Natural product–drug conjugates for modulation of TRPV1-expressing tumors

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ABSTRACT

We report the design, synthesis and biological evaluation of natural product–drug conjugates for treatment of prostate cancers over-expressing the transient receptor potential vanilloid 1 (TRPV1) channel. We validate the relevance of TRPV1 as a target in prostate cancer patients by using a bioinformatics approach and provide proof-of-concept for the drug delivery strategy through bioorthogonal chemistry and stability assays under simulated physiological conditions. In cell-based assays, the constructs displayed modest activity. Moreover, we serendipitously discover that a stoichiometric combination of a TRPV1 agonist with a small, positively charged cytotoxic may provide new research avenues in personalized medicines for prostate cancer.

1. Introduction

Cancer remains a major health issue worldwide and is responsible for substantial mortality and morbidity burdens. Therefore, therapies displaying not only high clinical efficacy, but also intervening through novel mechanisms of action – ideally differentiated from healthy cell metabolism – and/or displaying high selectivity are constantly sought after. With the advent of biologics for cancer therapy, antibody–drug conjugates (ADCs) have transformed the therapeutic armamentarium landscape, in particular for blood cancers where distribution of the drug to diseased cells is not a limiting step. Indeed, there are currently four FDA-approved ADCs, of which three present a blood cancer indication and only one for solid tumors. As a result, engineering the selective cancer recognition feature of ADCs, with subsequent controlled drug/payload release, into small molecule constructs offers a solution to mitigate the aforementioned limitations and improve clinical outcomes.

The transient receptor potential channels (TRP) are a superfamily of mono and divalent ion transporters that are chiefly responsible for sensory functions, namely relaying pain signals. Despite the limited knowledge of (patho)physiology regulated by TRP channels there is growing interest in exploiting their signaling pathways in the clinic. In particular, the vanilloid 1 receptor (TRPV1) regulates calcium signaling and disruption of its homeostasis is known to affect proliferation, apoptosis and migration of cancer cells. Importantly, TRPV1 is overexpressed in a range of cancer types, including prostate cancer. As such, TRPV1 small molecule modulators may find applicability as cancer therapeutics. Capsaicin (1, Fig. 1) is a natural product extracted from chili peppers with moderate anticancer activity by enhancing calcium influx via TRPV1. As a prototypical and high affinity TRPV1 agonist, we envisaged that 1 could serve as a cancer recognition chemotype for the selective delivery of potent payloads and construction of personalized medicines.

Herein we provide preliminary validation that TRPV1 can be used as an “antigen” for the recognition of diseased cells. Additionally, we harness the potential of such finding by synthesizing and evaluating in vitro different capsaicin–drug conjugates. We show through these unprecedented small molecule–drug conjugates that exploiting TRPV1 biology is a viable strategy for the development of cancer therapeutics and probes for imaging in surgery in select cancer types.
Expression of TRPV1 was observed for healthy prostate tissue. Conversely, prostate cancer samples from The Cancer Genome Atlas (TCGA) displayed a disparate pattern (Fig. 2b). The latter overexpresses TRPV1, which is in line with previous reports.\(^1\) Moreover, when analyzing the survival rate of prostate cancer patients grouped by TRPV1 expression, we found that higher TRPV1 expression associates with poor prognosis \((p < 0.01, \text{Wilcoxon rank-sum test})\), suggesting that TRPV1 can be used as a marker to select targets for aggressive prostate cancers. Nonetheless, it should be noted that in some instances, prostate cancers also display low expression of TRPV1 (Fig. 2b), which suggests that TRPV1-targeting agents are unwarranted in those cases. Still, patients in this category typically have a better prognosis and the survival probability is close to 100\% \((p > 0.001, \text{log-rank test; Fig. 2c})\), suggesting that TRPV1 can be used as a marker to selectively target aggressive prostate cancers.

Next, to assess whether capsaicin–drug conjugates could enter cancer cells prior to releasing the payload, we designed and synthesized a capsaicin analogue as an imaging probe precursor (Fig. 3a). Considering that the vanilloid head of 1 is critical for molecular recognition of TRPV1,\(^2\) we installed an alkyne handle in the hydrophobic tail of 1 (Fig. 3a). Conjugating 1 with different glioma grades with normal brain tissue one can conclude that down-regulation of TRPV1 is concomitant with tumor progression (Fig. 2d). Hence, TRPV1 ligand–drug conjugates would not present a viable alternative to current glioblastoma therapeutics.

Following identical synthetic routes, we synthesized conjugates 9–11 using commercially available building blocks and standard coupling conditions. First, the appropriate methylene amine starting material was coupled to N-protected carboxylic acids of different lengths to afford intermediates 6–8. After Boc deprotection, intermediates 6–8 were reacted with hydrolyzed TMZ to yield conjugates 9–11. To ascertain that conjugation with TMZ did not disrupt the binding affinity to TRPV1 channels, we profiled our minimal constructs in calcium imaging assays, as previously conducted. Our data show that all conjugates act as TRPV1 agonists and that all three entities evoke calcium influx similarly, i.e. independently of the linker length (Fig. 5a).
Though, the obtained responses for compounds 9–11 were ca. 40% of the control compound 1. Our results suggest that TRPV1 does not fully tolerate a fragment-like payload, such as TMZ, attached to the tail region of 1. Moreover, meticulous molecular design will be key to improve the binding affinity of capsaicin–drug conjugates to TRPV1, in the future. Recent progress in cryo-electron microscopy has enabled the understanding of function and dynamics of TRP channels, including TRPV1.21,22 However, the current state-of-the-art still does not enable receptor-based drug design due to low resolution of the structures. Given these limitations, we used the synthesized constructs for subsequent proof-of-concept studies.

Prior to performing viability assays with cancer cells, we assessed the stability of the constructs to ascertain that the alkylating methyl-diazonium moiety could be efficiently released from the TMZ prodrug. Upon incubation of compounds 9–11 at 37°C in phosphate buffered saline (PBS), we observed a time-dependent hydrolysis of the western

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**Fig. 3.** Design and in vitro assessment of a capsaicin (1) analogue as imaging probe (2). a) Retrosynthetic analysis for compound 2. Highlighted in red is the head region, responsible for directed interactions with TRPV1. b) Imaging of calcium influx mediated by TRPV1 and evoked by chemical matter (5 µM). Data show comparable influx of calcium evoked by 1 and 2 in TRPV1-RFP transfected HEK-293 cells. No effects were observed for non-transfected cells. 10 best responding cells from each well selected, n = 5–7 wells. c) Scheme of the in situ azide-alkyne cycloaddition reaction. d) Imaging of U251 cells displaying staining of click adduct in the intracellular compartment and not co-localized with anti-TRPV1 polyclonal antibody. Treated: alkyn (20 µM), azide-488 (10 µM). Control: azide-488 (10 µM).

**Fig. 4.** Capsaicin–temozolomide (TMZ) conjugates. a) Design of conjugates with non-cleavable linker. b) Synthesis of capsaicin–TMZ conjugates. Reagents and conditions: i) carboxylic acid, HATU, DIEA, r.t., 16 h; ii) TFA/CH₂Cl₂ (1:4), r.t., 1 h; iii) TMZ-CO₂H, HATU, DIEA, r.t., 16 h.
Capsaicin–temozolomide (TMZ) conjugates kill prostate cancer cells. a) Conjugates evoke influx of calcium mediated by TRPV1 at a concentration of 50 µM. 10 best responding cells from each well selected, n = 2–6 wells. b) Stability of conjugates 9–11 under simulated physiological conditions (PBS pH 7.4; 37 °C), as assessed by LC-MS traces. Stability of 9–11 is comparable to TMZ and a time course experiment shows hydrolysis and loss of a methyldiazonium unit. c) Conjugates 9–11 show a time dependent effect at a concentration of 200 µM. At 72 h post incubation the effect of the conjugates is identical to that of TMZ. A stoichiometric combination of capsaicin and TMZ shows synergism. *p < 0.01; ****p < 0.0001 (n = 9; 2-way ANOVA with Tukey post hoc test).

Finally, we used the prostate cancer PC3 cells to query our conjugates in viability studies at different time points while using the CellTiter Blue assay. PC3 cells have been shown to express TRPV1,19 which motivates its use as a model system from both mechanistic and disease vantage points. Treatment of PC3 cells with 200 µM of each conjugate showed a time-dependent effect, similar to TMZ alone (Fig. 5c) and indistinguishable between them (p = 0.06–0.85; n = 9; 2-way ANOVA with Tukey post hoc test). The result corroborates the previous observation that, in this particular case, linkers of different lengths have limited impact on bioactivity. Curiously, a stoichiometric combination of capsaicin and TMZ afforded synergistic results, significantly different from either TMZ alone or conjugates 9–11 (p = 0.0001; n = 9; 2-way ANOVA with Tukey post hoc test). It has been shown that TRPV1 can mediate the transport of small, yet charged molecules through its pore domain.28 Considering the fragment-like nature of TMZ and a calculated pKa value of 10.5 (source DrugBank) it is feasible that a higher concentration of TMZ is achieved in the intracellular compartment when co-administered with 1, as a result of TRPV1-mediated transport, and hence the higher activity (p = 0.01; n = 9; 2-way ANOVA with Tukey post hoc test). Indeed, this serendipitous finding ought to be explored in future drug delivery studies.

3. Conclusions

Herein, we explored TRPV1 channels as antigens for the selective delivery of payloads to cancer cells and provide initial proof-of-concept that such strategy may afford novel personalized medicines. Moreover, we show how minimal constructs with non-cleavable linkers afford viable chemotypes, notwithstanding being recently neglected from exploratory and development pipelines. Despite the modest activity of the reported conjugates, a similar activity profile to the parent FDA-approved drug and suitable stabilities were achieved, which will motivate further studies. In addition, administration of stoichiometric amounts of TRP channel agonist with an ionizable fragment-like drug may provide unexpected avenues in cancer research.

4. Experimental section

4.1. Immunofluorescence assays

A healthy human tissue array (T6234700-5, amsbio) was defrosted at room temperature for 5 min then immersed in Dako wash buffer for 5 min. Samples were blocked with Dako Protein Block for 40 min at room temperature then incubated with anti-TRPV1 antibody (1:100 in PBS, ab34878, abcam) for 1 h at room temperature. The slides were washed with Dako wash buffer for 3 × 5 min then incubated with a goat anti-rabbit 488 antibody at room temperature for 1 h (1:1000, Abcam, ab150077). After three more washes with Dako wash buffer, samples were incubated with Dako wash buffer, samples were incubated with Hoechst (1:1000) for 10 min. Confocal images were acquired with a Zeiss 880 microscope.

4.2. Bioinformatics

The normalized TRPV1 gene expression (quantified through RNA-seq by Expectation Maximization – RSEM29) and clinical data for pancreatic tumor and matched-normal samples from The Cancer Genome Atlas (TCGA; https://cancergenome.nih.gov/) were downloaded from Firebrowse (http://firebrowse.org/). Survival analysis was done using the R package surviALL.30 Here, tumor samples were divided in two groups based on their TRPV1 expression and the prognostic significance was estimated using a log-rank test. We tested all possible points-of-separation and, after correcting for multiple testing through the false discovery rate method, the one that optimized the prognostic value of TRPV1 was used.

TRPV1 gene expression (TPM, Transcripts Per Million) for brain tumors from TCGA and normal brain tissues from Genotype-Tissue Expression (GTEx; https://www.gtexportal.org/) project were retrieved from Toil.31

4.3. Chemistry

Building blocks and solvents were purchased from ABR Chemicals, Sigma Aldrich, Alfa Aesar, Acros, Fluka or TCI Deutschland and used without further purification. Proton and carbon nuclear magnetic resonance (1H and 13C NMR) spectra were recorded on a Bruker AVANCE 300 or 400 MHz spectrometers. All chemical shifts are quoted on the δ scale in ppm using with TMS as an internal reference. Coupling constants (J) are reported in Hz with the following splitting abbreviations:
s = singlet, d = doublet, t = triplet, m = multiplet. All compounds present ≥95% purity unless otherwise stated.

4.3.1. N-(4-hydroxy-3-methoxybenzyl)undec-10-ynamide (2)

Thionyl chloride (0.13 mL, 1.8 mmol) was added to (10-undecynoic acid (36.5 mg, 0.2 mmol) and refluxed at 65 °C for 1 h. Excess thionyl chloride was evaporated under argon and the crude product was dissolved in 1.5 mL of chloroform. In a separate flask, water (2 mL) and sodium bicarbonate (67 mg, 0.8 mmol) was added to 3-hydroxy-4-methoxybenzylamine hydrochloride (38 mg, 0.2 mmol) and the mixture was stirred for 30 min. Chloroform (1 mL) was added and the flask was de-gassed. Undec-10-ynyl chloride was slowly added to the amine. The chloroflake was rinsed with a total of 1.5 mL chloroform. The reaction was followed by TLC. The organic layer was separated and the aqueous layer was washed with chloroform 3×10 mL). The organics was stirred for 30 min. Chloroform (1 mL) was added and the flask was evaporated under argon and the crude product was dissolved in ethyl acetate (2×50 mL), dried with anhydrous MgSO₄, filtered and concentrated in vacuum. The residue was purified by preparative HPLC (column: YMC-Actus Triart C18 150 × 30 mm × 5 μm; mobile phase: [water (0.225% formic acid) – acetonitrile]: 8%: 22%–45%, 11 min) to give the desired product as a white solid (50 mg, 103.8 μmol, 7% yield). 1H NMR (400 MHz, DMSO-d₆) δ 8.84 (s, 1H), 8.83 (s, 1H), 8.49 (t, J = 5.9 Hz, 1H), 8.18 (t, J = 5.8 Hz, 1H), 6.80 (d, J = 1.7 Hz, 1H), 6.73–6.67 (m, 1H), 6.67–6.58 (m, 1H), 4.14 (d, J = 5.9 Hz, 2H), 3.87 (s, 3H), 3.73 (s, 3H), 3.28 (q, J = 6.6 Hz, 2H), 2.16–2.07 (m, 2H), 1.52 (br. t, J = 6.7 Hz, 4H), 1.35–1.25 (m, 4H). 13C NMR (75 MHz, DMSO-d₆) δ 172.68, 160.09, 147.84, 145.71, 131.61, 130.92, 128.80, 120.10, 115.60, 112.04, 55.97, 42.28, 36.58, 35.82, 39.25, 28.85, 26.63, 25.80. LC-MS: Rₚ = 1.976 min; m/z 458.1 (M + H)⁺.

4.3.4. N-(4-hydroxy-3-methoxybenzyl)-7-((2-(3-methyl-4-oxo-3,4-dihydroimidazo[5,1-d][1,2,3,5]tetrazin-8-yl)-2-oxoethyl)amino) heptanamide (9)

To a mixture of the required intermediate (400 mg, 1.43 mmol, 1 M eq.) and carboxylic acid TMZ (271.31 mg, 1.50 mmol, 1 M eq.) in DMF (10 mL) was added DIEA (921.97 mg, 7.13 mmol, 1.24 mL, 5 M eq.). The mixture was stirred at 0 °C for 5 min and added HATU (569.61 mg, 1.50 mmol, 1.05 M eq.) in one portion at 30 °C. The mixture was stirred at 30 °C for 1 h. The reaction was followed by LCMS. The aqueous phase was extracted with ethyl acetate (2 × 50 mL), dried with anhydrous MgSO₄, filtered and concentrated in vacuum. The residue was purified by preparative HPLC (column: YMC-Actus Triart C18 150 × 30 mm × 5 μm; mobile phase: [water (0.225% formic acid) – acetonitrile]: 8%: 22%–45%, 11 min) to give the desired product as a white solid (50 mg, 103.8 μmol, 7% yield). 1H NMR (400 MHz, DMSO-d₆) δ 8.84 (s, 1H), 8.83 (s, 1H), 8.49 (t, J = 5.9 Hz, 1H), 8.18 (t, J = 5.8 Hz, 1H), 6.80 (d, J = 1.7 Hz, 1H), 6.73–6.67 (m, 1H), 6.67–6.58 (m, 1H), 4.14 (d, J = 5.9 Hz, 2H), 3.87 (s, 3H), 3.73 (s, 3H), 3.28 (q, J = 6.6 Hz, 2H), 2.16–2.07 (m, 2H), 1.52 (br. t, J = 6.7 Hz, 4H), 1.35–1.25 (m, 4H). 13C NMR (75 MHz, DMSO-d₆) δ 172.68, 160.09, 147.84, 145.71, 131.61, 130.92, 128.80, 120.10, 115.60, 112.04, 55.97, 42.28, 36.58, 35.82, 39.25, 28.85, 26.63, 25.80. LC-MS: Rₚ = 1.976 min; m/z 458.1 (M + H)⁺.
4.3.11. Stability of conjugates

Stock solutions of all compounds were made in DMSO and then diluted in PBS to give a final concentration of (0.2 mM) with 2% DMSO. The solutions were then incubated at 37 °C. At regular time points a sample was taken and analyzed via LC-MS. (4µM samples of the reaction mixture were prepared in water and 50 µL was injected into the system. A gradient of 95:5 – 5:95 H2O ( + 0.225% formic acid): acetonitrile was used as the mobile phase. The area under the peak corresponding to the starting material was calculated and plotted to give stability of the conjugates.

4.4. Biology

4.4.1. In situ click reaction

U251 cells (50,000 per well) were seeded on coverslips in a 24 well plate. After 24 h compound 2 was added. After 24 h cells were fixed with 4% formaldehyde in PBS for 15 min at room temperature. Cells were washed 3 times with PBS then permeabilized with 0.3% Tween, 20% FBS in PBS for 15 min at room temperature. Cells were washed 3 times then blocked with 20% FBS in PBS at 4 °C overnight. Cells were washed 3 times with PBS and click reaction cocktail was prepared according to manufacturer’s instructions [870 µL Buffer A, 20 µL CuSO4, 100 µL additive C, 10 µL azide (500 or 1000 µM to give 5 or 10 µM final concentration)]. The 200 µL of reaction cocktail were added to each well and incubated for 90 min before measuring fluorescence intensity.

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