For T2R14 detection at the protein level, immunohistochemistry was carried out on TMAs. Tissue samples from 102 patients with PDAC, including invasive carcinoma, precursor lesions and non-neoplastic tissue from each patient, were analyzed. Patient data are summarized in Table I. All tumors exhibited T2R14 positivity. T2R14 expression was additionally seen in non-cancerous tissue and precursor lesions (Fig. 1C and D); however, stromal components revealed no marked expression. The vast majority of patient tissue samples expressed T2R14 at a high level (85%; Fig. 1E). Within PDAC, the expression patterns did not differ between the tumor center and the periphery. T2R14 protein was localized not only on the cell surface but also in the cytoplasm. These findings are in accordance with a previous report on T2R10 protein expression patterns in human PDAC (17). Regarding immunohistochemistry results, there was no association between T2R14 expression and clinicopathological parameters (data not shown).

Subsequently, five human pancreatic cancer cell lines, one normal pancreatic cell line (HPDE) and a breast cancer cell line (MCF-7) were tested for T2R14 expression at mRNA and protein levels. T2R14 mRNA expression was detected by RT-qPCR in all cell lines and varying expression levels were detected (Fig. 2A). Notably, the pancreatic cancer cell line SU.86.86 exhibited the highest T2R14 mRNA expression. For the detection of T2R14 protein expression, flow cytometric analyses were carried out. In line with the immunohistochemical findings, both normal pancreatic and tumor-derived cell lines expressed T2R14, with SU.86.86 and PANC-1 cells exhibiting the strongest signal (Fig. 2B and C). For further validation, immunofluorescence was used to visualize T2R14 protein expression (Fig. 2D-F). T2R14 staining was located both on the cell membrane and the cytoplasm, which is in concordance with the results of the aforementioned immunohistochemical staining (Fig. 2F). For subsequent studies, the two cell lines with the highest expression levels of T2R14, SU.86.86 and PANC-1, were chosen. PANC-1 is a primary

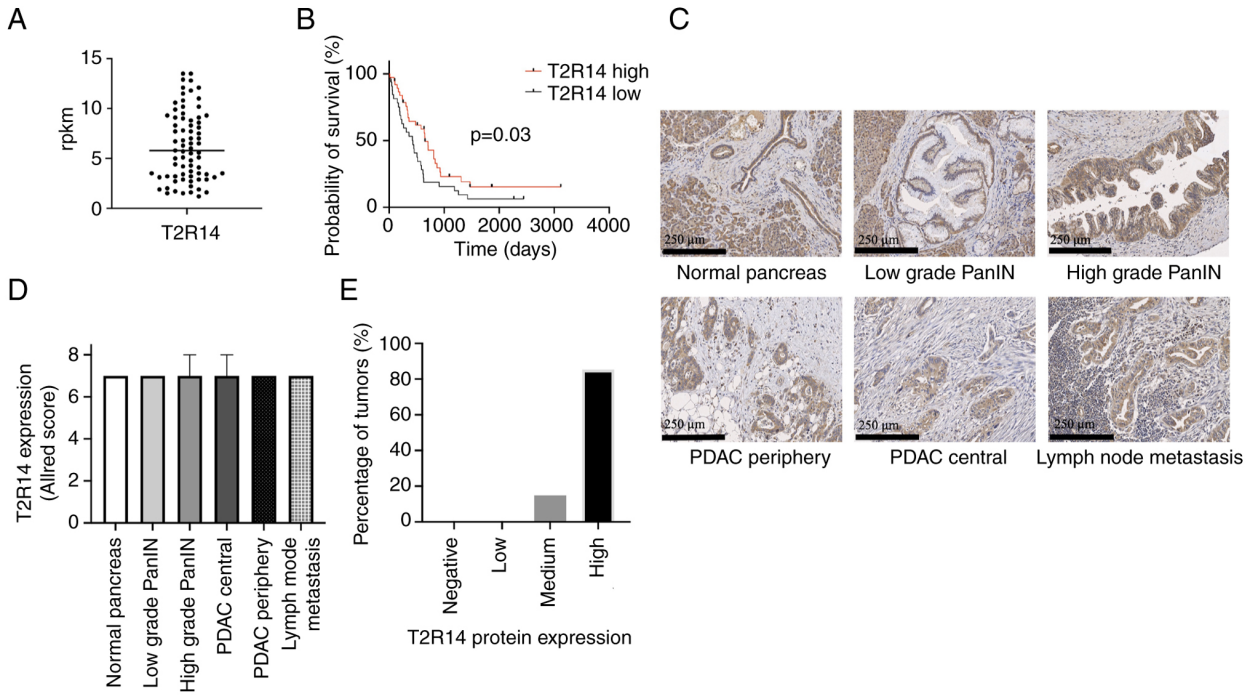

Figure 1. Expression of T2R14 in human pancreatic tissue. (A) T2R14 mRNA transcripts from 75 patients with PDAC derived from the HIPO databank (median, 5.8 rpkm). Data are presented as quantified transcript levels in rpkm. (B) Kaplan-Meier univariate survival analysis comparing patients with PDAC and high (≥5.8 rpkm; n=38) or low (<5.8 rpkm; n=37) T2R14 expression levels of T2R14. The median T2R14 expression level was used as the cut-off. (C) Representative images of T2R14 staining (brown) in normal pancreas, low grade PanIN, high grade PanIN, PDAC from the periphery and the center, and lymph node metastasis tissue specimens. (D) Allred scoring system was used to semi-quantify T2R14 protein expression in 102 PDAC tumor samples. Data are presented as the median ± IQR (E) Distribution of T2R14 protein expression intensity in PDAC tumors. Samples were categorized according to the Allred scoring system: Negative, score 0; low, score 2-3; medium, score 4-6; high, score 7-8. Bars represent the proportion of PDAC tumors with respective Allred scores. HIPO, Heidelberg Institute of Personalized Oncology; PanIN, pancreatic intraepithelial neoplasia; PDAC, pancreatic ductal adenocarcinoma; rpkm, reads per kilobase million; T2R14, bitter taste receptor 14.

pancreatic tumor cell line, whereas the SU.86.86 cell line was derived from PDAC liver metastasis.

Apigenin inhibits cell viability in SU.86.86 and PANC-1 cells in a dose-dependent manner. The present study evaluated the effects of apigenin, a known T2R14 agonist, on the chemoresponsiveness of PDAC cells. The effects of apigenin on the viability of SU.86.86 and PANC-1 pancreatic cancer cell lines was determined using the MTS assay. Treatment with apigenin inhibited the viability of cells; with an IC_{50} of 42 μM in SU.86.86 cells and 75 μM in PANC-1 cells (Fig. 3).

Effects of apigenin on cell viability are independent of T2R14. To assess whether the cytotoxic effects induced by apigenin were mediated via T2R14 signaling, T2R14 was knocked down using shRNA. Knockdown efficacy was tested at the mRNA and protein levels. According to the results of RT-qPCR, the mRNA expression levels of T2R14 were reduced by 36% in SU.86.86 cells and 31% in PANC-1 cells compared with in cells transduced with control-shRNA (Fig. 4A and E). Flow cytometry demonstrated a reduction in T2R14 protein expression of 83% in Su.86.86 cells and 65% PANC-1 cells (Fig. 4B,C, F and G). In line with these results, immunofluorescence confirmed that silencing of the T2R14 protein resulted in a clearly visible decrease in fluorescence intensity (Fig. 4D and H).

Having confirmed effective knockdown of T2R14 expression via shRNA transduction, the effect of T2R14 silencing on apigenin-induced cytotoxicity was examined. In both cell lines there was no significant difference in the IC_{50} values between cells in the control-shRNA and T2R14-shRNA groups, suggesting apigenin-induced cytotoxicity may be mediated by alternative pathways other than T2R14 (Fig. 5).

Apigenin synergistically attenuates the cytotoxic effects of 5-FU and FOLFIRINOX in SU.86.86 cells, independent of T2R14. To assess the potential effects of T2R14 activation on chemoresistance, cells were pretreated with apigenin and then exposed to various concentrations of clinically used chemotherapeutic drugs. Apigenin was applied at a concentration (10 μM) that had previously been shown to induce no relevant cytotoxic effect on SU.86.86 or PANC-1 cells. For chemotherapeutic treatment, gemcitabine, 5-FU and the combination chemotherapy regimen FOLFIRINOX were applied since these are the most frequently used cytotoxic agents in the treatment of patients with PDAC (2,3).

In SU.86.86 cells, exposure to 10 μM apigenin significantly reduced the IC_{50} of FOLFIRINOX by 61% ($P<0.0001$; Fig. 6A). The CDI for SU.86.86 cells was <1 suggesting a synergistic behavior between apigenin and FOLFIRINOX for all concentrations of FOLFIRINOX except for the highest (Table II). The highest level of synergy, represented by the lowest CDI (0.74), was thereby achieved when FOLFIRINOX was used at concentrations around its IC_{50} value (1.5X). A similar trend was observed for the combination treatment with 5-FU and apigenin. In the presence of apigenin, the IC_{50} of 5-FU was reduced by 42% ($P<0.05$; Fig. 6B). CDI values of

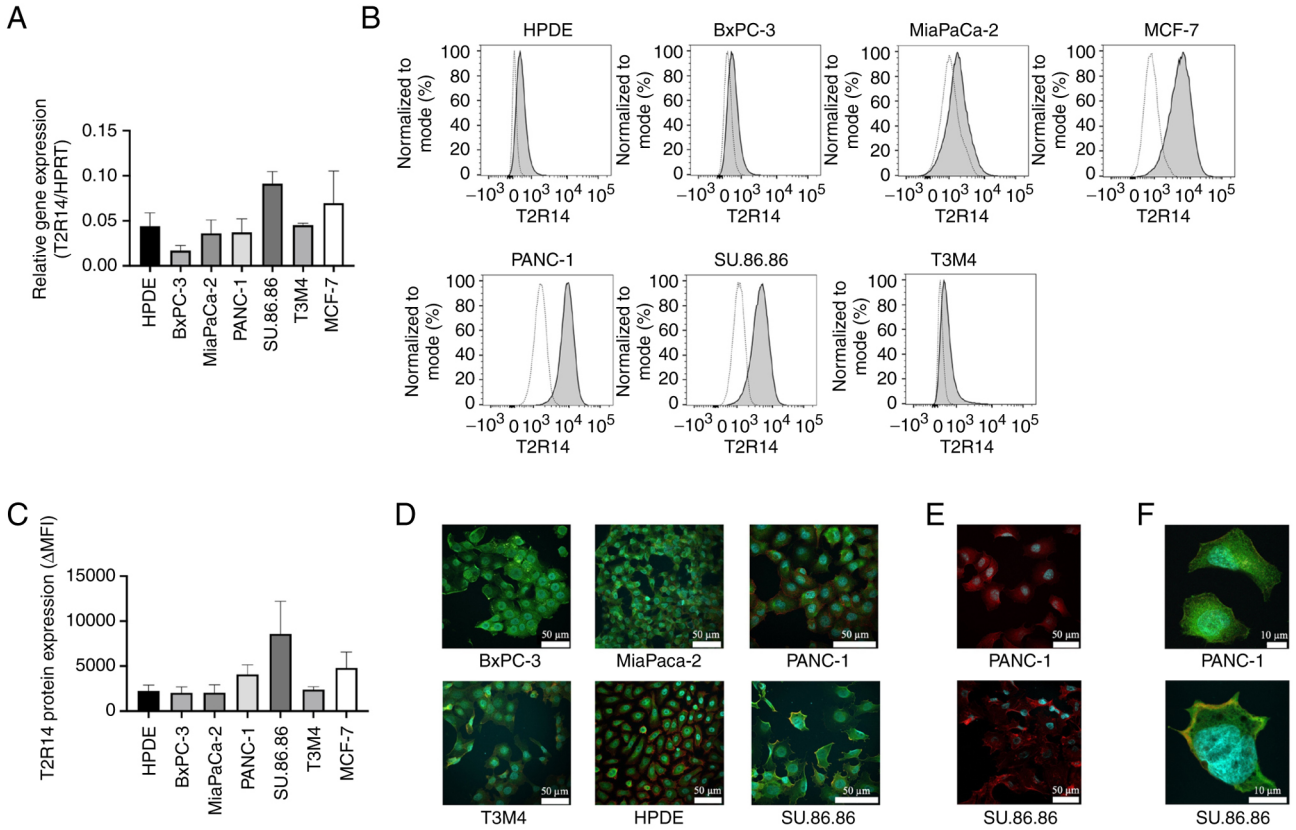

Figure 2. Expression of T2R14 in human pancreatic cell lines. (A) mRNA expression levels of T2R14 were analyzed in various pancreatic cancer cell lines by reverse transcription-quantitative polymerase chain reaction and were normalized to HPRT mRNA levels. SU.86.86 cells exhibited the highest expression levels. The MCF-7 breast cancer cell line served as a positive control. Data are presented as the mean ± SD of three independent experiments. (B) Flow cytometry was used to detect T2R14 surface protein expression in pancreatic cell lines. The MCF-7 breast cancer cell line served as a positive control. Solid lines represent T2R14 antibody binding and the dotted lines represent the IgG isotype control. (C) Quantification of T2R14 protein expression as the mean fluorescent intensity normalized to mode. Out of the pancreatic cancer cell lines, the highest T2R14 protein expression levels were found in SU.86.86 and PANC-1 cells. The MCF-7 breast cancer cell line served as a positive control. Data are presented as the mean ± SD of three independent experiments. (D) Representative confocal images showing human pancreatic tumor cells exhibiting immunoreactivity to T2R14 (green staining). Nuclear DNA was stained with DAPI (blue) and F-actin with Texas Red™-X Phalloidin (red). (E) Representative images of IgG isotype staining used as negative control to exclude non-specific T2R14 antibody binding. (F) High magnification images of immunofluorescence staining in SU.86.86 and PANC-1 cells demonstrating robust expression of T2R14 on the cell membrane and in the cytoplasm. MFI, mean fluorescence intensity; T2R14, bitter taste receptor 14.

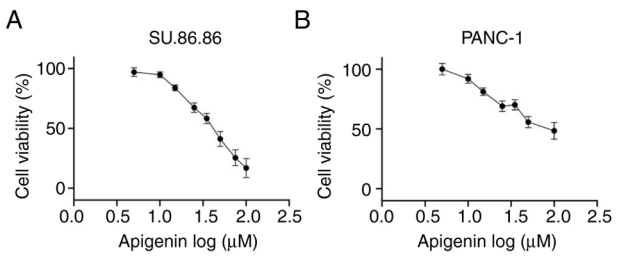

Figure 3. Apigenin inhibits the viability of SU.86.86 and PANC-1 cells in a dose-dependent manner. (A) SU.86.86 and (B) PANC-1 cells were treated with a range of concentrations of apigenin for 72 h. Cell viability was assessed using the MTS assay. All values were normalized to the group of cells treated with PBS/DMSO only. Data are presented as the mean ± SD of three independent experiments.

<1 for all concentrations of 5-FU except for one, suggested a predominantly positive synergistic interaction in SU.86.86 cells. The lowest CDI values (0.86) were detected when 5-FU was used at concentrations around its IC_{50} value (5 μM). By contrast, SU.86.86 cells became less susceptible to gemcitabine when exposed to apigenin, but this effect was not statistically significant (P>0.05) (Fig. 6C). Silencing T2R14 did not eliminate the apigenin-induced chemosensitization towards FOLFIRINOX or 5-FU (Fig. 6D and E); however, the level of synergy between apigenin and FOLFIRINOX was slightly reduced, as indicated by higher CDI values for FOLFIRINOX at concentrations ranging from 0.5 to 2.5X.

For PANC-1 cells, pretreatment with apigenin did not have any apparent effect on the chemoresistance towards any of the three tested chemotherapeutic agents, with CDI values ~1 (Fig. 7; Table III). In addition, knockdown of T2R14 had no effect on chemosensitivity.

Apigenin inhibits cell migration independent of T2R14 activation. A previous study on PDAC cell lines have reported the inhibitory effects of apigenin on migration (46). To evaluate whether T2R14 mediated these effects in SU.86.86 and PANC-1 cells, a migration assay was carried out following treatment with a range of concentrations of apigenin. In both SU.86.86 and PANC-1 cells, apigenin inhibited migration in a concentration-dependent manner. Again, apigenin was applied at a concentration (10 μM) that had previously been shown to induce no relevant cytotoxic effect on SU.86.86 or PANC-1

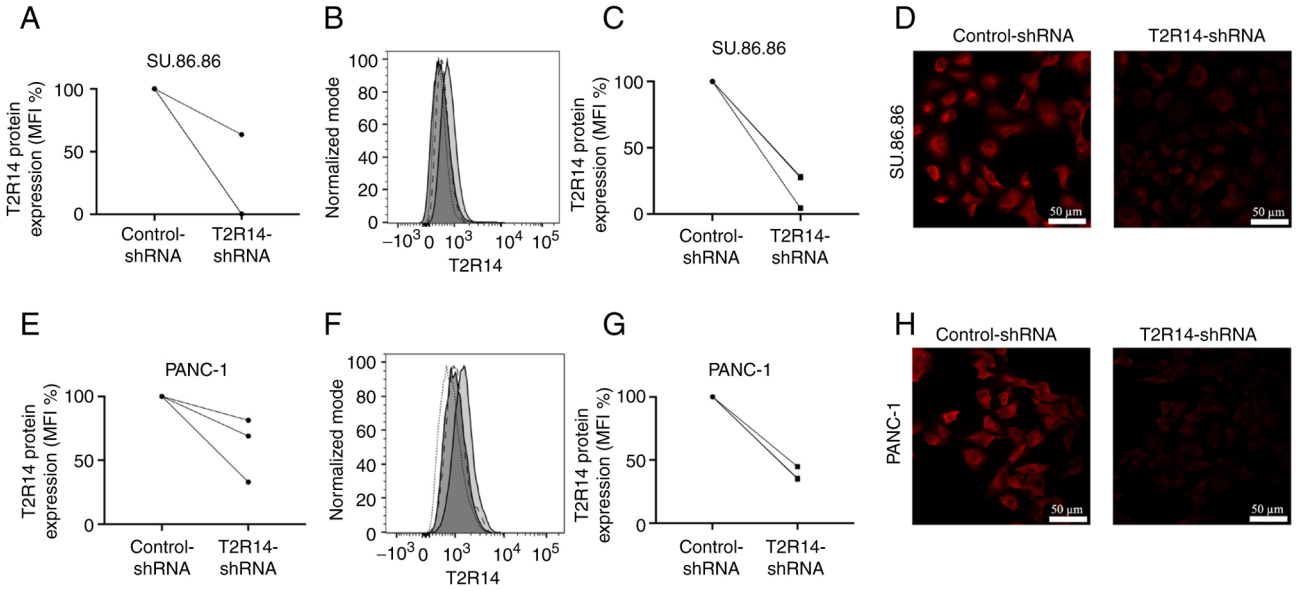

Figure 4. Efficacy of shRNA-mediated silencing of T2R14. (A and E) Reverse transcription-quantitative polymerase chain reaction was used to assess T2R14 mRNA expressions levels in cells transduced with T2R14-shRNA and control-shRNA. The expression levels of cells transduced with control-shRNA was set at 100%. T2R14 mRNA was reduced in T2R14-shRNA-transduced PANC-1 and SU.86.86 cells (three independent experiments are shown, of which two data points are overlaid and displayed as single points in the graph). (B and F) Cell surface expression of T2R14 protein in PDAC cells transduced with control-shRNA (light grey) or T2R14-shRNA (dark grey) was measured by flow cytometry. Solid lines represent specific T2R14-antibody signal and dotted/dashed lines represent the isotype control. (C and G) Quantification of MFI showed a reduction in T2R14 expression in PANC-1 and SU.86.86 cells following shRNA transduction. The MFI for cells transduced with control-shRNA was set at 100%. Three independent experiments are shown. Two data points are overlaid and thus displayed as single points in the graph. (D and H) Representative images of immunofluorescence (red staining) in cells transduced with T2R14-shRNA and control-shRNA. Reduced immunofluorescence staining in silenced cells confirming a sufficient knockdown of T2R14 at the protein level. MFI, mean fluorescence intensity; shRNA, short hairpin RNA; T2R14, bitter taste receptor 14.

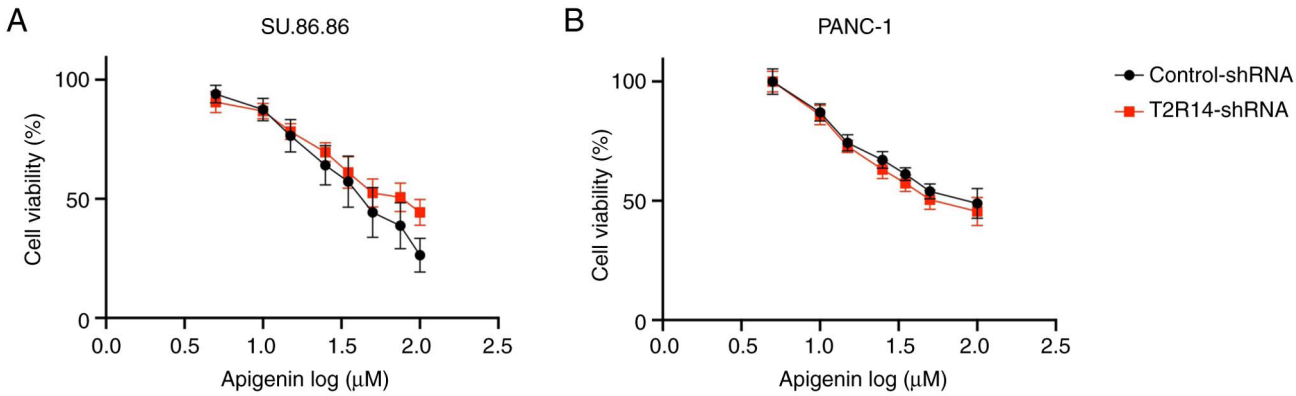

Figure 5. Cytotoxic effects of apigenin are independent of T2R14 expression. (A) SU.86.86 and (B) PANC-1 cells were exposed to increasing concentrations of apigenin. Cell viability was assessed using the MTS assay. Cells in the T2R14-shRNA group were compared with those in the control-shRNA group. All values were normalized to the group of cells treated with PBS/DMSO only. Data are presented as the mean ± SD of three independent experiments. shRNA, short hairpin RNA; T2R14, bitter taste receptor 14.

cells. In the presence of 10 μM apigenin, cell migration was slightly reduced; however, not to a statistically significant level (Fig. 8). Further increasing the concentration of apigenin to 30 and 50 μM resulted in a significant decrease in cell migration by 51% (P<0.001) and 68% (P<0.0001) for PANC-1 cells, and 44% (P<0.0001) and 70% (P<0.0001) for SU.86.86 cells, respectively (Fig. 8A and D). However, at these concentrations, cytotoxic side effects contributing to the reduced number of migrating cells cannot be ruled out conclusively. Silencing T2R14 did not affect the anti-migratory effects induced by apigenin, neither in PANC-1 or in SU.86.86 cells (Fig. 8B and E).

Discussion

Pancreatic cancer remains a fatal disease with a lack of sufficient systemic treatment options due to early and aggressive progression, late diagnosis and intrinsic chemoresistance (2,4). To overcome the obstacle of chemoresistance, combination treatment approaches have been intensively studied with the aim of improving therapeutic efficacy. In this context, beyond the regularly used cytotoxic agents, various bitter compounds have been reported to exhibit chemosensitizing effects through signaling via T2Rs (7-9). T2Rs have recently emerged as potential therapeutic targets in the context of anticancer

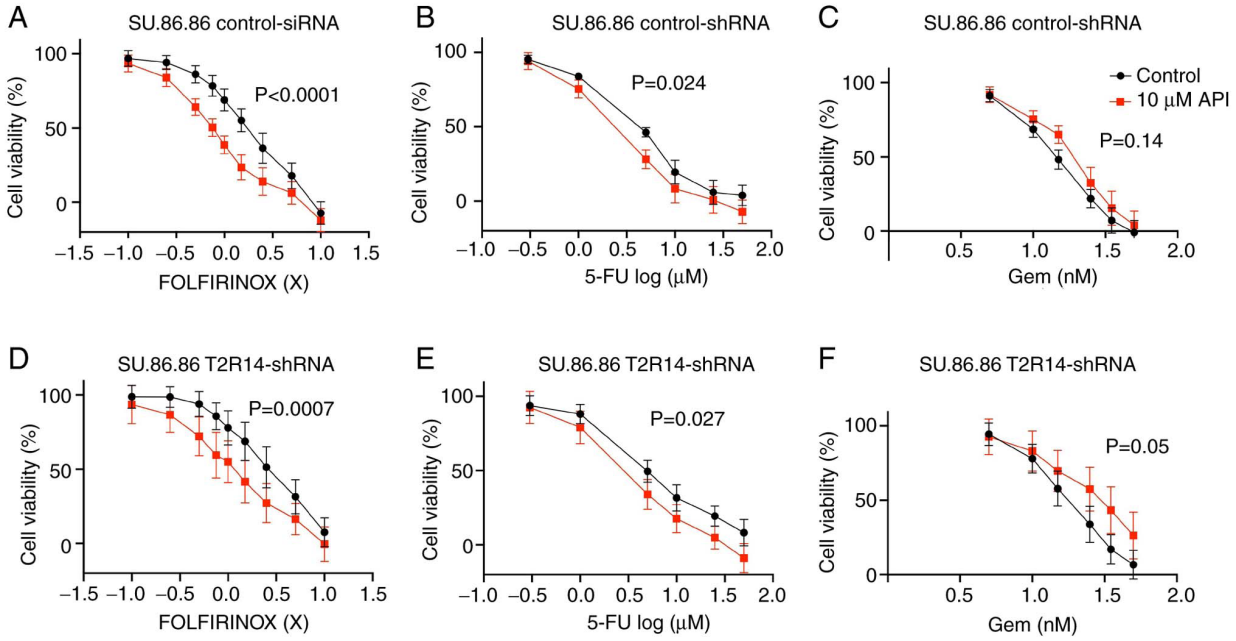

Figure 6. Apigenin synergistically enhances the cytotoxicity of FOLFIRINOX and 5-FU in SU.86.86 cells. (A-C) SU.86.86 cells were exposed to a range of concentrations of chemotherapeutic treatments (Gem, 5-FU and FOLFIRINOX), in the presence or absence of 10 μM apigenin. (D-F) T2R14-shRNA-transfected cells were exposed to a range of concentrations of chemotherapeutic treatments (Gem, 5-FU and FOLFIRINOX), in the presence or absence of 10 μM apigenin. MTS assays were applied for the determination of cell viability. Values were normalized to untreated cells. Data are presented as the mean ± SD of at least three independent experiments, each of them performed in triplicate. Statistical significance was assessed using the extra sum-of-squares F test. 5-FU, 5-fluorouracil; FOLFIRINOX, 5-FU, leucovorin, irinotecan and oxaliplatin; Gem, gemcitabine; shRNA, short hairpin RNA; T2R14, bitter taste receptor 14.

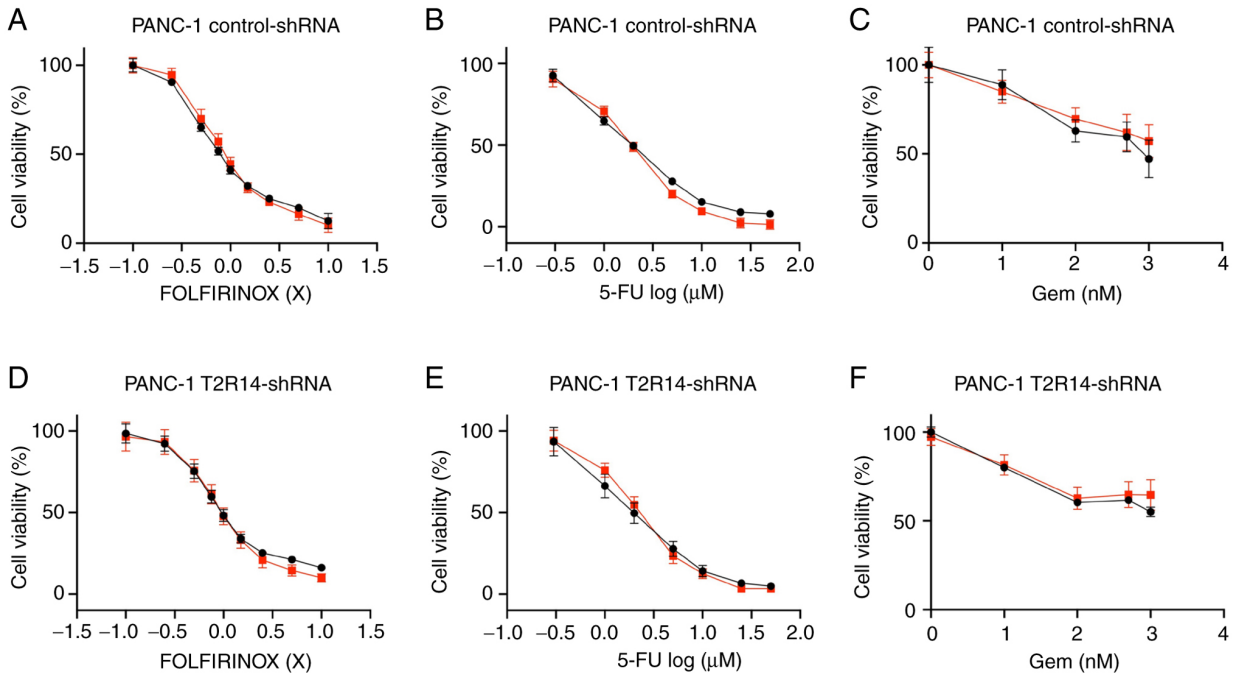

Figure 7. Apigenin does not affect the cytotoxicity of FOLFIRINOX, 5-FU or Gem in PANC-1 cells. (A-C) PANC-1 cells were exposed to a range of concentrations of chemotherapeutic treatments (Gem, 5-FU and FOLFIRINOX), in the presence or absence of 10 μM apigenin. (D-F) T2R14-shRNA-transfected cells were exposed to a range of concentrations of chemotherapeutic treatments (Gem, 5-FU and FOLFIRINOX), in the presence or absence of 10 μM apigenin. MTS assays were applied for the determination of cell viability. Values were normalized to untreated cells. Data are presented as the mean ± SD of at least three independent experiments, each of them performed in triplicate. Statistical significance was assessed using the extra sum-of-squares F test. 5-FU, 5-fluorouracil; FOLFIRINOX, 5-FU, leucovorin, irinotecan and oxaliplatin; Gem, gemcitabine; shRNA, short hairpin RNA; T2R14, bitter taste receptor 14.

therapy (13,14). The present study aimed to investigate whether a certain type of T2R, namely T2R14, was expressed in PDAC and to assess its functional abilities. To test this hypothesis, the expression of T2R14 was detected in PDAC tissues and pancreatic cancer-derived cell lines. Subsequently, the low toxicity T2R agonist apigenin was used, which has been

Table II. Synergistic effect of apigenin and FOLFIRINOX, 5-FU or Gem in SU.86.86 cells.

A, FOLFIRINOX

Concentration of chemotherapeutic agent	CDI value	
	Control-shRNA	T2R14-shRNA
0.1X	0.98	0.97
0.25X	0.93	0.93
0.5X	0.85	0.87
0.75X	0.8	0.84
1X	0.77	0.86
1.5X	0.74	0.82
2.5X	0.79	0.83
5X	0.91	0.91
10X	1.04	1.01

B, 5-FU

Concentration of chemotherapeutic agent	CDI value	
	Control-shRNA	T2R14-shRNA
0.3 μM	0.99	1
1 μM	0.95	0.95
5 μM	0.86	0.92
10 μM	0.92	0.93
25 μM	1.02	0.93
50 μM	0.92	0.9

C, Gem

Concentration of chemotherapeutic agent	CDI value	
	Control-shRNA	T2R14-shRNA
5 nM	1.01	0.99
10 nM	1.07	1.05
15 nM	1.2	1.13
25 nM	1.21	1.32
35 nM	1.25	1.45
50 nM	1.23	1.44

CDI values were calculated for the assessment of synergy between apigenin and chemotherapeutic agents (<1, synergistic; 1, additional; >1, antagonistic). 5-FU, 5-fluorouracil; CDI, coefficient of drug interaction; FOLFIRINOX, 5-FU, leucovorin, irinotecan and oxaliplatin; Gem, gemcitabine; shRNA, short hairpin RNA.

Table III. Synergistic effects of apigenin and FOLFIRINOX, 5-FU or Gem in PANC-1 cells.

A, FOLFIRINOX

Concentration of chemotherapeutic agent	CDI value	
	Control-shRNA	T2R14-shRNA
0.1X	0.99	0.99
0.25X	1.02	1.01
0.5X	1.05	1.03
0.75X	1.07	1.06
1X	1.06	1.06
1.5X	1.03	1.09
2.5X	1.03	1.07
5X	1.01	1.05
10X	1.04	1.07

B, 5-FU

Concentration of chemotherapeutic agent	CDI value	
	Control-shRNA	T2R14-shRNA
0.3 μM	0.99	1.01
1 μM	1.07	1.11
2 μM	1.04	1.1
5 μM	1	1.06
10 μM	1.05	1.13
25 μM	1.04	1.14
50 μM	1.05	1.18

C, Gem

Concentration of chemotherapeutic agent	CDI value	
	Control-shRNA	T2R14-shRNA
1 nM	1.01	0.98
10 nM	0.99	1.02
100 nM	1.08	1.05
500 nM	1.05	1.05
1,000 nM	1.13	1.12

CDI values were calculated for the assessment of synergy between apigenin and chemotherapeutic agents (<1, synergistic; 1, additional; >1, antagonistic). 5-FU, 5-fluorouracil; CDI, coefficient of drug interaction; FOLFIRINOX, 5-FU, leucovorin, irinotecan and oxaliplatin; Gem, gemcitabine; shRNA, short hairpin RNA.

reported to exert anticancer effects on pancreatic tumor cells, albeit via as yet unknown mechanisms (31,33-36).

The present study demonstrated that T2R14 was expressed in PDAC tissue, and also in cell lines derived from primary or metastatic tumors. All tumor samples were revealed to be T2R14 positive. In contrast to previous reports on other members of the T2R family in PDAC, namely T2R10 and T2R38 (15,17), T2R14 was present in normal pancreatic tissue, precursor lesions and pancreatic cancer. Furthermore, patients with PDAC and with relatively high T2R14 expression displayed a significantly longer survival time compared with that of patients with low T2R14 expression, suggesting a functional role for T2R14 in PDAC.

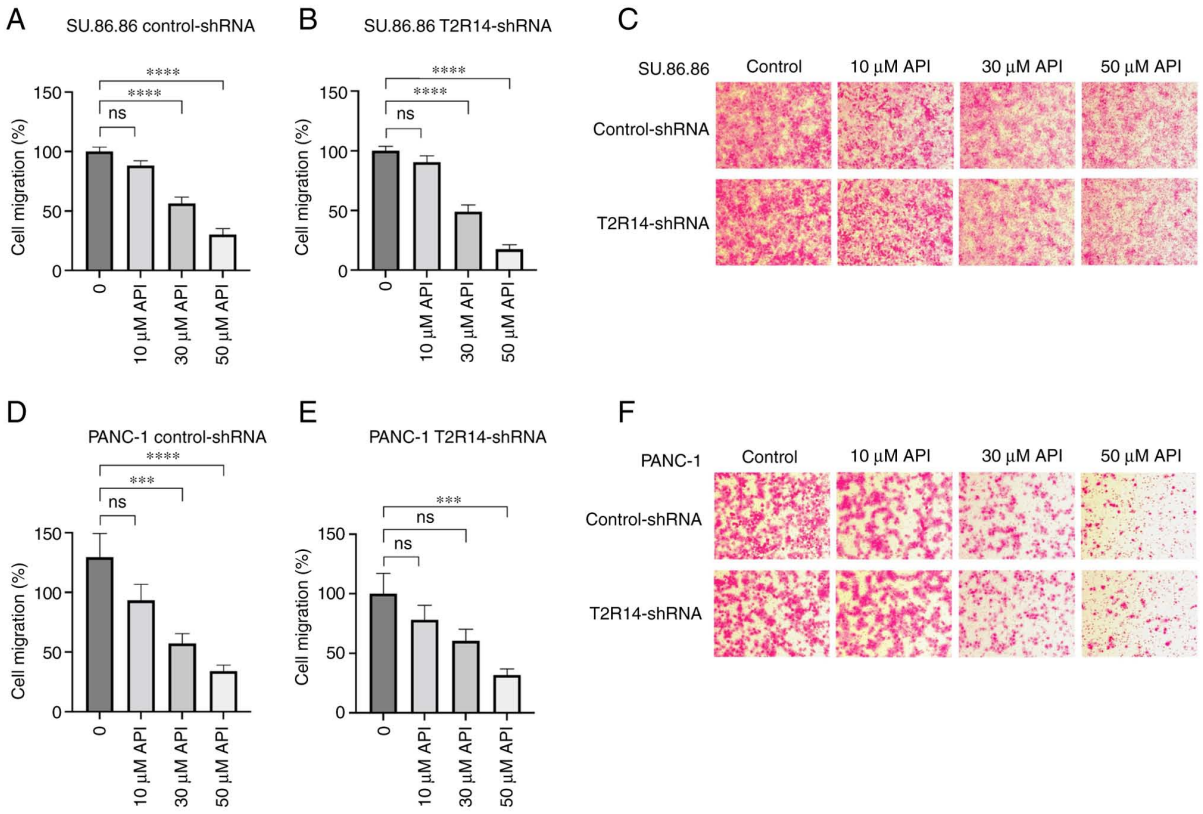

Figure 8. API inhibits the migration of PANC-1 and SU.86.86 cells in a concentration-dependent manner. (A and D) Migratory capacity of SU.86.86 (upper panel) and PANC-1 (lower panel) cells. (B and E) Migratory capacity of T2R14-shRNA cells. Migration of control cells treated with PBS only was set as 100%. Semi-quantification of the relative numbers of migrated cells exhibited a gradual decrease in migration with increasing API concentration in both cell lines with silenced T2R14 and control cells. The number of migrated cells was normalized to the untreated cell groups. Data are presented as the mean ± SEM of three independent experiments. Statistical significance was assessed using one-way ANOVA and Bonferroni's multiple comparison test. ***P<0.001: ****P<0.0001. (C and F) Representative images of the migrated SU.86.86 and PANC-1 cells stained with crystal violet. Cells were treated with three concentrations of API. Relative migration decreased with rising apigenin concentration. Magnification, x200. API, apigenin; shRNA, short hairpin RNA; T2R14, bitter taste receptor 14.

To investigate the potential effects of T2R14 activation on PDAC, two pancreatic cancer cell lines with the highest expression levels of T2R14, but differing in their origin, were chosen: PANC-1, a cell line derived from primary PDAC in the head of the pancreas (47), and SU.86.86, which was initially obtained from a liver metastasis of pancreatic cancer (48). In both cell lines, apigenin inhibited cell viability in a dose-dependent manner. Furthermore, the presence of apigenin significantly enhanced the responsiveness of SU.86.86 cells to FOLFIRINOX and 5-FU in a synergistic manner; however, the chemoresponsiveness of cells to gemcitabine was not affected. To the best of our knowledge, the present study is the first to show the chemosensitizing effects of apigenin towards 5-FU and FOLFIRINOX in a pancreatic cancer cell line. FOLFIRINOX was first introduced to clinical practice in 2010 and has since rapidly emerged as the standard treatment option for patients with PDAC due to its superior response rates compared with previously established chemotherapeutic regimens (6,49). In PANC-1 cells, no substantial modulation of chemoresistance was detected. This may result from the fact that PANC-1 cells display a very high intrinsic chemoresistance compared with other cell lines (17).

To assess whether the effects of apigenin were mediated by T2R14, the expression of T2R14 was silenced via the transduction of cells with shRNA-containing lentiviral particles. The knockdown of T2R14 did not modify the cytotoxic or antimigratory activity of apigenin. Furthermore, apigenin-induced chemosensitization in SU.86.86 cells was not fully dependent on T2R14 expression. However, there was a reduced synergistic effect between apigenin and FOLFIRINOX or 5-FU in SU.86.86 cells in which T2R14 expression was silenced compared with that in the control-shRNA-transduced cells. Notably, T2R14-shRNA transduction resulted in incomplete knockdown of T2R14; therefore, the remaining T2R14 may still have participated. Whilst this is a possibility, the results suggested that alternative signaling pathways of apigenin independent of T2R14 may also be involved, since apigenin has other biotargets. In particular, apigenin has been reported to activate another T2R subtype, T2R39 (28), which is not expressed in PDAC tissue according to our own not yet published data. Recently, the effects of apigenin were assessed on all human T2Rs; this previous study confirmed that it could activate T2R14, but not T2R39, and identified T2R43 as the only additional T2R activated by apigenin (50). However, T2R43 was activated by 30 μM apigenin and not by 10 μM, which is the concentration that was used in the present study. In addition, several other mechanisms underlying the antiproliferative and chemosensitizing effects of apigenin have been proposed in previous studies: In PDAC, apigenin has been reported to inhibit the activation of NF-κB,

HIF-1α and AKT (37,38,51,52), all of which are involved in key oncogenic signaling pathways that are relevant to chemoresistance (18,53). Moreover, apigenin has been shown to induce cell cycle arrest and apoptosis via activation of the oncosupressor gene p53 (36). Thus, T2R14 may synergize with multiple mechanisms to induce chemosensitizing effects.

For various natural bitter compounds exhibiting chemosensitizing effects, the modulation of ABC transporters has been proposed as a common molecular mechanism. Notably, xanthines have been reported to downregulate ABCG2 in breast cancer and choriocarcinoma cell lines (7). Similarly, bitter melon extract has been shown to reduce the expression of ABCG2 and increase the efflux of doxorubicin in colon cancer cells (8). In line with this, our previous studies demonstrated that T2R10 and T2R38 could affect ABCG2 and ABCB1 in pancreatic adenocarcinoma cell lines, as determined following activation with their respective natural bitter agonists (15,17). Therefore, we tested the expression of multiple ABC transporters in response to the T2R14 agonist apigenin; however, no significant change in expression levels was found (data not shown). An investigation of potential alternative downstream targets of T2R14 is required to gain a better understanding of the underlying molecular mechanisms. Studying cells overexpressing T2R14 could also potentially provide additional information on the involvement of T2R14 in apigenin-induced effects. Furthermore, a major challenge in applying natural compounds as chemosensitizing treatments is to achieve a biologically active concentration at the target site. Therefore, despite the good bioavailability and low toxicity of apigenin it remains to be determined as to whether a sufficient concentration can be reached in the pancreas of human patients (29,54). In addition to that, the exact serum level of the different components of the multi-component treatment FOLFIRINOX are not well studied and could differ from the *in vitro* conditions established in a previous study (55). Therefore, *in vivo* experiments are required to gain a better understanding and confirm these *in vitro* data. Furthermore, testing the relationship between T2R14 expression in PDAC and responsiveness to chemotherapy in patients could be informative for evaluating the clinical impact of the *in vitro* findings described in this study.

In conclusion, to the best of our knowledge, the present study is the first to detect T2R14 expression in PDAC, both at the mRNA and protein levels. Notably, the findings of prolonged survival in patients with relatively high T2R14 expression, compared with that of patients with low T2R14 expression, strongly suggested a functional role of T2R14 in pancreatic cancer. Furthermore, the known T2R14 agonist, apigenin, was shown to elicit cytotoxic, anti-migratory and chemosensitizing effects on pancreatic cancer cells; however, this was not exclusively dependent on T2R14 expression. Therefore, identifying other possible involvements of T2R14 in PDAC requires further study.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LS mainly conceived the study, analyzed the data, and wrote and edited the manuscript. MMG and TG made substantial contributions to the conception and design of the study, and critically reviewed and edited the manuscript. LFB performed the majority of the experiments, contributed to the conception and design of the study and edited the manuscript. AG and TZ assisted with the PCR. AH performed the immunohistochemistry. SH made substantial contributions to tissue acquisition and assisted in interpretation of data. NG and TH provided access to expression data and were involved in data interpretation and revision of the manuscript critically for important intellectual content. JRI, MR, CG, MRG, CN, RF and MYN assisted in data interpretation and revised the article critically. All authors have read and approved the final manuscript. LS and LFB confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was approved by the ethics committee of the University Medical Center Mainz (approval mo. 2019-14390; Rhineland-Palatinate Medical Association) and written informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interest.

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