The central role of NFAT signalling in the mechanism of action of the TRPV6 oncochannel inhibitor and clinical candidate SOR-C13

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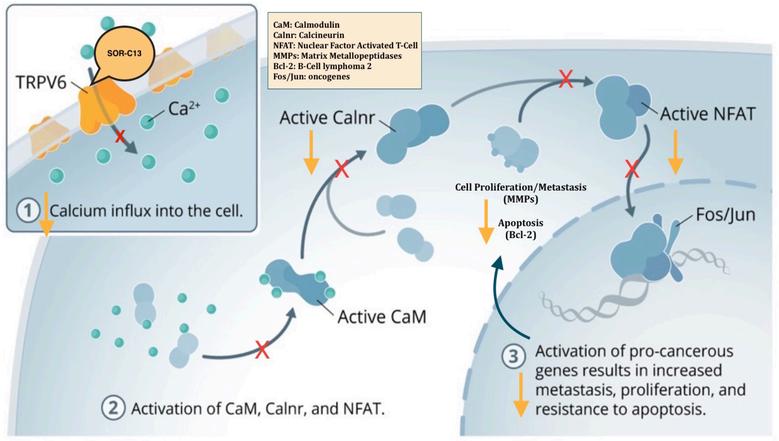
ABSTRACT: SOR-C13, a 13-mer peptide derived from soricidin, the paralytic peptide in saliva of the Northern Short-tailed shrew, has completed an open-label, all comers Phase I Clinical Trial for the treatment of epithelially-derived cancers. SOR-C13 is a first-in-class drug candidate for the treatment of solid tumors. SOR-C13 specifically targets and inhibits the TRPV6 calcium channel - a recognized oncochannel whose over-expression in a number of epithelial cancers (e.g. breast, ovarian, prostate) is associated with poor prognosis. An increase in TRPV6 facilitates calcium influx into the cell, which activates the calcium binding protein calmodulin. This complex in turn activates calcineurin that de-phosphorylates and activates Nuclear Factor of Activated T-cells (NFAT), a gene transcription factor involved in multiple oncogenic processes. To demonstrate the role of NFAT activation in the mechanism of action (MOA) of SOR-C13, breast cancer cells (T-47D) were transfected with a NFAT dual reporter plasmid, treated with the peptide, and NFAT activation monitored. Additionally, RT-qPCR TaqMan® array profiling was performed on prostate (PC-3), breast (T-47D), ovarian (OVCAR-3 and SK-OV-3), and pancreatic (BxPC-3 and SU.86.86) cancer cell lines treated with SOR-C13 compared with untreated cells. The TaqMan® array panel consisted of 187 genes involved in cancer calcium signalling associated with the MOA of TRPV6 and directly, or indirectly, involved in NFAT signalling. SOR-C13 treatment significantly inhibited NFAT activation ($p < 0.05$) in T-47D cells transfected with a NFAT reporter plasmid. Molecular profiling of the 6 cancer cell lines showed that many genes involved in NFAT signalling were modulated by SOR-C13 treatment. Affected genes included genes involved in cell proliferation (e.g. TGF-beta, TLR9), metastasis (e.g. ADAM12, CEACAM6), resistance to apoptosis (e.g. WISP1) and angiogenesis (e.g. FLT4). Cytokines (e.g. IL-6) and chemokines (e.g. CXCL12), involved in cancer progression and associated with NFAT signalling, as well as transcription factors (e.g. GATA4, NF-kB) were down regulated upon SOR-C13 treatment. These results highlight NFAT signalling in the mechanism of action of SOR-C13. The impact of SOR-C13 on the expression of genes involved in resistance to apoptosis, proliferation, metastasis and angiogenesis as well as on the expression of immune cytokines and transcription factors in multiple cancer cells, makes SOR-C13 an attractive, novel anti- cancer drug.

Introduction

SOR-C13 is a novel 13-mer synthetic peptide derived from soricidin, a proprietary paralytic peptide isolated from the saliva gland of the Northern Short-tailed shrew. Our previous studies have shown that SOR-C13 specifically targets, strongly binds to, and inhibits the TRPV6 calcium channel with an IC_{50} of 14 nM (**Bowen et al. 2013**). TRPV6 is a recognized oncochannel over-expressed in many epithelial cancers including Breast/Prostate/Pancreatic/Ovarian and is correlated with poor outcomes. TRPV6 plays a central role in a biochemical cascade that results in the up-regulation of an array of pro-cancerous genes maintaining a high proliferation rate, increasing cell survival and apoptosis resistance through calcineurin/NFAT pathways. SOR-C13 binds with high affinity and selectivity disrupting the function of TRPV6 and curtailment of downstream calcineurin/NFAT signalling pathways.



1) SOR-C13 inhibits the TRPV6 channel
-Ca^{2+} is decreased into the cell.

2) Decreased influx of Ca^{2+}:
-Cannot activate CaM.
-Reduces phosphatase Calnr activity.
-NFAT is left phosphorylated and not able to translocate to the nucleus.

3) NFAT pathway associated gene expression are de-regulated, allowing for reductions in NFAT and pro-cancerous gene events:
-Anti-apoptotic (Bcl-2)
-Metastatic (MMPs)
-Cell proliferation

Results

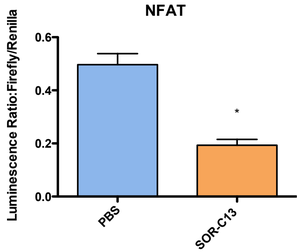
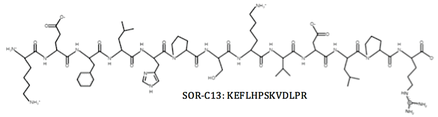


Figure 1: Effect of SOR-C13 on NFAT activation in T-47D cells treated with SOR-C13 (500 µM): A significant difference (*:p<0.05) was observed in NFAT activation between the SOR-C13-treated cells vs. PBS (no treatment).



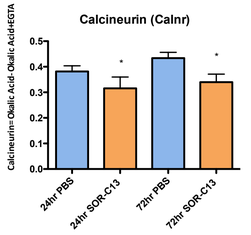


Figure 2: Calcineurin activity inhibition by SOR-C13 (500 µM) in BxPC-3 cell lysates (*:p<0.05) at 24 hrs and 72 hrs.

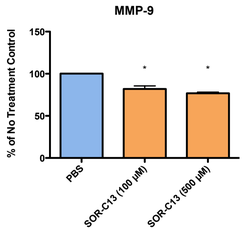


Figure 3: Total MMP-9 (% NT ctrl) in BxPC-3 cells treated for 96 hrs daily with SOR-C13 (100, 500 µM) (*:p<0.05).

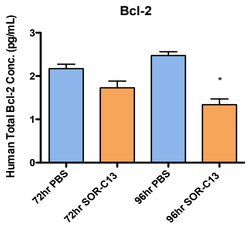


Figure 4: Bcl-2 expression in BxPC-3 cells treated with SOR-C13 (500 µM). A significant decrease (*:p<0.05) was observed at 96 hrs.

Materials & Methods

Peptide: SOR-C13, its modified form was synthesized by CanPeptide (QC).
Cell cultures: Breast (T-47D), Prostate (PC-3) and Pancreatic (BxPC-3, SU.86.86) cancer cell lines were obtained from ATCC and cultured as recommended.
Cells: Cells lines were selected for analysis based on their TRPV6 mRNA expression as either high/low and to cell types that responded in SOR-C13 Phase 1 Clinical Trial.

***In vitro* NFAT Analysis:**
Transfection: T-47D cells were plated at 1 x 10^4 cells/well in a 96-well plate and cultured overnight. Cells were transfected for 24 hrs at 37°C/5 % CO_2 using Liptofectamine 3000 (0.3 µL/well)/P3000 Reagent (1 µL/well) in Opti-MEM medium and with a 500 ng of NFAT dual reporter plasmid (Cignal NFAT Reporter, Qiagen).
Drug Dosing: After transfection the T-47D cells were dosed daily with NT (s.f. PBS) or 500 µM SOR-C13 (prepared fresh daily in f.s. PBS) for 72 hrs. Once dosed the 96-well plate was incubated at 37°C/5 % CO_2 for the duration dosing period of 72 hrs. Dosing was repeated every 24 hrs for 72 hrs.
Plate Assay: Luminescence was monitored using Dual-Glo® Luciferase assay (Promega) to measure changes in NFAT luminescence expression (related to pKC pathway) between PBS and SOR-C13 treated T-47D cells. Firefly and Renilla Luminescence was measured using a molecular devices microplate reader.

***In vitro* Calnr and Bcl-2 Analysis:**
Drug Dosing: Cells were plated at 5 x 10^5 cells/well in a 6-well plate. After 24 hrs incubation the BxPC-3 cells were dosed daily with NT (s.f. PBS) or 500 µM SOR-C13 (prepared fresh daily in f.s. PBS) for 72 hrs. Once dosed cells were incubated at 37°C/5 % CO_2 for the duration dosing period of 72 hrs. Dosing was repeated every 24 hrs for 72 hrs. Every 24 hrs protein lysates (RIPA) were prepared from cells and de-salted (7 kDa MWCO, Zebra spin columns, Fisher). Bcl-2 assay was a 96 hrs dosing parameter.
Plate Assay (Calnr): Cellular Calcineurin concentration was monitored at 620 nm using the Calcineurin Cellular (PP2B) Phosphate Activity Assay kit (EMD Millipore). Results were interpolated from standard curve for amount of Phosphate released.
Plate Assay (Bcl-2): Human total Bcl-2 concentration was performed using DuoSet® Human Total BcL-2 sandwich ELISA (R&D Systems). Bcl-2 concentration for BxPC-3 cells was extrapolated from (4-PL) standard curve Bcl-2.

***In vitro* MMP-9 Analysis:**
Drug Dosing: : BxPC-3 cells were plated at 2 x 10^4 cells/well in a 96-well plate and cultured overnight. Cells were dosed daily with NT (s.f. PBS) 100, 500 µM SOR-C13 (prepared fresh daily in f.s. PBS) for 96 hrs. Dosing was repeated every 24 hrs for 96 hrs.
Plate Assay: MMP-9 concentration was monitored using the MMP-9 Cytoglow ELISA (Assay Biotech). Cells were fixed to plate, and ELISA was performed, MMP-9 expression was calculated as % of No Treatment control.

RT-qPCR TaqMan array NFAT/Ca^{2+} gene pathway profiling:
mRNA Expression: The expression NFAT/Ca^{2+} related genes mRNA levels were determined in 6 Cancer Cell lines treated with SOR-C13 (500 µM) for 120hrs using a qPCR TaqMan® Array panel consisting of 187 genes (ThermoFisher). Cells were harvested with lysis buffer from the PureLink RNA Mini Kit and then RNA isolation carried out as per manufacturers instructions (ThermoFisher Scientific). RNA was quantified using the Qubit Fluorometer. Reverse Transcription was performed and cDNA created using SuperScript™ IV VILO Master Mix with ezDNase Enzyme (ThermoFisher Scientific). Each port of the Custom TaqMan® Array Card was loaded with 500 ng of cDNA and 1X TaqMan Fast Advanced Master Mix. RT-qPCR was performed using the Quantstudio™ 7Flex and analysis performed by ThermoFisher cloud software using GUSB and HRPT1 as endogenous controls.

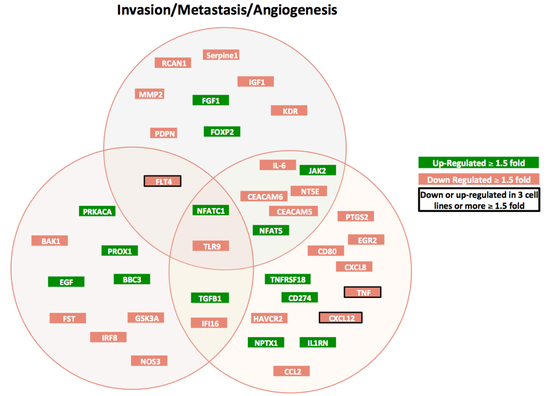


Figure 5: Up and down-regulated genes (> 1.5 fold change in expression) from a 187 gene panel in T-47D cells lines treated with SOR-C13. These genes are up or down-regulated in at least one of the 4 other cancer cell lines tested (BxPC-3, PC-3, SKOV-3 and SU. 86.86).Genes involved in Calcineurin/NFAT pathway that are shown to be affected: NFATC1, MMP-2, GSK3A and RCAN1. Interestingly, Bcl-2 and MMP-9 were down-regulated (app. 0.6 CT) in BxPC-3 (not shown), in line with the protein expression data (Figure 4).

Summary

- SOR-C13 peptide targets and inhibits TRPV6 channels, and has shown decreases with treatment in the NFAT signalling molecular pathway targets (Calnr, Bcl-2, ATX, MMP-9 and NFAT) in *in vitro* dosing experiments.
- Up and Down-regulation of NFAT/Ca^{2+} signalling genes indicates SOR-C13 MOA involvement in the NFAT/Ca^{2+} signalling pathways, along with other downstream molecular interactions associated with oncogenesis.
- The impact of SOR-C13 on the expression of genes involved in resistance to apoptosis, proliferation, metastasis and angiogenesis as well as on the expression of immune cytokines and transcription factors in multiple cancer cells, makes SOR-C13 an attractive, novel anti- cancer drug.
- SOR-C13 provides a unique mechanism for anticancer activity through inhibition of TRPV6 calcium channel. Based on good tolerability and manageable safety profile and with promising anti-tumour activity (**Fu et al. 2017**), further studies with SOR-C13 as an anti-cancer agent are planned, as it is the first highly specific TRPV6 inhibitor to be identified and to be taken into clinical development.

References:
Bowen, C.V., DeBay, D., Ewart, H.S., et al. 2013. In vivo Detection of Human TRPV6-Rich Tumors with Anti-Cancer Peptides Derived from Soricidin. PLOS ONE 10.1371/journal.pone.0058866.

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