Cancer cell binding, uptake and localization of a novel therapeutic peptide SOR-C13 targeting the TRPV6 oncochannel

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Abstract No:
LB-051

ABSTRACT: SOR-C13, a 13-mer peptide derived from soricidin, the paralytic protein component of saliva of the Northern Short-tailed shrew, has recently completed a Phase I clinical trial for the treatment of epithelial-derived cancers. SOR-C13 specifically targets and inhibits the TRPV6 calcium channel - a recognized oncochannel over-expressed in a number of epithelial cancers (*e.g.* breast, ovarian, prostate). SOR-C13 is the first TRPV6 targeting drug to enter clinical development. Xenograft and fluorescent imaging studies provide *in vivo* evidence of SOR-C13 efficacy in a range of epithelial tumours as well as SOR-C13's preferential accumulation at tumour sites. To demonstrate the specificity of the peptide for TRPV6 and determine its fate, modified SOR-C13 peptide was labeled with fluorescein to enable monitoring of cellular binding and internalization in high versus low TRPV6 cancer cell lines. Various organelle-specific fluorescent trackers for nuclei, actins, lysosomes, and endoplasmic reticula were paired with labeled SOR-C13 peptides and anti-TRPV6 antibody in fluorescence imaging studies to monitor intracellular trafficking. Rapid peptide binding and internalization was observed on incubation of fluorescently labeled SOR-C13 with a breast cancer cell line (T-47D) expressing high levels of the ion channel. Significantly less SOR-C13 binding was observed in an ovarian cancer cell line (SKOV-3) with low TRPV6 levels. Fluorescently labeled SOR-C13 scramble peptide was included as a control and did not bind to high TRPV6 cells. Co-localization studies did not demonstrate a clear association of the fluorescently labeled SOR-C13 with lysosomes, the ER or actin. This study demonstrates that SOR-C13 not only specifically binds to TRPV6-expressing cancer cells according to their level of TRPV6 expression but is also internalized. The correlation of TRPV6 expression in tumours and clinical efficacy will be evaluated during the next SOR-C13 clinical trials. Additionally, these results set the stage for further development of SOR-C13-based peptide drug conjugates for TRPV6 targeted anti-cancer therapies.

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 and ovarian. Xenograft and fluorescent imaging studies provide *in vivo* evidence of SOR-C13 efficacy and accumulation at tumour sites. Fluorescence imaging studies were performed to demonstrate peptide binding specificity for TRPV6 and to follow uptake and trafficking of labeled SOR-C13 by cell lines expressing low and high levels of the oncochannel. The study not only demonstrated that labeled SOR-C13 peptide was internalized by cancer cells, but that the quantities taken up correlated with TRPV6 expression levels. These results suggest a link between clinical SOR-C13 efficacy and TRPV6 expression in tumours. Furthermore, internalization of SOR-C13 by cancer cells makes the peptide an attractive candidate for anti-cancer TRPV6 targeted peptide-drug conjugate design.

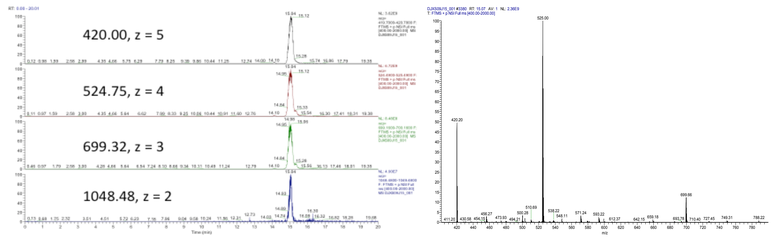
Materials & Methods

Peptide: SOR-C13, its modified form and scrambled SOR-C13 were synthesized by CanPeptide (QC).
Fluorescent Labeling of Peptides: Modified SOR-C13 peptide dissolved in PBS was mixed with 1.3 molar equivalents of fluorescein 5-maleimide (5FM, Invitrogen) dissolved in DMSO and reacted for 2 h in the dark at room temperature. The reaction was monitored by HPLC with triple absorbance at 207, 254 and 440nm. Fluorescent peptides were purified and concentrated by ultrafiltration (Vivaspin 2, 2kDa MWCO, Sartorius). Peptide-5FM conjugate purity and correct molecular mass were verified by LC/MS.
Biodistribution in Tumour-Bearing Mice: SKOV-3 ovarian tumour xenografts were established in CD-1 female nude mice (n=3; 5-6 week old, Charles River) by injection of $4x10^6$ cells/graft subcutaneously at the lower left flank. 100µg of fluorescent peptide was administered by i.v. injection via tail vein and biodistribution evaluated at various time points from 10min to 72 h post-injection by whole-body scanning (small animal imager; Xenogen, IVIS Lumina II). (see ***Bowen et al. 2013*** reference)
Fluoresence Microscopy: T-47D breast cancer cells were plated at 2 x 10^5 cells/well in two-well Lab-Tek II Permanox coverslides (Nunc, Inc.) and cultured overnight. Cells were then incubated with fluorescent peptides in medium at 37°C/5% CO_2 for 1h. The peptide-containing media was removed, the cells washed, and then stained with organelle-specific fluorescent trackers (Invitrogen), including Hoechst 33342 (10ug/mL), LysoTracker DND-99 (75nM), and ER-Tracker Red (1uM), in FL-DMEM for 30min at 37°C. Subsequently, cells were rinsed three times with PBS and fixed in 4% (v/v) formaldehyde in PBS for 15 min at room temperature (RT). Where indicated, the fixed cells were incubated with ActinRed 555 reagent in PBS for 30min at RT. For labeling of TRPV6 at the membrane surface, fixed cells were blocked with 3% BSA, and then incubated with affinity purified rabbit anti-TRPV6 antibody (10ug/mL; Agrisera, Sweden) for 1 h at RT. Goat anti-rabbit IgG conjugated to TexasRed was used for detection of bound TRPV6 antibody. Stained cells were mounted under No. 1 glass coverslips using ProLong Diamond Antifade mountant and imaged using the EVOS FL™ Cell Imaging System (ThermoFisher).
Plate Assay: T-47D and SKOV-3 cells were plated at 5 x 10^4 cells/well in a 96-well plate and cultured overnight. Cells were then incubated with 0, 10, 25 and 50µM fluorescently labeled SOR-C13 or scrambled SOR-C13 peptides for 2 h at 37°C/5% CO_2. The peptide-containing media was removed, cells washed, and external fluorescence quenched with 0.1% Trypan Blue. Fluorescence was measured using a BioTek Synergy HT reader (Ex490/Em525). Results were normalized against cell number and background fluorescence.

Acknowledgements:
LC/MS analysis was performed by Dr. David Barnett at the Atlantic Cancer Research Institute (Moncton, NB)

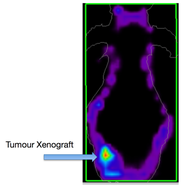
Results

LC/MS Analysis of Peptide-5FM



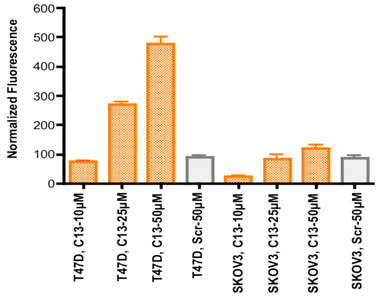
Purified peptide-5FM conjugate was analyzed using a Q Exactive Orbitrap LC-MS/MS system (ThermoFisher). Sample was injected on a microcapillary (75 micron dia.) C18 column. One LC peak was detected for the peptide-5FM conjugate. The mass spectrum clearly showed 3 distinct charge states that indicated a molecular weight of 2095 Da (matching the predicted size for the conjugate).

Biodistribution



Accumulation of fluorescent peptide in SKOV-3 tumour xenografts. Representative *in vivo* whole body scan at 48h post i.v. injection of peptide. Preferential accumulation of fluorescence in the tumour starts within 1 hour post injection. ***Bowen et al. 2013***

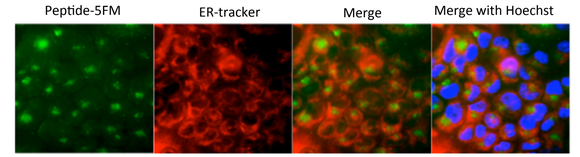
Peptide Uptake Assay

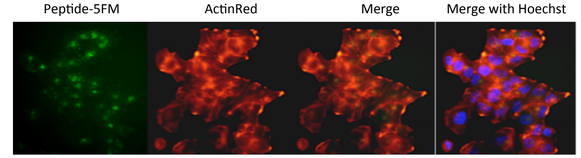


Measurement of SOR-C13 internalization by T-47D and SKOV-3 cells. T-47D breast cancer cells (**HIGH** TRPV6 expression) and SKOV-3 ovarian cancer cells (**LOW** TRPV6 expression) were incubated with fluorescein-labeled SOR-C13 (at 10, 25 and 50µM) and scrambled SOR-C13 peptides (at 50µM, Scr) in a 96-well plate assay. After quenching external fluorescence using trypan blue, internalized signal was measured. The data showed internalized SOR-C13 peptide amounts correlated with cell line TRPV6 expression level. There was minimal uptake of the scrambled peptide.

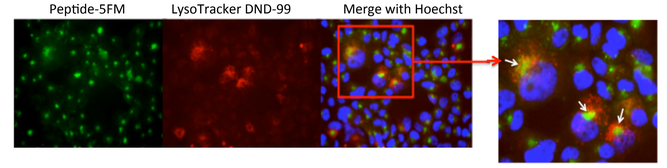
Results Continued

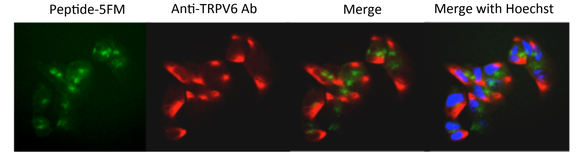
Fluorescence Microscopy

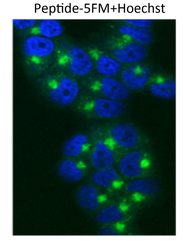




T-47D breast cancer cells incubated with peptide-5FM. After 1h incubation, internalized peptide appears to be concentrated close to the nucleus. The peptide does not co-localize with ER or Actin (Panels A and B, 400x). A small amount of peptide may be associated with lysosomes (Panel C). After 1h incubation, the peptide appears to be completely internalized. No co-localization with TRPV6 at the cell membrane is observed (Panel D). Panel E shows a high resolution confocal image of peptides heavily concentrated (likely within vesicle structures) close to the nucleus (600x).







Summary:
- SOR-C13 peptides target and inhibit TRPV6 channels, and preferentially accumulate at tumour-bearing sites in xenograft studies.
- The fluorescently tagged SOR-C13 peptide is internalized by T-47D breast cancer cells. Amount of SOR-C13 uptake correlates with level of TRPV6 expression.
- Further co-localization and extended time course studies are required to clarify intracellular trafficking of peptides (towards rationale peptide-drug conjugate design).

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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